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Original article

Interlaboratory validation of the modified murine local lymph node assay based on adenosine triphosphate measurement

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ABSTRACT

Introduction: The murine local lymph node assay (LLNA) is a well-established alternative to the guinea pig maximization test (GPMT) or Buehler test (BT) for the assessment of the skin sensitizing ability of drugs and chemicals. Daicel Chemical Industries Ltd. has developed a modified LLNA based on the adenosine triphosphate (ATP) content (LLNA-DA). We conducted 2 interlaboratory validation studies to evaluate the reliability and relevance of LLNA-DA. **Methods:** The experiment involved 17 laboratories, wherein 14 chemicals were examined under blinded conditions. In the first study, 3 chemicals were examined in 10 laboratories and the remaining 9 were examined in 3 laboratories. In the second study, 1 chemical was examined in 7 laboratories and the remaining 4 chemicals were examined in 4 laboratories. The data were expressed as the ATP content for each chemical-treated group, and the stimulation index (SI) for each chemical-treated group was determined as the increase in the ATP content relative to the concurrent vehicle control group. An SI of 3 was set as the cut-off value for exhibiting skin sensitization activity. **Results:** The results of the first study obtained in the experiments conducted for the 3 chemicals that were examined in all the 10 laboratories and for 5 of the remaining 9 chemicals were sufficiently consistent with small variations in their SI values. The sensitivity, specificity, and accuracy of LLNA-DA against those of GPMT/BT were 7/8 (87.5%), 3/3 (100%), and 10/11 (90.9%), respectively. In the second study, all the 5 chemicals studied demonstrated acceptably small interlaboratory variations. **Discussion:** In the first study, a large variation was observed for 2 chemicals; in the second study, this variation was small. It was attributed to the

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application of dimethylsulfoxide as the solvent for the metallic salts. In conclusion, these 2 studies provide good evidence for the reliability of the LLNA-DA.

1. Introduction

Skin sensitization (allergic contact dermatitis (ACD)) is an immunologically mediated cutaneous reaction to a drug or chemical. It is known that detecting and evaluating the immune-based adverse effects that are collectively referred to as hypersensitivity reactions is a very difficult task, particularly during the drug approval process, because of the lack of adequate non-clinical models and the low incidence rate of reactions (Hastings, 2001). However, there are several adequate and predictive methods for modeling ACD. For several decades, tests involving guinea pigs, such as the guinea pig maximization test (GPMT) or the Buehler test (BT), have been used for assessing the skin sensitization potential of chemicals (OECD, 1992).

The local lymph node assay (LLNA) employs a mouse model for assessing the relative sensitization potential; it is a well-established alternative method for determining whether a chemical causes ACD. Although GPMT and BT can be viewed as phenomenological methods in which the clinical signs are modeled, LLNA was developed on the basis of a mechanistic understanding of immune-based contact dermatitis (Hastings, 2001). In addition, this method also offers important animal welfare benefits. The use of LLNA has been successfully validated by several studies (Basketter et al., 2002; Basketter, Gerberick, Kimber, & Loveless, 1996; Basketter & Scholes, 1992; Gerberick, Ryan, Kimber, Dearman, & Basketter, 2000; Haneke, Tice, Carson, Margolin, & Stokes, 2001). Recently, it has been recommended that this method be formally adopted by the Organization for Economic Co-operation and Development (OECD), according to the guidelines for testing chemicals 406 and 429 (OECD, 1992, 2002), and that it be accepted by the EU and US as a suitable method for classifying the skin sensitizing ability of chemicals (Basketter, Casati, Gerberick, Griem, Philips, & Worth, 2005; Dean, Twerdok, Tice, Sailstad, Hattan, & Stokes, 2001; Sailstad, Hattan, Hill, & Stokes, 2001). The LLNA was specifically designed to identify contact allergens. The assay was not intended to facilitate the detection of low molecular weight chemicals associated with systemic sensitization or drug allergies (Kimber, 2001). However, an investigation, which was designed to explore the ability of LLNA to identify pharmaceutical process intermediates known to cause contact allergy in humans, provided evidence that the assay is a useful method for hazard identification (Durand, De Burlet, Virat, & Nauman, 2003). Furthermore, presently, the use of the method, along with the use of GPMT and BT, is recommended for the determination of the skin sensitization potential of new drugs (FDA, 2002).

The original LLNA uses [^{3}H]-methyl thymidine to measure lymphocyte proliferation; this hinders it use, particularly in Japan, because being a radioisotope (RI)-based method, it requires special facilities. Several authors have been conducting investigations for the development of an alternative non-RI method for performing LLNA (Dearman, Hilton, Basketter, & Kimber., 1999; Ehling et al., 2005a, 2005b; Hatao, Hariya, Katsumura, & Kato, 1995; Lee, Park, Park, Kim, & Oh, 2002; Takeyoshi, Yamasaki, Yakabe, Takatsuki, & Kimber, 2001).

Daicel Chemical Industries Ltd. proposed a modification of LLNA, which involves the measurement of the adenosine triphosphate (ATP) content instead of [^{3}H]-methyl thymidine incorporation for assessing lymphocyte proliferation (Idehara, Yamagishi, Yamashita, & Ito, in press; Yamashita, Idehara, Fukuda, Yamagishi, & Kawada, 2005). This modified assay method is designated as the LLNA modified by Daicel, based on the ATP content (LLNA-DA).

Although LLNA-DA essentially involves the same procedure as LLNA, the evidence available is insufficient for validating the assay method through interlaboratory evaluation. Therefore, we conducted 2 interlaboratory validation studies for LLNA-DA.

In the first study, 2 metallic salts—cobalt chloride and nickel sulfate—dissolved in dimethylsulfoxide (DMSO) produced inconsistent results across the laboratories. We assumed that the inconsistency factor would be due to one of the following 2 reasons: (1) DMSO was used as the vehicle in the control group for the 2 metallic salts, and DMSO application in mice is difficult as compared with acetone–olive oil (AOO) or acetone (ACE) application or (2) LLNA-DA is unsuitable for use with metallic salts, and both the chemicals used were metallic salts. Therefore, a second study employing additional metallic salt with DMSO was planned in order to ascertain the hypothesis.

The primary objectives of the first study were (1) to evaluate the extent of interlaboratory variation with regard to LLNA-DA and (2) to ascertain whether the results of LLNA-DA are comparable with those of LLNA. The primary objective of the second study was to examine the reliability of the LLNA-DA method when metallic salts were tested with DMSO.

2. Methods

2.1. Organization

This study was organized by researchers belonging to the committee for the validation of the assay. The research team comprised

Table 1(a)
Selected chemicals with their corresponding vehicles, the referenced results of LLNA and GPMT/BT, and the allocation of chemicals for the LLNA-DA experiments in the first study

Chemical	CASRN[a]	Vehicle[b]	LLNA	GPMT/BT[c]	Laboratory[d]									
					1	2	3	4	5	6	7	8	9	10
A: 2,4-Dinitrochlorobenzene	97-00-7	AOO	+	+	□	□	□	□	○	△	□	□	△	○
B: Hexyl cinnamic aldehyde	101-86-0	AOO	+	+	○	○	△	△	△	□	△	○	○	△
C: 3-Aminophenol	591-27-5	AOO	+	+nonstd	□		○					□		
D: Glutaraldehyde	111-30-8	ACE	+		△	△			□					
E: Cobalt chloride	7646-79-9	DMSO	+	+				○		○		△		
F: Isoeugenol	97-54-1	AOO	+	+				□	○				△	
G: Formaldehyde	50-00-0	ACE	+	+	△	△			□					
H: Dimethyl isophthalate	1469-93-4	AOO	−	−	□		□				□			
I: Isopropanol	67-63-0	AOO	−	−	○	○	△	△	△	□	△	○	○	△
J: Nickel sulfate	10101-97-0	DMSO	−	+				○		○		△		
K: Abietic acid	514-10-3	AOO	+	+		□				△	○			
L: Methyl salicylate	119-36-8	AOO	−	−			○				○			○

[a] The Chemical Abstract Services Registry Number.
[b] ACE, acetone; AOO, acetone–olive oil; DMSO, dimethylsulfoxide.
[c] Judgment based on the guinea pig maximization test or the Buehler test; "nonstd" indicates a nonstandard animal that was not tested for chemical G.
[d] Allocated pairs for the LLNA-DA experiments in a laboratory; ○, experiment 1; △, experiment 2; □, experiment 3.

Table 1(b)
Selected chemicals with their corresponding vehicles, the referenced results of LLNA and GPMT/BT, and the allocation of chemicals in the second study

Chemical	CASRN[a]	Vehicle[b]	LLNA	GPMT/BT[c]	Laboratory[d]						
					11	12	13	14	15	16	17
B: Hexyl cinnamic aldehyde	101-86-0	AOO	+	+	○	○	○	○	○	○	○
E: Cobalt chloride	7657-79-9	DMSO	+	+	□		△	△			△
J: Nickel sulfate	10101-97-0	DMSO	−	+	□	△		△		△	
M: Lactic acid	598-82-3	DMSO	−	−	△		△		△	△	
N: Potassium dichromate	7778-50-9	DMSO	+	+	△	△			△		△

[a] The Chemical Abstract Services Registry Number.
[b] ACE, acetone; AOO, acetone–olive oil; DMSO, dimethylsulfoxide.
[c] Judgment based on guinea pig maximization test or Buehler test.
[d] Allocated pairs for an experiment in a laboratory; ○, experiment 1; △, experiment 2; □, experiment 3.

representatives from each experimental laboratory, toxicologists as the chemical selectors and as distributors of the chemicals and materials, biostatisticians, and the study manager. All the experimentations were performed by the toxicologists of the experimental laboratories. In the first study, participation was limited to 10 experimental laboratories with sufficient experience in the use of the LLNA and/or its modifications; however, this was not a limiting factor in the second study, in which 7 additional experimental laboratories were included. A total of 17 different experimental laboratories participated in these 2 studies.

Research teams of all the experimental laboratories obtained ethical approval for each standard operational procedure conducted in their laboratories.

2.2. Technology transfer

A 1-day technology-transfer seminar was held by the LLNA-DA developer for each study, which was attended by at least 1 toxicologist from each experimental laboratory. Participants learned the method of conducting the assay according to the standard protocol. In addition, in the second study, the operation of LLNA-DA with DMSO was also included in the seminar (Omori et al., 2008).

2.3. Preliminary tests

Prior to each study, a preliminary test was conducted by researchers from all the experimental laboratories, who used only the positive control chemical, namely, 25% hexyl cinnamic aldehyde. The purpose of these preliminary tests was to ascertain whether the standard protocol was being documented sufficiently and to confirm the sensitivity of LLNA-DA (Omori et al., 2008).

The results of both preliminary tests revealed that the standard protocol was essentially valid and required few modifications.

2.4. Chemical selection and allocation

The chemical selectors chose 20 candidate chemicals that were previously used in LLNA and whose test results had been documented (Basketter & Scholes, 1992; Basketter, Gerberick, & Kimber, 1998; Basketter, Lea, Cooper, et al., 1999; Basketter, Lea, Dickens, 1999; Basketter, Blaikie, Dearman, Kimber, Ryan, Gerberick, et al., 2000; Gerberick et al., 2004; Haneke et al., 2001; Kimber et al., 1998; Loveless et al., 1996). On the basis of these literature data and solubility of the chemicals, the chemical selectors selected vehicles and prepared 3 fixed doses (low, medium, and high) for each chemical; subsequently, the chemicals were transported from the chemical and material distributors to the experimental laboratories.

In the first study, 12 of the 20 candidate chemicals were selected and classified as strong, mild, or weak sensitizers or non-sensitizers on the basis of LLNA. In order to reduce the number of animals used, pairs comprising groups treated with 2 or 3 chemicals and the same vehicle control group were employed; in other words, in each laboratory, 2 or 3 chemicals were simultaneously tested with 1 negative control and 1 positive control for every experiment. Of the 12 chemicals, 3 were dispatched to all the 10 participating experimental laboratories, and the remaining 9 were randomly allocated to the laboratories by a biostatistician and dispatched to each of the 3 experimental laboratories.

In the second study, 5 of the 20 candidate chemicals were selected. To determine whether the results from the 7 new laboratories would be similar to those obtained in the first study, the chemical selectors chose a single chemical that had been tested by all the 10 laboratories in the first study. The remaining 4 chemicals selected by the chemical selectors comprised 3 metallic salts—cobalt chloride, nickel sulfate, and potassium dichromate—and lactic acid with DMSO as the vehicle control. Pairs comprising groups treated with 2 of the 4 chemicals and

Table 2(a)
Body weight (g) [day 1]

Laboratory	n	Mean	SD	Min	Med	Max
1	120	22.0	1.5	19.3	21.8	27.1
2	108	22.5	1.3	19.4	22.6	25.0
3	108	22.0	1.2	18.2	22.0	24.8
4	108	22.7	1.4	20.0	22.5	26.7
5	108	21.6	1.1	19.1	21.6	24.4
6	108	21.7	1.4	19.3	21.7	24.9
7	108	22.8	1.4	18.5	22.8	25.9
8	108	23.4	1.5	20.5	23.3	28.6
9	72	23.0	1.2	20.1	22.9	26.5
10	72	22.6	1.4	19.8	22.5	25.8
11	96	22.9	1.3	19.9	22.9	26.5
12	60	21.6	1.0	18.8	21.7	24.1
13	60	22.2	1.1	19.5	22.1	24.8
14	60	21.8	1.5	18.7	21.8	24.3
15	60	22.5	1.1	20.0	22.5	25.2
16	60	22.3	1.5	18.8	22.6	25.5
17	60	22.1	1.4	19.5	22.3	26.4

Table 2(b)
Body weight (g) [day 8]

Laboratory	n	Mean	SD	Min	Med	Max
1	120	22.1	1.5	19.0	22.0	26.1
2	108	23.4	1.4	20.6	23.3	26.7
3	108	23.2	1.4	19.8	23.2	26.6
4	104	23.4	1.4	20.4	23.3	27.1
5	108	23.0	1.3	20.1	23.0	25.8
6	108	22.2	1.4	19.2	22.2	25.6
7	108	23.0	1.5	17.1	23.0	26.0
8	108	23.9	1.8	20.1	24.0	29.2
9	72	23.9	1.3	20.9	23.9	27.0
10	72	23.3	1.3	20.7	23.3	26.8
11	96	23.4	1.3	21.1	23.3	27.1
12	60	23.1	1.2	20.4	23.2	26.5
13	60	22.9	1.3	20.2	22.7	26.2
14	59	22.3	1.9	16.3	22.4	25.9
15	60	23.8	1.3	21.3	23.6	26.6
16	60	23.3	1.6	19.1	23.4	27.0
17	60	23.1	1.4	19.7	23.3	26.7

Table 3(a)
Mean and SD for the ATP content and SI values obtained in all the laboratories in the first study

Vehicle/ concentration	1		2		3		4		5		6		7		8		9		10	
	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI
A: 2,4-Dinitrochlorobenzene																				
AOO	27,188±10,027	–	26,159±2157	–	35,610±7212	–	42,866±9956	–	11,899±7366	–	13,910±3921	–	22,466±3515	–	20,576±5546	–	26,842±9515	–	53,350±14,893	–
0.03%	77,305±25,181	2.8	60,843±19,746	2.3	80,548±34,265	2.3	127,990±23,651	3.0	18,107±3203	1.5	38,247±10,833	2.7	86,083±21,219	3.8	49,730±22,738	2.4	75,290±20,086	2.8	62,000±23,941	1.2
0.10%	147,161±32,102	5.4	70,451±26,337	2.7	150,579±23,446	4.2	210,206±57,119	4.9	45,691±21,305	3.8	59,302±19,598	4.3	121,021±23,461	5.4	62,571±30,199	3.0	112,282±36,388	4.2	112,163±22,420	2.1
0.30%	325,485±46,981	12.0	241,465±73,709	9.2	354,678±27,371	10.0	365,768±51,573	8.5	166,224±43,333	14.0	210,636±46,213	15.1	296,024±33,270	13.2	259,203±105,308	12.6	292,230±5423	10.9	251,172±40,569	4.7
B: Hexyl cinnamic aldehyde																				
AOO	24,583±5761	–	41,189±17,452	–	35,652±12,253	–	43,007±8931	–	19,146±6582	–	16,375±3953	–	29,925±6142	–	12,207±4127	–	29,602±8049	–	29,077±2876	–
5%	33,196±6535	1.4	56,291±5484	1.4	48,383±14,959	1.4	64,212±6709	1.5	23,417±6260	1.2	27,369±8594	1.7	46,148±14,005	1.5	16,616±4630	1.4	25,602±11,242	0.9	40,685±14,674	1.4
10%	73,884±14,255	3.0	109,204±15,298	2.7	82,040±12,032	2.3	138,873±51,932	3.2	35,432±14,357	1.9	38,327±9530	2.3	126,755±35,639	4.2	50,829±8197	4.2	65,640±27,871	2.2	79,321±10,548	2.7
25%	142,130±29,633	5.8	198,520±40,800	4.8	158,304±26,958	4.4	219,687±29,834	5.1	76,029±5733	4.0	90,067±27,828	5.5	212,285±50,835	7.1	124,803±34,287	10.2	114,791±13,669	3.9	101,984±21,546	3.5

C: 3-Aminophenol

Vehicle/concentration	1		3		8	
	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI
AOO	27,188±10,027	–	24,047±3932	–	20,576±5546	–
1%	47,591±2668	1.8	33,875±4945	1.4	25,167±4299	1.2
3%	63,021±9400	2.3	42,352±11,487	1.8	40,921±10,896	2.0
10%	76,927±15,323	2.8	41,759±8243	1.7	49,037±8244	2.4

D: Glutaraldehyde

Vehicle/concentration	1		2		5	
	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI
ACE	17,947±4929	–	38,044±13,217	–	16,439±6488	–
0.05%	25,594±9403	1.4	28,096±9168	0.7	17,024±5163	1.0
0.15%	72,748±20,584	4.1	48,980±8745	1.3	40,319±17,078	2.5
0.50%	89,767±21,798	5.0	129,110±31,985	3.4	42,237±6048	2.6

E: Cobalt chloride

Vehicle/concentration	4		6		8	
	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI
DMSO	100,396±24,632	–	4184±2395	–	19,803±4451	–
0.30%	–±–	–	44,002±30,922	10.5	87,562±13,336	4.4
1.00%	203,895±24,479	2.0	44,465±23,293	10.6	131,004±34,534	6.6
3.00%	267,172±52,088	2.7	85,978±24,933	20.6	159,808±13,473	8.1

F: Isoeugenol

Vehicle/concentration	4		5		9	
	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI
AOO	42,866±9956	–	11,899±7366	–	26,842±9515	–
1%	125,838±22,236	2.9	22,896±7449	1.9	69,256±20,292	2.6
3%	175,277±10,289	4.1	23,619±8830	2.0	86,598±20,489	3.2
10%	262,118±34,406	6.1	117,098±5209	9.8	190,392±38,486	7.1

G: Formaldehyde

Vehicle/concentration	1		2		5	
	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI
ACE	17,947±4929	–	38,044±13,217	–	16,439±6488	–
0.5%	52,214±10,965	2.9	64,467±11,056	1.7	19,510±5239	1.2
1.5%	51,405±13,007	2.9	115,143±20,638	3.0	30,959±12,804	1.9
5.0%	86,934±33,582	4.8	120,966±21,688	3.2	44,219±7822	2.7

H: Dimethyl isophthalate

Vehicle/concentration	1		3		7	
	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI
AOO	27,188±10,027	–	35,610±7212	–	22,466±3515	–
5%	36,534±10,199	1.3	35,710±8126	1.0	28,306±4047	1.3
10%	31,200±10,875	1.1	34,357±8364	1.0	25,555±3074	1.1
25%	30,030±10,456	1.1	23,900±3733	0.7	23,583±3751	1.0

I: Isopranol

Vehicle/concentration	1		2		3		4		5		6		7		8		9		10	
	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI
AOO	24,583±5761	–	41,189±17,452	–	35,652±12,253	–	43,007±8931	–	19,146±6582	–	16,375±3953	–	29,925±6142	–	12,207±4127	–	29,602±8049	–	29,077±2876	–
10%	37,756±12,448	1.5	37,286±9163	0.9	36,155±7444	1.0	67,307±12,946	1.6	10,106±3170	0.5	32,233±26,281	2.0	43,446±17,986	1.5	14,797±2984	1.2	18,791±7645	0.6	26,480±4594	0.9
25%	27,101±2623	1.1	35,024±4878	0.9	23,465±7953	0.7	38,859±7172	0.9	14,531±1549	0.8	14,762±5342	0.9	27,285±10,469	0.9	12,387±3421	1.0	20,627±6175	0.7	30,676±5707	1.1
50%	28,723±3313	1.2	33,259±7651	0.8	30,822±4465	0.9	34,382±6421	0.8	13,581±3696	0.7	21,360±4957	1.3	24,776±3613	0.8	13,551±1001	1.1	15,039±3697	0.5	36,216±4960	1.2

J: Nickel sulfate

Vehicle/concentration	4		6		8	
	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI
DMSO	100,396±24,632	–	4184±2395	–	19,803±4451	–
1%	116,266±22,468	1.2	21,990±7141	5.3	69,077±14,602	3.5
3%	153,074±35,051	1.5	27,966±6162	6.7	60,881±7880	3.1
10%	103,595±20,343	1.0	49,303±14,901	11.8	50,568±9846	2.6

K: Abietic acid

Vehicle/concentration	2		6		7	
	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI
AOO	26,159±2157	–	13,910±3921	–	21,546±13,493	–
5%	55,039±8805	2.1	25,277±9139	1.8	40,328±8389	1.9
10%	91,706±17,069	3.5	57,615±12,621	4.1	85,821±24,030	4.0
25%	121,351±36,474	4.6	110,697±29,265	8.0	81,818±24,819	3.8

L: Methyl salicylate

Vehicle/concentration	3		7		10	
	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI
AOO	24,047±3932	–	21,546±13,493	–	53,350±14,893	–
5%	25,764±7330	1.1	23,459±7751	1.1	33,663±5192	0.6
10%	26,361±6381	1.1	38,158±6803	1.8	41,698±7559	0.8
25%	37,359±10,622	1.6	29,881±11,569	1.4	44,426±13,600	0.8

(continued on next page)

Table 3(a) (*continued*)

Positive control (hexyl cinnamic aldehyde)

Vehicle/ concentration	1		2		3		4		5		6		7		8		9		10	
	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI
AOO	23,639±5906	–	30,284±11,576	–	25,429±5894	–	44,371±9224	–	15,183±5554	–	10,447±4413	–	25,112±8035	–	18,428±4503	–	26,327±5484	–	22,309±6293	–
25%	147,032±30,059	6.2	153,995±35,670	5.1	144,091±18,550	5.7	243,877±42,495	5.5	72,877±19,820	4.8	84,748±16,459	8.1	136,327±26,932	5.4	101,382±22,894	5.5	140,388±23,895	5.3	113,209±18,835	5.1

ACE, acetone; AOO, acetone–olive oil; DMSO, dimethylsulfoxide.
Number of animals: 4 for all the tested chemicals, 8 for the positive controls of laboratories 9 and 10, and 12 for the positive controls of laboratories 1–8.

the same vehicle control group were employed. These 4 chemicals were randomly allocated by a biostatistician.

In order to avoid predicting the severity of the effects of each chemical, all the chemical names were coded into alphabetic characters, and they were labeled as low, medium, and high in terms of the concentration that enabled blinded distribution for both the studies. However, prior to the study, the researchers and toxicologists of the respective laboratories were informed of the identity of the 20 candidate chemicals and the corresponding control vehicles. This was done in order to ensure the safety of the chemists performing the experiments (e.g., with regard to proper disposal of the chemicals) and to prevent any anxiety that they would experience while handling unknown chemicals.

2.5. Development of LLNA-DA

The original LLNA measures the proliferation of draining lymph node cells (LNCs) via the incorporation of [^{3}H]-methyl thymidine into DNA and β scintillation counting. Although this approach to measure the activity of LNC is well established through many studies on the original LLNA, alternative approaches that do not require the use of radioisotopes are expected to be beneficial.

ATP is the main energy source for a majority of cellular functions, and it is an essential molecule for living cells. ATP activity is known to indicate the number of living cells. Therefore, measurement of the ATP content in the lymph node by a luciferin–luciferase assay is considered to be one of the surrogates of altered lymph node cellularity. The measurement of the ATP content of the lymph node involves determination of the cell number at the end of cell proliferation, while the measurement of [^{3}H]-methyl thymidine incorporation involves determination of the endpoint of cell proliferation. One of the benefits of measuring the ATP content is that it allows the use of commercially available reagent kits; in this method, the ATP content is expressed in terms of the chemiluminescence (relative light units, RLU) induced by the luciferin–luciferase reaction.

Yamashita, Idehara, Fukuda, Yamagishi, and Kawada (2005) used 3 chemicals to study the approach involving the measurement of the ATP content. They found that when the dosing schedule of the original LLNA was followed, the ATP measurement approach as well as the flow cytometric analysis of LNCs (Hatao, Hariya, Katsumura, & Kato, 1995) or the assessment of 5-bromo-2′-deoxyuridine (BrdU) incorporation into LNCs (Takeyoshi, Yamasaki, Yakabe, Takatsuki, & Kimber, 2001) tended to show lower stimulation indices (SIs) than the original LLNA. Hence, in order to increase lymph node proliferation, Yamashita et al. proposed pretreatment with 1% sodium lauryl sulfate (SLS) prior to the application of the test chemicals and an additional treatment with the tested chemical. Through their studies, these authors successfully increased the sensitivity of the ATP measurement approach, and the SI value of 3 obtained with this approach was considered to be comparable to that of the original LLNA. Additionally, these authors conducted 6 independent experiments using eugenol to determine the intralaboratory variation in the SI values of the ATP measurement approach. The mean and coefficient of variance of the SI values were 4.0% and 17.3%, respectively.

Daicel Chemical Industries Ltd. refined the ATP measurement approach, which was designated LLNA-DA. In addition to the original LLNA procedure, this ATP content measurement assay includes pretreatment with 1% SLS solution along with its application of the test chemicals on the seventh day; this strategy was expected to yield similar SI values, i.e., approximately 3, to those of the original LLNA. Therefore, this additional step enabled the use of the same cut-off point as that of the original LLNA. By the time the first validation study was conducted, Daicel Chemical Industries Ltd. had obtained some results for LLNA-DA by using the abovementioned cut-off point, in which the correlation coefficient of the EC3 value for LLNA and LLNA-DA for 10 chemicals was 0.90,

Table 3(b)
Mean and SD for the ATP content and SI values obtained in all the laboratories in the second study

B: Hexyl cinnamic aldehyde

Vehicle/ concentration	11		12		13		14		15		16		17	
	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI
AOO	21,328±8537	–	27,436±7629	–	24,739±6350	–	24,348±8236	–	31,189±10,511	–	28,421±8943	–	23,888±10,275	–
5%	32,306±7470	1.5	45,178±8970	1.6	35,059±13,111	1.4	50,408±15,075	2.1	46,853±7275	1.5	65,209±12,332	2.3	31,668±6045	1.3
10%	70,689±7059	3.3	94,494±20,913	3.4	110,638±34,223	4.5	88,935±40,202	3.7	78,471±11,510	2.5	146,720±30,935	5.2	110,331±13,800	4.6
25%	95,348±32,502	4.5	156,615±19,035	5.7	133,833±22,340	5.4	185,142±43,204	7.6	122,146±25,678	3.9	239,220±35,785	8.4	154,106±28,583	6.5

E: Cobalt chloride

Vehicle/concentration	11		13		14		17	
	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI
DMSO	82,093±26,296	–	81,326±13,350	–	41,770±12,971	–	50,815±5671	–
1%	122,193±21,742	1.5	133,890±34,318	1.6	97,101±15,349	2.3	148,776±68,574	2.9
3%	141,919±33,024	1.7	199,335±5756	2.5	171,272±19,452	4.1	216,116±18,966	4.3
5%	165,350±10,204	2.0	206,394±16,349	2.5	177,705±46,577	4.3	256,978±54,531	5.1

J: Nickel sulfate

Vehicle/concentration	11		12		14		16	
	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI
DMSO	82,093±26,296	–	83,046±6308	–	41,770±12,971	–	76,153±28,228	–
1%	53,652±8085	0.7	82,896±14,003	1.0	77,804±25,666	1.9	90,029±11,264	1.2
3%	65,034±25,414	0.8	103,345±24,614	1.2	65,200±11,620	1.6	118,932±13,811	1.6
10%	60,451±17,784	0.7	80,596±21,515	1.0	88,990±14,982	2.1	88,482±19,237	1.2

M: Lactic acid

Vehicle/concentration	11		13		15		16	
	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI
DMSO	65,060±9211	–	81,326±13,350	–	49,353±21,291	–	76,153±28,228	–
5%	60,576±20,296	0.9	80,639±18,883	1.0	45,730±8622	0.9	69,247±15,579	0.9
10%	49,033±11,761	0.8	55,369±7627	0.7	47,928±15,171	1.0	60,621±11,273	0.8
25%	52,131±16,088	0.8	60,124±13,945	0.7	35,259±2939	0.7	69,108±14,746	0.9

N: Potassium dichromate

Vehicle/concentration	11		12		15		16	
	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI
DMSO	65,060±9211	–	83,046±6308	–	49,353±21,291	–	50,815±5671	–
01%	123,936±17,967	19	157,464±29,682	19	131,244±35,222	27	165,248±46,056	33
0.3%	145,833±41,893	2.2	217,061±37,807	2.6	191,819±51,627	3.9	257,138±29,816	5.1
1.0%	311,009±24,188	4.8	338,610±33,485	4.1	296,431±75,377	6.0	323,834±60,878	6.4

Positive control (hexyl cinnamic aldehyde)

Vehicle/ concentration	11		12		13		14		15		16		17	
	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI	Mean±SD	SI
AOO	25,807±8795	–	30,147±6951	–	24,943±6509	–	27,245±7022	–	33,713±7937	–	37,383±5294	–	17,417.3±7195	–
25%	102,118±22,127	4.0	142,679±50,388	4.7	136,950±22,057	5.5	184,010±31,146	6.8	143,322±31,990	4.3	268,199±47,663	7.2	138,799±25,305	8.0

ACE, acetone; AOO, acetone–olive oil; DMSO, dimethylsulfoxide.
Number of animals: 4 for all the tested chemicals, 8 for the positive controls of laboratories 12–17, and 12 for the positive control of laboratory 11.

and the accuracy of LLNA-DA against LLNA for 18 chemicals was 89% (16/18) (in-house data).

The ATP content value is influenced by time, that is, it decreases over time. This is not emerge in the original LLNA since it involves the measurement of [^{3}H]-methyl thymidine incorporation. Daicel Chemical Industry Ltd. investigated it and found that the ATP content value is not influenced by a 10- to 20-min delay, while this value would be reduced to approximately 50% of its original value with a 2-h delay. Therefore, Daicel Chemical Industry Ltd. recommends that when LLNA-DA is conducted, all the procedural steps from lymph node excision to the determination of the ATP content be performed rapidly and without delay.

Very recently, Idehara et al. (in press) reported the details of the intralaboratory study on LLNA-DA.

2.6. Standard protocol of LLNA-DA for the studies

The standard protocol for the assay was prepared prior to the preliminary test and determined according to the time of commencement of the study. Three doses were prepared for each of the test chemicals.

The groups of female CBA/JNCrlj mice (n=4; Charles River Japan Inc., Kanagawa) were treated with the topical application of 25 μL of 1 of the 3 doses of the test chemicals or the vehicle control exclusively on the dorsum of both ears. Following pretreatment with 1% SLS for 1 h, daily treatments with the chemicals were performed for the first 3 days and, subsequently, on day 7. On day 8, the treated mice were sacrificed, and the draining auricular lymph nodes were excised. After recording the lymph node weight (LNW), the LNCs were ground

between 2 slide glasses and subsequently suspended in 1 mL of phosphate-buffered saline (PBS) with a cell scraper. The LNC suspension was mixed and diluted to 1% with PBS. The ATP content was determined using a commercially available kit (Kikkoman Co., Tokyo). ATP was extracted from 0.1 mL of the diluted LNC suspension for 20 s, following which 0.1 mL of a reagent containing luciferase was added and the bioluminescence (RLU) in 10 s was measured with a luminometer (Lumitester C-100; Kikkoman Co., Tokyo). A point to note is that after the death of the animal, the ATP content of the lymph node decreases over time. It is therefore desirable that the series of procedures from lymph node excision to the determination of the ATP content must be performed rapidly and without delay.

2.7. Database

A biostatistician created a database containing the LNW and ATP content data obtained for each mouse in all the experimental laboratories. For comparison, data from studies on the original LLNA were collected and included in the database.

2.8. Statistical methods

For each experimental group, the SI was defined as the increase in the ATP content in the chemical-treated group relative to that in the vehicle control group. An SI of 3 was defined as the cut-off value for the skin sensitization potential. In order to demonstrate the variability within the SI values, the confidence interval of the SI values was calculated (Omori & Sozu, 2007). A variance component, τ^2, estimated by a random effect model for the log-transformed SI, was used as a measure of the interlaboratory variations; this is similar to the meta-analysis technique used in clinical studies (Normand, 1999). Using the abovementioned random effect model, we estimated the weighted average as an overall estimate of the SI value recorded for each chemical dose. The EC3 is defined as the estimated concentration that yields an SI value of 3. The EC3 of the weighted average was estimated and classified into the appropriate chemical category (Gerberick et al., 2004). Finally, the sensitivity, specificity, accuracy, positive predictivity, and negative predictivity were calculated as measures of relevance on the basis of the weighted averages in order to assess the concordance of the LLNA-DA results with the LLNA or GPMT/BT results (OECD, 2005). These measures were not calculated in the second study because of a shortage of chemicals.

3. Results

3.1. Chemical selection

Tables 1(a) and 1(b) show the selected chemicals, the results of LLNA and GPMT/BT as references, and the results obtained for the chemicals allocated for the LLNA-DA experiments of both the studies.

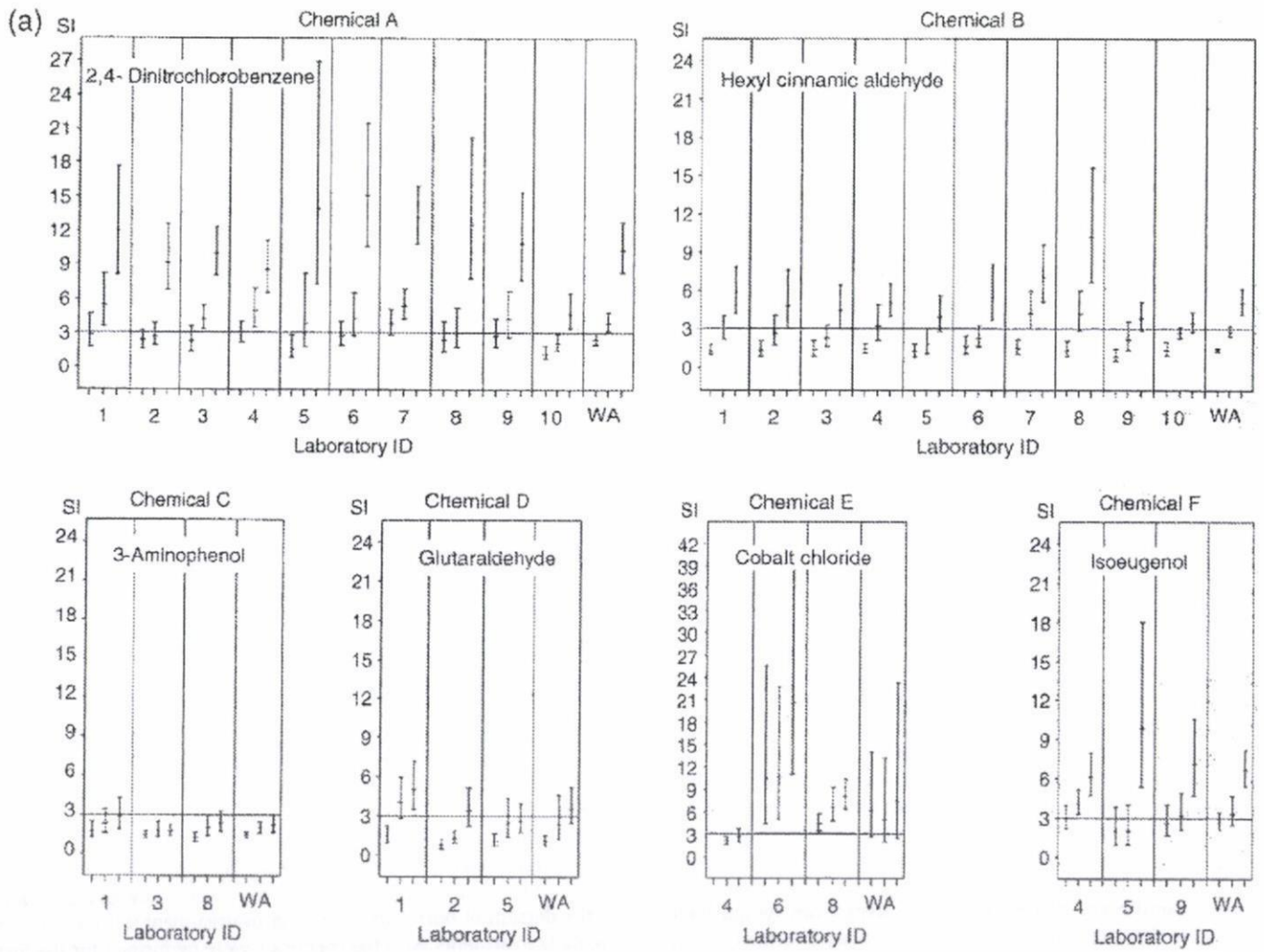

Fig. 1. (a). Dose–response relationships of the SI values with 95% confidence intervals for each chemical analyzed in all the laboratories. "WA" indicates the weighted average of the SI values obtained by meta-analysis using the random effect model in the first study. (b). Dose–response relationships of the SI values with 95% confidence intervals for each chemical analyzed in all the laboratories. "WA" indicates the weighted average of the SI values obtained by meta-analysis using the random effect model in the second study.

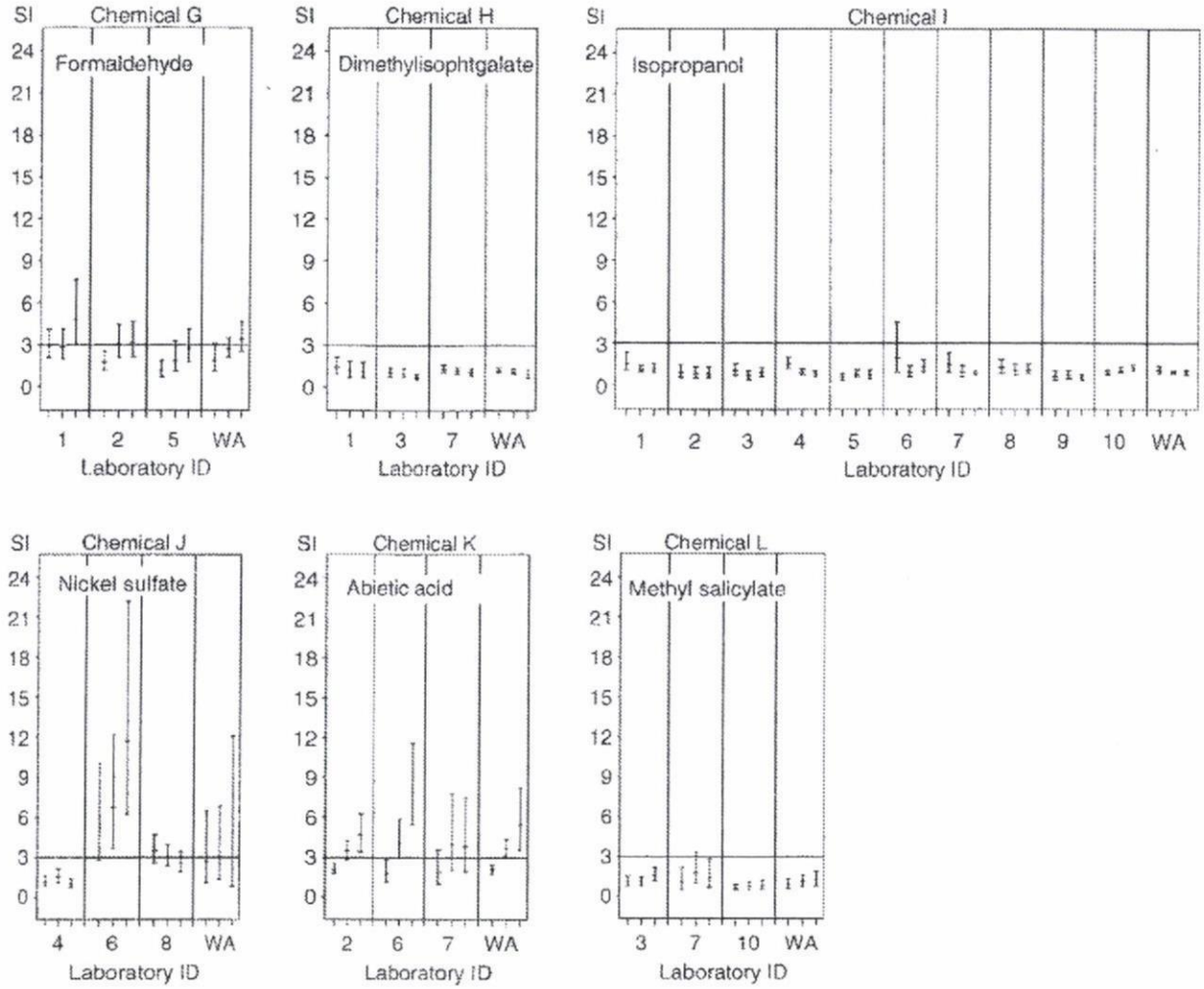

Fig. 1 (*continued*).

The GPMT/BT results for chemical D (glutaraldehyde) are not listed in Table 1(a) because the data were not available at the time the list was prepared.

The chemical selectors initially set the dose concentrations of chemical E (cobalt chloride) at 1%, 3%, and 10%. However, during the first round of the experiments in a laboratory in the first study, 2 of the 4 mice treated with the 10% dose concentration died, while the other 2 exhibited signs of hypokinesia. Since only the laboratory had conducted the experiment using this chemical concentration at the time, the chemical selectors decided to alter the dose concentrations. Then, the dose concentrations of chemical E were subsequently set at 0.3%, 1%, and 3% in a blinded manner for the remaining 2 laboratories in the first study. However, after several considerations, the chemical selectors adopted different doses in the second study, i.e., 1%, 3%, and 5%.

3.2. Body weights

Tables 2(a) and 2(b) summarize the body weight statistics observed on days 1 and 8 in each laboratory, respectively. No substantial interlaboratory variations were observed with regard to the body weights.

3.3. ATP content and SI values

The ATP content and SI values recorded by the experimental laboratories for each of the test chemicals are summarized in Tables 3(a) and 3(b), and the dose–response relationships for the SI values are indicated in Fig. 1(a) and (b).

The results of the first study are shown in Table 3(a) and Fig. 1(a). For chemicals A (2,4-dinitrochlorobenzene), B (hexyl cinnamic aldehyde), F (isoeugenol), and K (abietic acid), dose–response relationships of the SI values were clearly evident in each laboratory, and the SI values for all the high-dose groups were greater than 3. The dose–response relationships for chemicals H (dimethyl isophthalate), I (isopropanol), and L (methyl salicylate) were unclear, and the laboratories that assessed these chemicals reported negative findings. The SI values obtained for chemical C (3-aminophenol) in all 3 laboratories were lower than 3, and the values obtained in laboratories 1 and 3 were approximately 3 for the high-dose group. Further, dose–response relationships of the SI values were observed for chemicals D (glutaraldehyde) and G (formaldehyde), whose SI values were also approximately 3 for the high-dose groups. The SI values were greater than 3 for the high-dose groups in laboratories 1 and 2 but not in laboratory 5. The SI values for chemicals E (cobalt chloride) and J (nickel sulfate) were inconsistent across laboratories; further, an inconsistency was observed in the ATP content values in the vehicle control group for these chemicals. In the case of chemical E, the dose–response relationship of the weighted average of the SI values yielded a v-shaped curve; therefore, it may be considered that the observed dose–response relationships based on the weighted average values for chemical E were inappropriate.

Table 3(b) and Fig. 1(b) describe the results of the second study. For chemicals B (hexyl cinnamic aldehyde) and N (potassium dichromate),

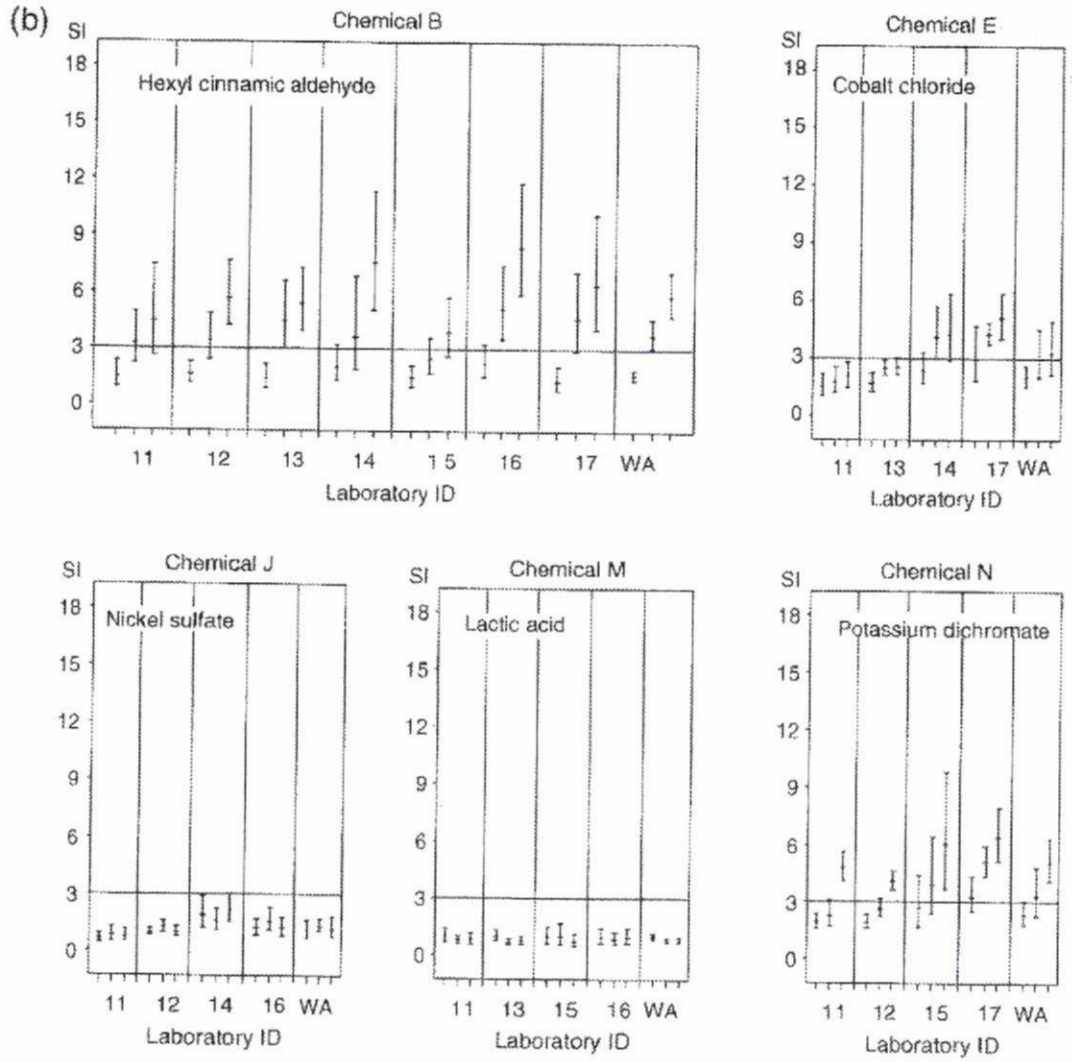

Fig. 1 (*continued*).

the dose–response relationships of the SI values were evident in each laboratory, and all the SI values of the high-dose groups were greater than 3. The SI values for chemicals J (nickel sulfate) and M (lactic acid) were lower than 3, and these chemicals tested negative in all the laboratories. The SI value for chemical E (cobalt chloride), which was inconsistent in the first study, was also inconsistent between different laboratories in the study. However, as opposed to the results of the first study, the dose–response relationships and ATP contents were considerably similar between laboratories.

3.4. ATP content and LNW

Fig. 2(a) and (b) shows the scatter plots of ATP content according to LNW for all the chemicals. Since the ATP content decreases with time, it is important for the scatter plot to demonstrate a linear relationship between the ATP content and LNW. This linear relationship can be used as a rough indicator of whether the experiments conformed to the protocol for measuring the ATP content. Since all the scatter plots demonstrated linearity, it can be concluded that all the experiments adhered to the protocol.

3.5. Assay sensitivity

We defined assay sensitivity as the ability to accurately detect the positive control chemical. Since a positive control was included in each experiment, we investigated whether the SI value assigned to the positive control group was greater than 3 in the experiments. Fig. 3(a) and (b) shows the SI values obtained for all the positive control groups with 95% confidence intervals. All the experiments in these studies were assay sensitive because all the SI values were greater than 3.

3.6. Intralaboratory variability

Although limited, the results obtained for the positive control groups allowed us to evaluate the intralaboratory variability of the assay. Fig. 3(a) and (b) also shows the variability of the SI values obtained for the positive control groups in each laboratory in both the studies. No large intralaboratory variation was observed in any of the laboratories.

3.7. Interlaboratory variability

The data shown in Fig. 1(a) and (b) were used to measure the interlaboratory variability in the SI values for all the chemical doses. Tables 4(a) and 4(b) show the weighted average of the SI values with 95% confidence intervals and a summary index of the interlaboratory variability, i.e., τ^2.

In the first study, all the doses of chemicals E (cobalt chloride) and J (nickel sulfate) and the intermediate dose of chemical D (glutaraldehyde) exhibited relatively large interlaboratory variations. On the

other hand, in the second study, no large interlaboratory variation was observed in any of the laboratories.

Tables 5(a) and 5(b) show the results of the judgments based on the cut-off value of 3 for the SI values obtained for all the chemicals in all the laboratories. In the first study, 4 chemicals, namely, D (glutaraldehyde), E (cobalt chloride), G (formaldehyde), and J (nickel sulfate), showed inconsistent results among the laboratories. For chemicals D (glutaraldehyde) and G (formaldehyde), the SI values for the high doses were approximately 3 among all 3 laboratories; thus, the variation was small. On the other hand, the values for chemicals E (cobalt chloride) and J (nickel sulfate) were inconsistent among the laboratories (Fig. 1(a)). In the second study, consistent results were observed for the 4 chemicals. Although an inconsistency was observed for chemical E (cobalt chloride), the dose–

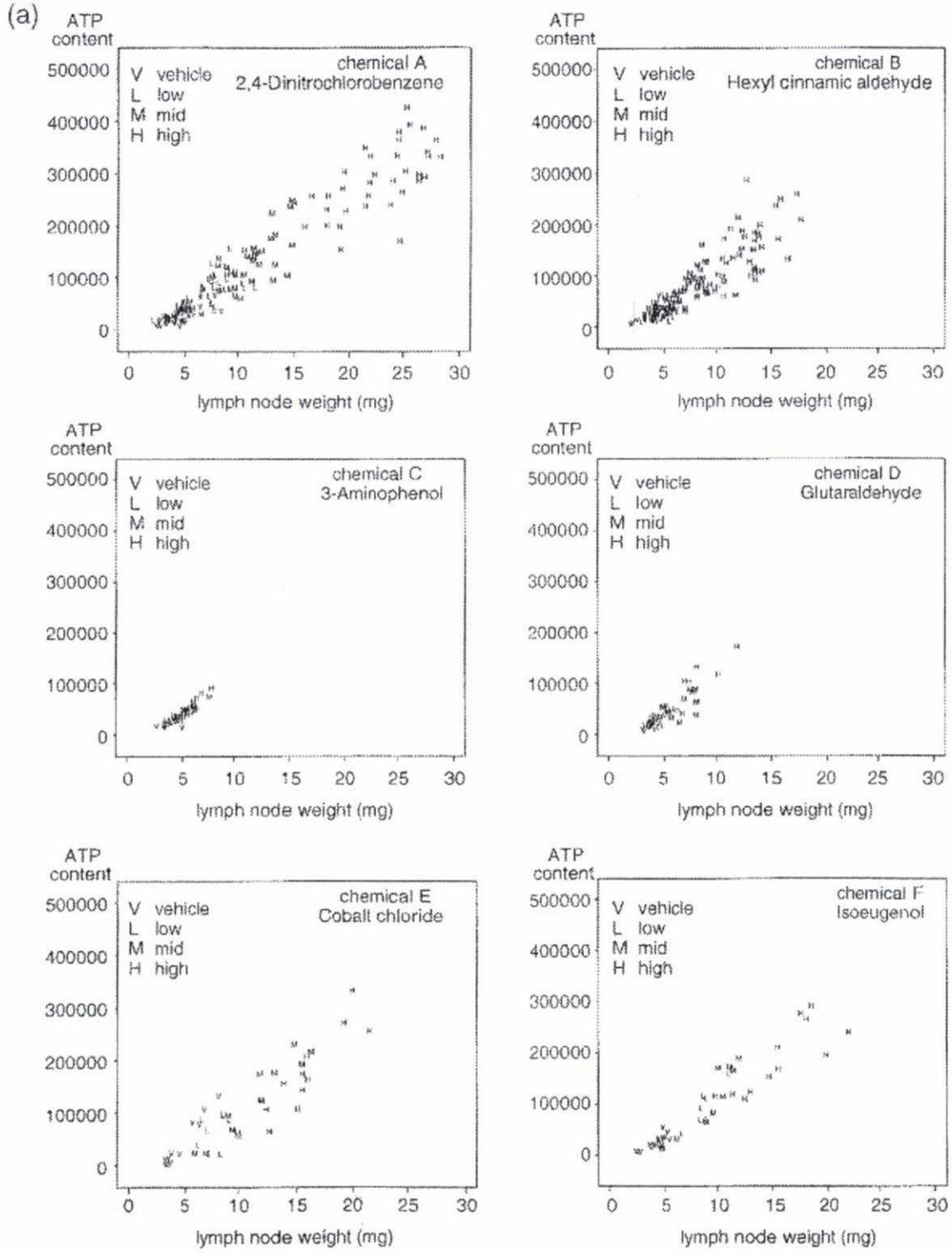

Fig. 2. (a). Scatter plots indicating the ATP content with the LNW (mg) recorded for the vehicle (V), low-dose (L), middle-dose (M), and high-dose groups (H) for each chemical in the first study. (b). Scatter plots indicating the ATP content with the LNW (mg) recorded for the vehicle (V), low-dose (L), middle-dose (M), and high-dose groups (H) for each chemical in the second study.

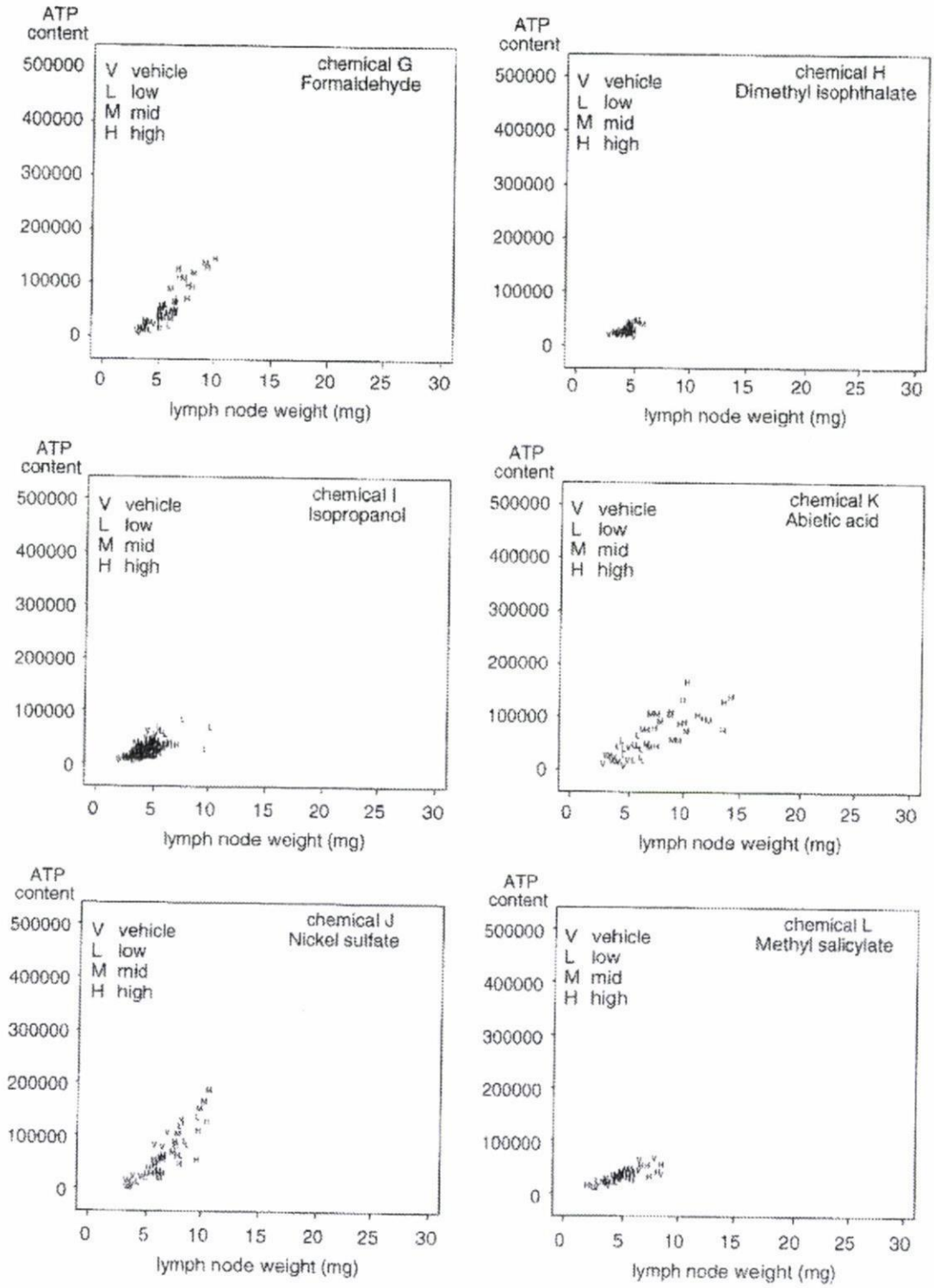

Fig. 2 (continued).

response relationships of the chemical were very similar between laboratories and the variation in the SI value for each dose was small, as mentioned above.

3.8. EC3 and measures of relevance

To avoid the problem of multiple counts of the same chemicals from different laboratories, the calculations of EC3 and sensitivity, specificity, accuracy, positive predictivity, and negative predictivity of LLNA-DA were based on the weighted averages of the SI values.

Tables 6(a) and 6(b) show the EC3 results and its classification for LLNA-DA based on the weighted averages for both the studies and the reported EC3 and its classification based on the reported values for LLNA.

The sensitivity, specificity, accuracy, positive predictivity, and negative predictivity of LLNA-DA with regard to the chemicals in the first study, as against those of GPMT/BT and LLNA are shown in Table 7.

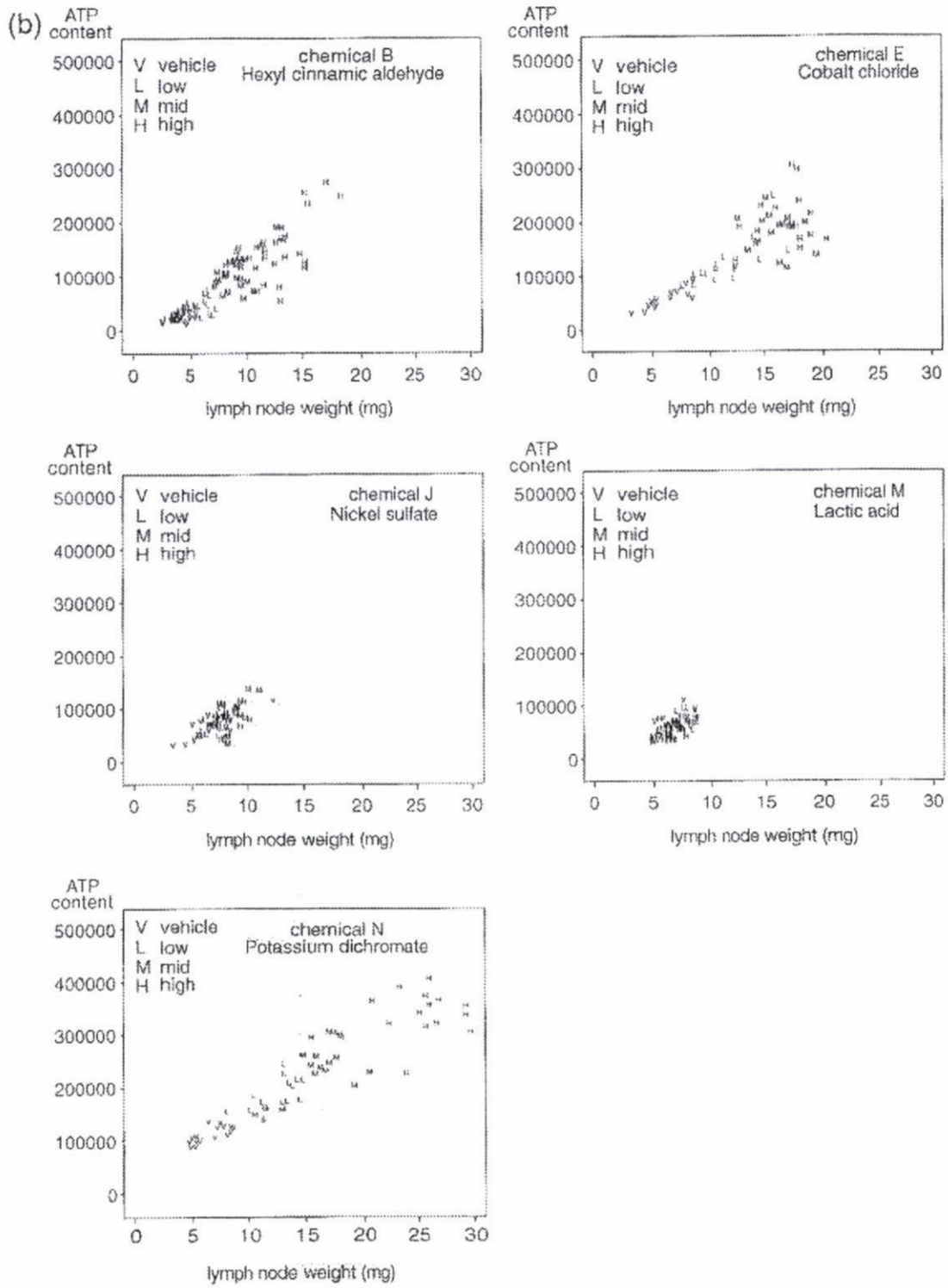

Fig. 2 (continued).

To enable comparison of the measurements of LLNA against those of GPMT/BT when the same chemicals were selected, these values calculated only on the basis of the referenced data are shown in Table 7.

The sensitivity, specificity, accuracy, positive predictivity, and negative predictivity values of LLNA-DA against those of GPMT/BT were similar to those of LLNA against those of GPMT/BT. Chemical C (3-aminophenol) was negative for LLNA-DA and positive for LLNA, and chemical J (nickel sulfate) was positive for LLNA-DA and negative for LLNA.

4. Discussion

Researchers have provided considerable evidence for the reliability of LLNA; however, limited evidence is available for the reliability of LLNA-DA. Since the methods involved in LLNA-DA and LLNA are essentially identical, the results of our study provide adequate evidence in support of LLNA-DA as an alternative assay method to LLNA.

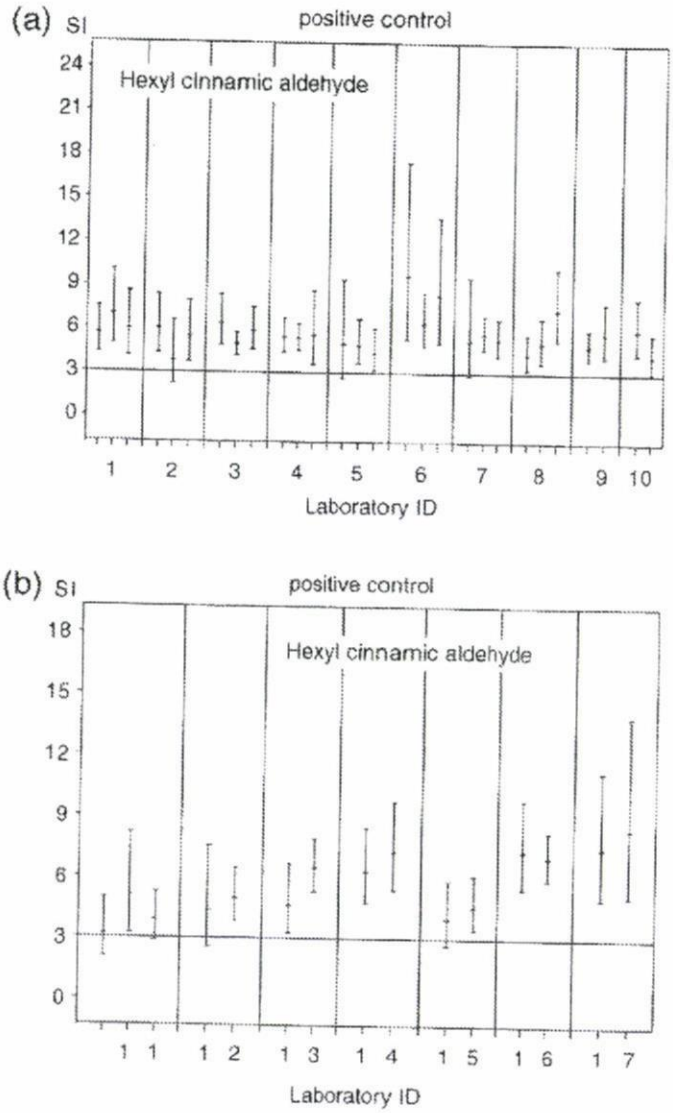

Fig. 3. (a). SI values with 95% confidence intervals obtained for the positive control (25% hexyl cinnamic aldehyde) groups in all the laboratories in the first study. (b). SI values with 95% confidence intervals obtained for the positive control (25% hexyl cinnamic aldehyde) groups in all the laboratories in the second study.

Although several interlaboratory studies on LLNA have been reported, they did not necessarily employ the same protocol; additionally, these studies were conducted by approximately 5 experimental laboratories (Basketter et al., 1991; Kimber et al., 1991, 1998, 1995; Loveless et al., 1996; Scholes et al., 1992). In contrast, one of the distinguishing features of the series of the present 2 studies is that 17 independent experimental laboratories used the same protocol to test chemicals. The fact that the interlaboratory variations were small for most of the chemicals is considered as a significant finding of this study. In particular, chemical B (hexyl cinnamic aldehyde) was tested by all the 17 laboratories; it was observed that the SI value of the interlaboratory variation was small and that the dose–response relationship was considerably similar. These results indicate that LLNA-DA is a robust technique.

In the first study, 2 of the 12 chemicals—chemicals E (cobalt chloride) and J (nickel sulfate)—demonstrated large interlaboratory variations. We considered that this might be attributed to the use of DMSO as the exclusive vehicle for these 2 chemicals. The fact that these 2 chemicals were the only metallic salts could be another reason for the large variations observed. Therefore, in the second study, it was necessary to examine interlaboratory variations with regard to other metallic salts with DMSO as the vehicle. The results of the second study, which used 5 chemicals including these 2 metallic salts, demonstrated small interlaboratory variations for all the chemicals. The small variation observed for the metallic salts could be due to the following reasons. (1) Prior to the study, the developer advised the toxicologists to carefully apply the DMSO solution onto the ears since it is highly hydrophilic, and the presence of moisture in the ears could lead to considerable variation in the results. (2) During the technical-transfer seminar, the participating toxicologists were well trained in all aspects of the experiment, including the application of DMSO solution onto the ears of the mice. Thus, our present finding is that the large variation for the 2 metallic salts in the first study was caused by inappropriate DMSO application, which in comparison with AOO or ACE has unique physical properties in terms of the difficulty involved in its application to the dorsum of the ears. Therefore, this factor was considered when the metallic salts were assessed with LLNA-DA.

Furthermore, these 2 studies provided substantial historical data with regard to the ATP content for the vehicle control group that used AOO, ACE, or DMSO with LLNA-DA. These data could be referred to by new laboratories that are considering the use of this assay. As in the case of LLNA studies, data from these studies regarding DMSO appear to demonstrate the toxicity of the chemical (Wright et al., 2001). We observed a higher ATP content when DMSO was used as a solvent than when AOO or ACE was used. This tendency may cause the SI values to change depending on the vehicle used in the experiment because a high ATP content in the vehicle control group would lead to relatively low SI values.

These studies also present certain limitations. First, the results are representative of only 14 chemicals. Although it may be recommended that the assay be tested using several chemicals, the

Table 4(a)
The weighted average of the SI values and the variance component, τ^2, in the first study

Chemical	Low-dose group			Middle-dose group			High-dose group		
	SI	95%CI	τ^2	SI	95%CI	τ^2	SI	95% CI	τ^2
A: 2,4-Dinitrochlorobenzene	2.5	(2.0, 3.0)	0.03	3.9	(3.1, 4.8)	0.03	10.3	(8.4, 12.8)	0.04
B: Hexyl cinnamic aldehyde	1.4	(1.2, 1.6)	0.00	2.8	(2.5, 3.3)	0.01	5.1	(4.2, 6.2)	0.03
C: 3-Aminophenol	1.4	(1.2, 1.7)	0.00	2.0	(1.6, 2.4)	0.00	2.2	(1.7, 2.9)	0.02
D: Glutaraldehyde	1.0	(0.7, 1.5)	0.02	2.3	(1.2, 4.6)	0.13	3.6	(2.4, 5.2)	0.03
E: Cobalt chloride	6.1	(2.7, 13.9)	0.11	5.0	(1.9, 13.2)	0.29	7.4	(2.4, 23.3)	0.42
F: Isoeugenol	2.7	(2.2, 3.4)	0.00	3.4	(2.4, 4.7)	0.02	6.7	(5.5, 8.3)	0.00
G: Formaldehyde	1.8	(1.1, 3.1)	0.07	2.7	(2.1, 3.4)	0.00	3.4	(2.5, 4.7)	0.01
H: Dimethyl isophthalate	1.2	(1.0, 1.4)	0.00	1.1	(0.9, 1.3)	0.00	0.9	(0.7, 1.2)	0.02
I: Isopropanol	1.1	(0.8, 1.4)	0.04	0.9	(0.8, 1.0)	0.00	0.9	(0.8, 1.1)	0.03
J: Nickel sulfate	2.7	(1.1, 6.5)	0.24	3.1	(1.4, 6.9)	0.20	3.1	(0.8, 12.1)	0.62
K: Abietic acid	2.1	(1.8, 2.4)	0.00	3.7	(3.1, 4.3)	0.00	5.4	(3.5, 8.3)	0.04
L: Methyl salicylate	0.9	(0.6, 1.3)	0.03	1.1	(0.7, 1.6)	0.04	1.2	(0.8, 1.9)	0.04

The variance component τ^2 represents the interlaboratory variance for the log-transformed SI, which is obtained by decomposing the total variance into the between variance and within variance by performing meta-analysis with a random effect model. Since τ^2 indicates variance, its value is greater than 0, and a higher value indicates greater interlaboratory variation.

Table 4(b)
The weighted averages of the SI values and the variance component,(τ^2, in the second study

Chemical	Low-dose group			Middle-dose group			High-dose group		
	SI	95%CI	τ^2	SI	95%CI	τ^2	SI	95% CI	τ^2
B: Hexyl cinnamic aldehyde	1.7	(1.4, 2.0)	0.00	3.8	(3.1, 4.6)	0.01	5.9	(4.8, 7.2)	0.01
E: Cobalt chloride	2.0	(1.5, 2.6)	0.02	3.0	(2.0, 4.5)	0.07	3.2	(2.1, 4.9)	0.07
J: Nickel sulfate	1.1	(0.7, 1.6)	0.06	1.3	(1.0, 1.6)	0.01	1.2	(0.8, 1.8)	0.07
M: Lactic acid	1.0	(0.8, 1.1)	0.00	0.7	(0.6, 0.9)	0.00	0.8	(0.7, 0.9)	0.00
N: Potassium dichromate	2.3	(1.8, 3.0)	0.02	3.3	(2.2, 4.8)	0.06	5.1	(4.1, 6.3)	0.02

The variance component τ^2 represents the interlaboratory variance for the log-transformed SI, which is obtained by decomposing the total variance into the between variance and within variance by using meta-analysis with a random effect model. Since τ^2 indicates variance, it takes on a value greater than 0, and a larger value indicates greater interlaboratory variation.

chemicals used in the present studies were selected from a wide range of chemicals, and their skin sensitization potentials were determined by the application of the LLNA method.

Further, the precision of the measurements of relevance was low because only 12 chemicals were tested by this assay method; therefore, even a difference in only a single chemical would affect the sensitivity. Since the study demonstrated the strong reliability of the assay, further assessments using other known chemicals should be conducted in other studies. Idehara et al. (in press) report the results of the intralaboratory study.

Another limitation is with regard to the quality of the data. It was extremely difficult to ensure complete compliance with good laboratory practice (GLP) in these studies. However, although the experiments involved in the studies were not conducted in complete accordance with GLP, the format file for data recording of individual experiments was devised at the planning stage of the study, and the data files collected for all the experiments complied with this format. Furthermore, since all the data used for the analyses were based on the database, if required, we can provide the database regarding the ATP content values obtained for the individual animals with the standard protocol that was used here.

Unlike LLNA, LLNA-DA measures the ATP content. It is an extremely simple method for measuring the ATP content during an experiment, and it yields quick results. However, since the ATP content of the LNCs decreases with time, while performing LLNA-DA, it is necessary to comply with the time of operation from lymph node excision to the determination of ATP content. Measuring the LNW as an internal control is recommended. The plot of ATP content against LNW, as in Fig. 2, might aid in roughly checking the compliance.

In conclusion, these 2 studies provide valuable evidence for the reliability of LLNA-DA.

Table 5(a)
Judgment based on SI values greater than 3 for LLNA and the referenced values for LLNA and GPMT/BT in the first study

Chemical	LLNA	GPMT/BT	Laboratory									
			1	2	3	4	5	6	7	8	9	10
A: 2,4-Dinitrochlorobenzene	+	+	+	+	+	+	+	+	+	+	+	+
B: Hexyl cinnamic aldehyde	+	+	+	+	+	+	+	+	+	+	+	+
C: 3-Aminophenol	+	+nonstd	−		−					−		
D: Glutaraldehyde	+		+	+			−					
E: Cobalt chloride	+	+				−		+		+		
F: Isoeugenol	+	+				+	+				+	
G: Formaldehyde	+	+	+	+			−					
H: Dimethyl isophthalate	−	−	−		−				−			
I: Isopropanol	−	−	−	−	−	−	−	−	−	−	−	−
J: Nickel sulfate	−	+				−		+		+		
K: Abietic acid	+	+		+				+	+			
L: Methyl salicylate	−	−			−				−			−

"nonstd" indicates a nonstandard animal.

Table 5(b)
Judgment based on SI values greater than 3 for LLNA and the referenced values for LLNA and GPMT/BT in the second study

Chemical	LLNA	GPMT/BT	Laboratory						
			11	12	13	14	15	16	17
B: Hexyl cinnamic aldehyde	+	+	+	+	+	+	+	+	+
E: Cobalt chloride	+	+	−		−	+			+
J: Nickel sulfate	−	+	−	−		−		−	
M: Lactic acid	−	−	−		−		−	−	
N: Potassium dichromate	+	+	+	+			+		+

Table 6(a)
EC3 and chemical classification in the first study

Chemical	LLNA-DA		LLNA	
	EC3	Classification	EC3	Classification
A: 2,4-Dinitrochlorobenzene	0.06	Extreme	0.04	Extreme
B: Hexyl cinnamic aldehyde	11.1	Weak	8.4	Moderate
C: 3-Aminophenol	–	Negative	3.2	Moderate
D: Glutaraldehyde	0.3	Strong	0.1	Extreme
E: Cobalt chloride	–	(Positive)[a]	<0.5	Strong
F: Isoeugenol	1.9	Moderate	1.8	Moderate
G: Formaldehyde	3.0	Moderate	0.7	Strong
H: Dimethyl isophthalate	–	Negative	–	Negative
I: Isopropanol	–	Negative	–	Negative
J: Nickel sulfate	2.7	Moderate	–	Negative
K: Abiet ic acid	7.9	Moderate	14.7	Weak
L: Methyl salicylate	–	Negative	–	Negative

The EC3 for LLNA-DA is based on the weighted average. The SI values obtained for chemical E (cobalt chloride) with LLNA-DA was greater than 3 for all the doses; however, since the dose–response relationship yielded a v-shaped curve, the EC3 could not be determined.

[a] Although the weighted averages of the SI values were greater than 3 for all the doses, the EC3 and classification were determined because the dose–response relationship exhibited a v-shaped curve.

Table 6(b)
EC3 and chemical classification in the second study

Chemical	LLNA-DA		LLNA	
	EC3	Classification	EC3	Classification
B: Hexyl cinnamic aldehyde	8.1	Moderate	8.4	Moderate
E: Cobalt chloride	3.0	Moderate	<0.5	Strong
J: Nickel sulfate	–	Negative	–	Negative
M: Lactic acid	–	Negative	>25	Negative
N: Potassium dicromate	0.2	Strong	0.1	Strong

Table 7
Sensitivity, specificity, accuracy, positive predictivity, and negative predictivity in the study

	n	Sensitivity	Specificity	Accuracy	Positive predictivity	Negative predictivity
LLNA-DA vs. GPMT/BT	11	87.5% (7/8)	100% (3/3)	90.9% (10/11)	100% (7/7)	75.0% (3/4)
LLNA-DA vs. LLNA	12	87.5% (7/8)	75.0% (3/4)	83.3% (10/12)	88% (7/8)	75.0% (3/4)
LLNA vs. GPMT/BT	11	87.5% (7/8)	100% (3/3)	90.9% (10/11)	100% (7/7)	75.0% (3/4)

For LLNA-DA, the judgment was based on the weighted average of the SI values. For LLNA and GPMT/BT, judgments were based on the referenced data.

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Skin sensitization potency of isoeugenol and its dimers evaluated by a non-radioisotopic modification of the local lymph node assay and guinea pig maximization test

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ABSTRACT: Allergic contact dermatitis is the serious unwanted effect arising from the use of consumer products such as cosmetics. Isoeugenol is a fragrance chemical with spicy, carnation-like scent, is used in many kinds of cosmetics and is a well-known moderate human sensitizer. It was previously reported that the dimerization of eugenol yielded two types of dimer possessing different sensitization potencies. This study reports the differences in skin sensitization potencies for isoeugenol and two types of dimer, β-O-4-dilignol and dehydrodiisoeugenol (DIEG), as evaluated by the non-radioisotopic local lymph node assay (non-RI LLNA) and guinea pig maximization test. In the guinea pig maximization test, isoeugenol, β-O-4-dilignol and DIEG were classified as extreme, weak and moderate sensitizers, respectively. As for the results of non-RI LLNA, the EC3 for isoeugenol, β-O-4-dilignol and DIEG were calculated as 12.7%, >30% and 9.4%, respectively. The two types of isoeugenol dimer showed different sensitizing activities similar to the case for eugenol dimers. A reduction of sensitization potency achieved by dimerization may lead to developing safer cosmetic ingredients. Isoeugenol dimers are not currently used for fragrance chemicals. However, the dimerization of isoeugenol may yield a promising candidate as a cosmetic ingredient with low sensitization risk. The data may also provide useful information for the structure-activity relationship (SAR) in skin sensitization. Copyright © 2007 John Wiley & Sons, Ltd.

KEY WORDS: isoeugenol; isoeugenol-dimer; local lymph node assay; guinea pig maximization test

Introduction

Allergic contact dermatitis is a serious unwanted effect arising from the use of consumer products such as cosmetics. Isoeugenol is a fragrance chemical with a spicy, carnation-like scent, and is used in many kinds of cosmetics (Rastogi *et al.*, 1998; Schnuch *et al.*, 2004). However, isoeugenol is a well-known moderate human sensitizer of human potency class II, and its sensitizing activity was confirmed in mice, guinea pigs and humans (Barratt and Basketter, 1992; Basketter *et al.*, 2005; Hilton *et al.*, 1996). Eugenol is also known as a human sensitizer and is used in various consumer products similar to isoeugenol (Rastogi *et al.*, 1998; Schnuch *et al.*, 2004). There are many publications concerning the sensitization potential of eugenol monomer, however, the sensitization potentials of eugenol dimers which are formed through its auto-oxidation process were not known. Recently the sensitization potential of eugenol dimers was investigated and it was found that the dimerization of eugenol, a moderate human skin sensitizer, yielded two types of dimer possessing different sensitization potencies, namely 2,2′-dihydroxyl-3,3′-dimethoxy-5,5′-diallyl-biphenyl as a weak sensitizer and 4,5′-diallyl-2′-hydroxy-2,3′-dimethoxy phenyl ether as an extreme sensitizer (Takeyoshi *et al.*, 2004).

Eugenol and isoeugenol are both important cosmetic ingredients with whitening and antibacterial effects (Yamazaki *et al.*, 1998; Yamazaki *et al.*, 2000). A reduction of sensitization potency achieved by dimerization may lead to the development of safer cosmetic ingredients. As in the case of eugenol, dimerization of isoeugenol yields two types of isoeugenol dimer. These isoeugenol dimers are not currently used for fragrance chemicals. However, these two isoeugenol dimers still retain their anti-oxidative and anti-bacterial effects similar to the monomer (unpublished data). Accordingly the dimerization of isoeugenol may yield a promising candidate for a cosmetic ingredient with low sensitization risk.

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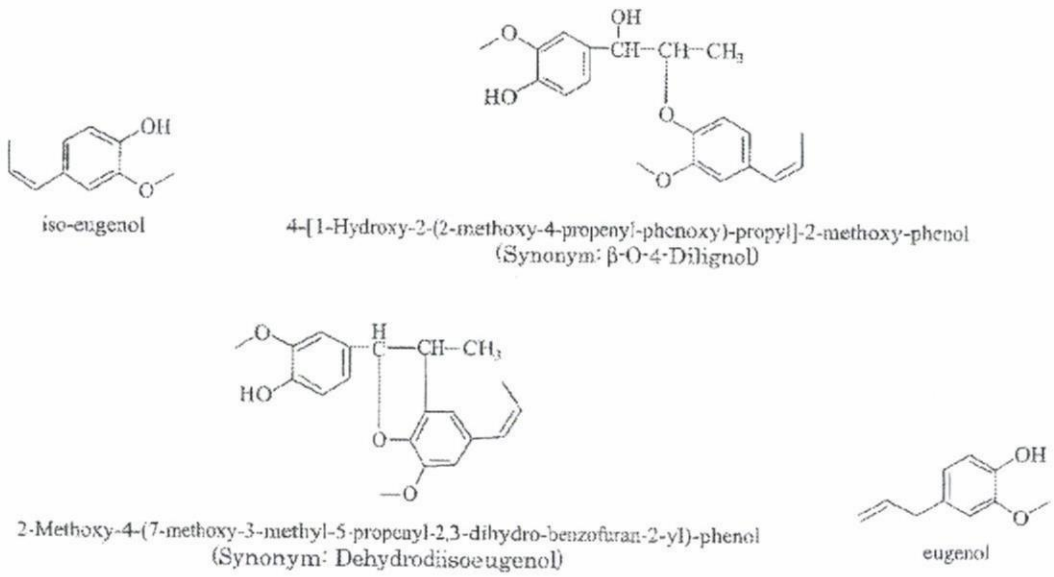

Figure 1. Chemical structures of isoeugenol, isoeugenol dimers and eugenol used in this study

This study employed two types of sensitization assay, i.e. a conventional guinea pig maximization test and non-radioisotopic local lymph node assay (non-RI LLNA) which can provide quantitative outcomes (Takeyoshi *et al.*, 2004) for evaluating the sensitization potency of related compounds. The possibility of a reduction of sensitization potency by dimerization of isoeugenol monomer was investigated.

Materials and Methods

Chemicals and Reagents

Isoeugenol and its dimers, β-O-4-dilignol and dehydrodiisoeugenol, were provided by Kanebo cosmetics, Inc. (Fig. 1, Kanagawa, Japan). Eugenol was obtained from Aldrich Chemicals Inc. All chemicals were confirmed to have >98% purity according to the HPLC analysis and were dissolved in olive oil for the GPMT, or in acetone:olive oil (AOO; 4:1) for the non-RI LLNA. 5-Bromo-2′-deoxyuridine (BrdU; Nacalai Tesque, Kyoto, Japan) was dissolved in physiological saline at a concentration of 10 mg ml^{-1}.

Animals

Female Hartley guinea pigs and CBA/JN strain mice were obtained from SLC Japan Ltd (Shizuoka, Japan) and Charles River Japan Ltd (Kanagawa, Japan), respectively. The animals were housed in animal rooms maintained at a temperature of 22 ± 3 °C, and at a relative humidity of 55 ± 15%. The rooms were ventilated at a frequency of 10–15 cycles h^{-1}, and lighted artificially for 12 h daily. Animals were allowed free access to laboratory diet (RC-4 for guinea pigs and MF for mice, Oriental Yeast Co., Tokyo, Japan) and tap water.

Guinea Pig Maximization Test (GPMT)

Guinea pigs were allocated randomly to three groups (10 animals/group). The test was conducted according to a method described previously (Magnusson and Kligman, 1969). Guinea pigs received a series of intradermal injections of eugenol, or of its dimers, in the shoulder region to induce sensitization. After 6–8 days, sensitization was boosted by a 48 h occluded patch of the same compound placed over the injection sites. Then 14 days later, the animals were challenged on a shaved flank by a 24 h occluded patch containing the same compound. All induction and challenge concentrations were set at 5% (maximum non-irritant concentration) in olive oil for all compounds as a result of preliminary dose finding tests. All compounds elicited apparent irritation at 10% in preliminary tests for intradermal injection and topical application, and then it was decided to use induction and challenge concentrations of 5% for all compounds to compare the sensitization potency of these three compounds. Chemicals were classified by the sensitization rate for each chemical, 0%–8%: weak, 9%–28%: mild, 29%–64%: moderate, 65%–80%: strong, 81%–100%: extreme.

Non-radioisotopic Local Lymph Node Assay (Non-RI LLNA)

The assay was conducted according to a method described previously (Takeyoshi *et al.*, 2001). Mice were

allocated randomly to 12 groups (4 animals/group). A 25 µl volume of test chemicals at concentrations of 3%, 10% or 30% was applied to the dorsum of both ears of the mice, daily for 3 consecutive days. The concentration ranges of each test chemical were decided according to the sensitization potencies classified by the results of GPMT. A single intraperitoneal injection (5 mg per mouse) of BrdU was then given on day 4. On day 5, the draining auricular lymph nodes were removed, weighed and stored at −20 °C until analysis using an enzyme-linked immunosorbent assay (ELISA) to measure the BrdU incorporation. The incorporation of BrdU into lymph node cells was determined using a commercial cell proliferation assay kit (Roche Applied Science Corp., Indianapolis, IN, USA; Cat. No. 1647229). The lymph nodes were crushed, passed through a 70 µm nylon mesh, and the lymph node cells were suspended in 15 ml of physiological saline individually. The cell suspension (100 µl) was added to the wells of a flat-bottom microplate in triplicate. After centrifugation (300 *g*, 10 min), the supernatants were removed. A 200 µl volume of Fix-Denat solution was added to each well, and then the plate was allowed to stand for 30 min at room temperature. After removing the Fix-Denat solution, diluted anti-BrdU antibody solution (100 µl, Boehringer Mannheim Corp.) was added to each well, and after rinsing three times with washing buffer (phosphate-buffered saline), 100 µl of substrate solution containing tetramethylbenzidine (TMB) was added and allowed to react for 15 min at room temperature. Absorbance at 370 nm was determined with a microplate reader (SpectraMAX™, Molecular Devices Inc., Sunnyvale, CA, USA) at a reference wavelength of 492 nm. The absorbance was defined as the BrdU labeling index and the stimulation index (SI) which is the ratio of individual labeling index of each animal against the mean labeling index for the concurrent vehicle control group was calculated. Then the estimated concentration of a chemical required to induce a stimulation index of 3 relative to vehicle-treated controls (EC3 value) was derived by linear interpolation as described previously (Basketter *et al.*, 2000). The EC3 value was calculated by interpolating between two points on the SI axis, one immediately above, and one immediately below, the SI value of 3. The vehicle-treated control value (SI = 1) cannot be used for the latter. Where the data points lying immediately above and below the SI value of 3 have the coordinates (a, b) and (c, d) respectively, then the EC3 value may be calculated using the following equation:

$$EC3 = c + [(3 - d)/(b - d)](a - c)$$

Statistical Analysis

The mean labeling index was calculated for vehicle control group. The stimulation index (SI) defended as a relative labeling index of the test group to the vehicle control value was then calculated. Data were analysed using a one-way analysis of variance (one-way ANOVA). If the one-way ANOVA produced a significant difference, the differences between the concurrent vehicle control group and each of the experimental groups were analysed using the Dunnett's multiple comparison tests (Bruning and Kintz, 1997).

Results

Guinea Pig Maximization Test (GPMT)

In the guinea pig maximization test for isoeugénol and its dimers, the sensitization response rates were as follows: isoeugenol 100%, β-O-4-dilignol 0%, dehydrodiisoeugenol 50% and eugenol 20%. According to convention (Magnusson and Kligman, 1969), isoeugenol was therefore classified as an extreme skin sensitizer; β-O-4-dilignol as a weak skin sensitizer, dehydrodiisoeugenol as a moderate skin sensitizer and eugenol as a mild sensitizer (Table 1).

Non-RI LLNA

The results are shown in Table 2. Dose dependent increases of the stimulation indices were noted in the animals tested with isoeugenol, dehydrodiisoeugenol and eugenol, and stimulation indices for 3%, 10% and 30% isoeugenol were 1.52 ± 0.49, 2.43 ± 0.45 and 6.73 ± 0.88, respectively. Stimulation indices for 3%, 10% and 30% β-O-4-dilignol were 1.02 ± 0.27, 1.19 ± 0.30 and 1.05 ± 0.20, respectively. Stimulation indices for 3%,

Table 1. Results of the guinea pig maximization test for isoeugenol, isoeugenol dimers and eugenol

Chemical name	Sensitization rate (%)	Grade[a]	Classification[a]
Isoeugenol	100	V	Extreme
β-O-4-Dilignol	0	I	Weak
Dehydrodiisoeugenol	50	III	Moderate
Eugenol	20	II	Mild

[a] Classifications were made according to the criterion of Magnusson and Kligmann (1969).