**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±33,682 | 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±6393 | – | | |
| 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±49,202 | 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, $\tau^2$, 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.

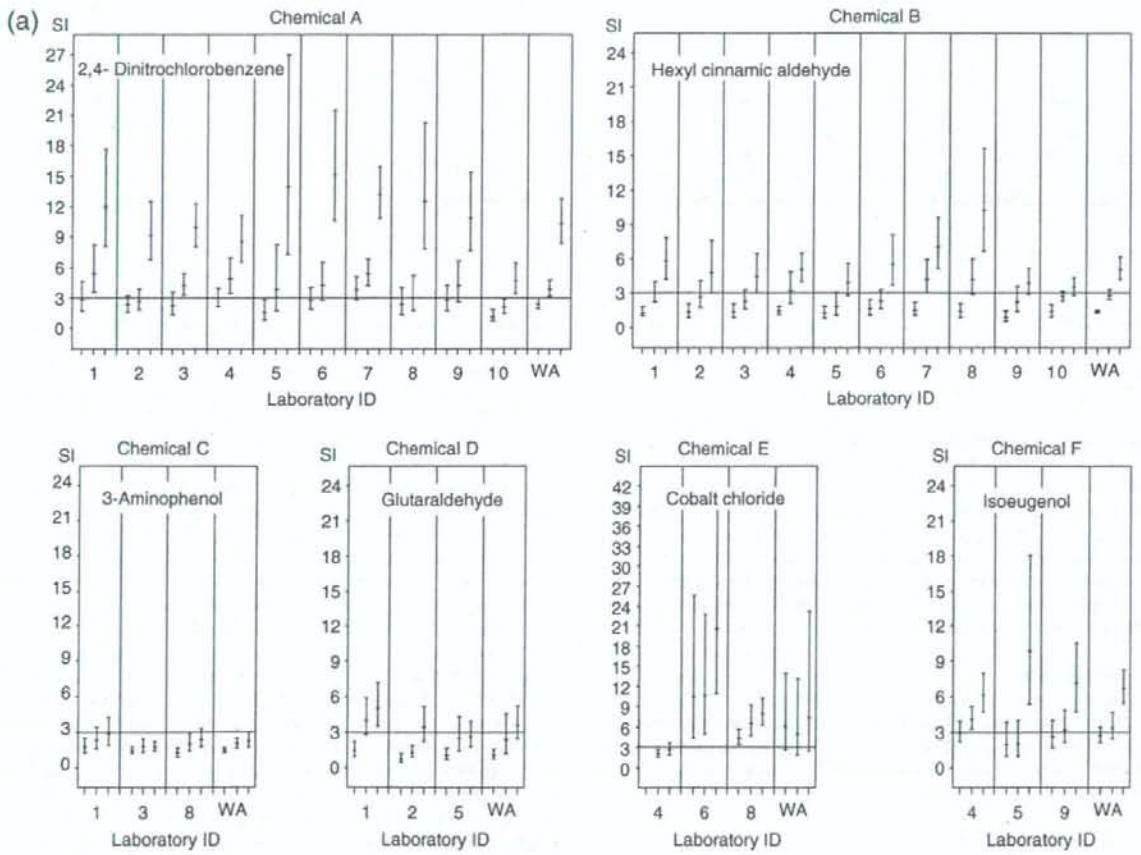


Fig. 1. (a). Dose–response relationships of the SI values with 95% confidence intervals for each chemical analyzed in all the laboratories. "WA" indicates the weighted average of the SI values obtained by meta-analysis using the random effect model in the first study. (b). Dose–response relationships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

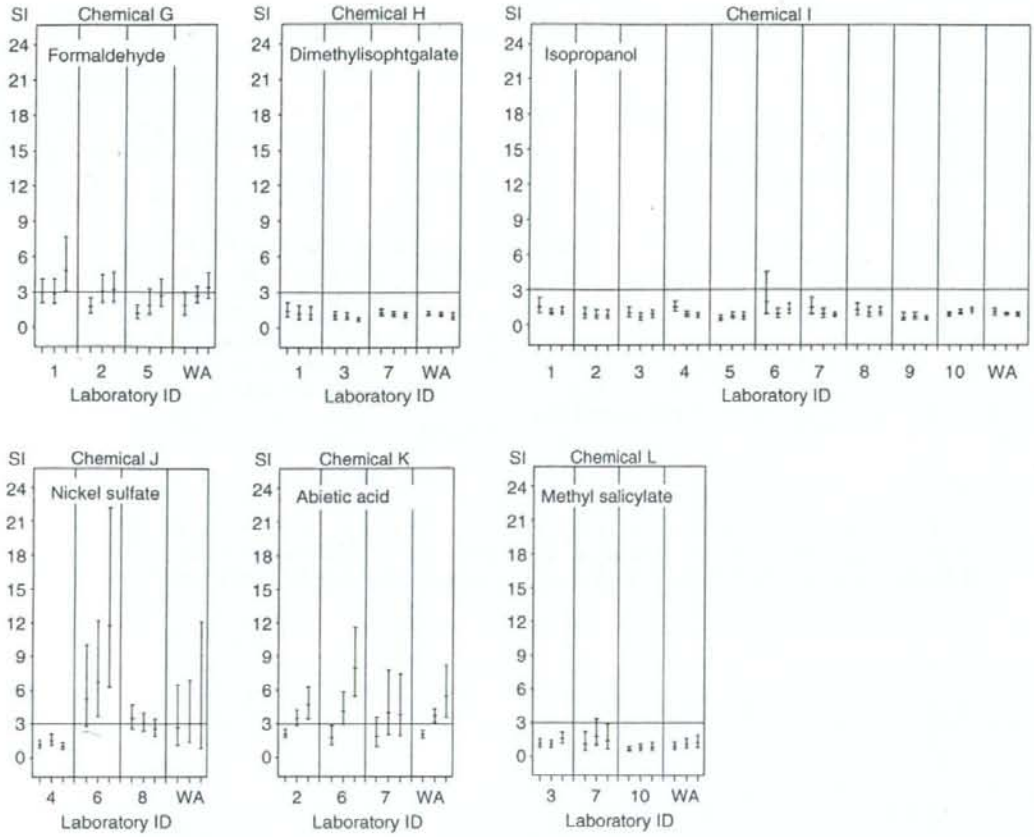


Fig. 1 (*continued*).

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),

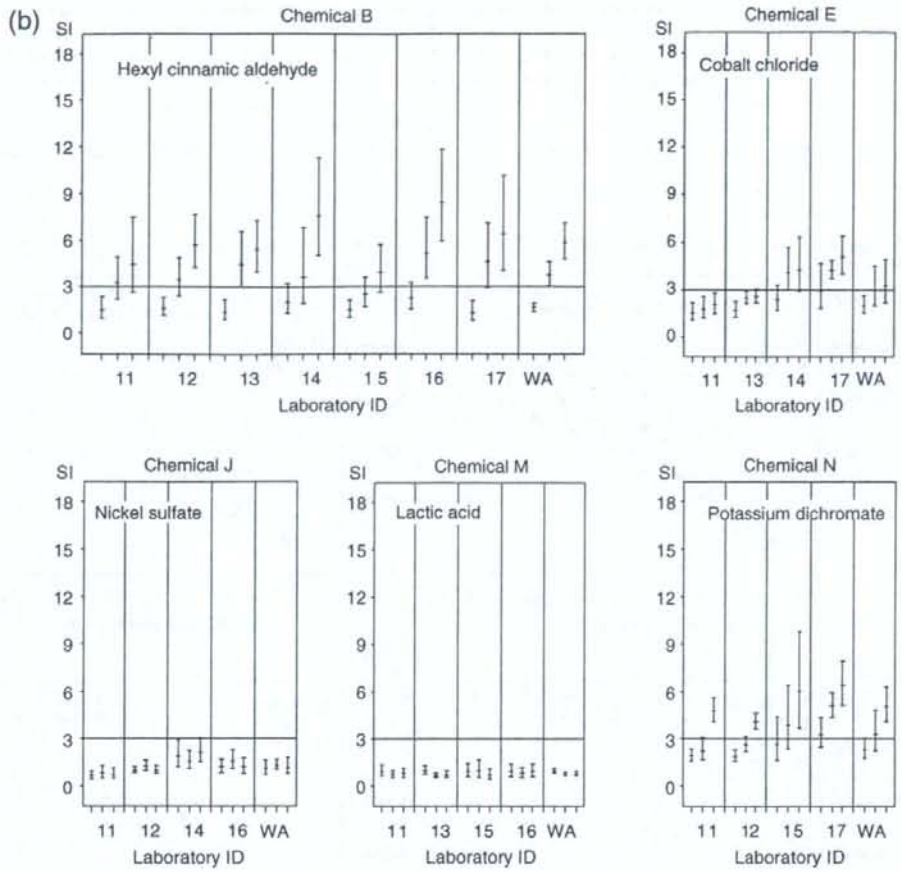


**Fig. 1** (*continued*).

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., $\tau^2$.

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-

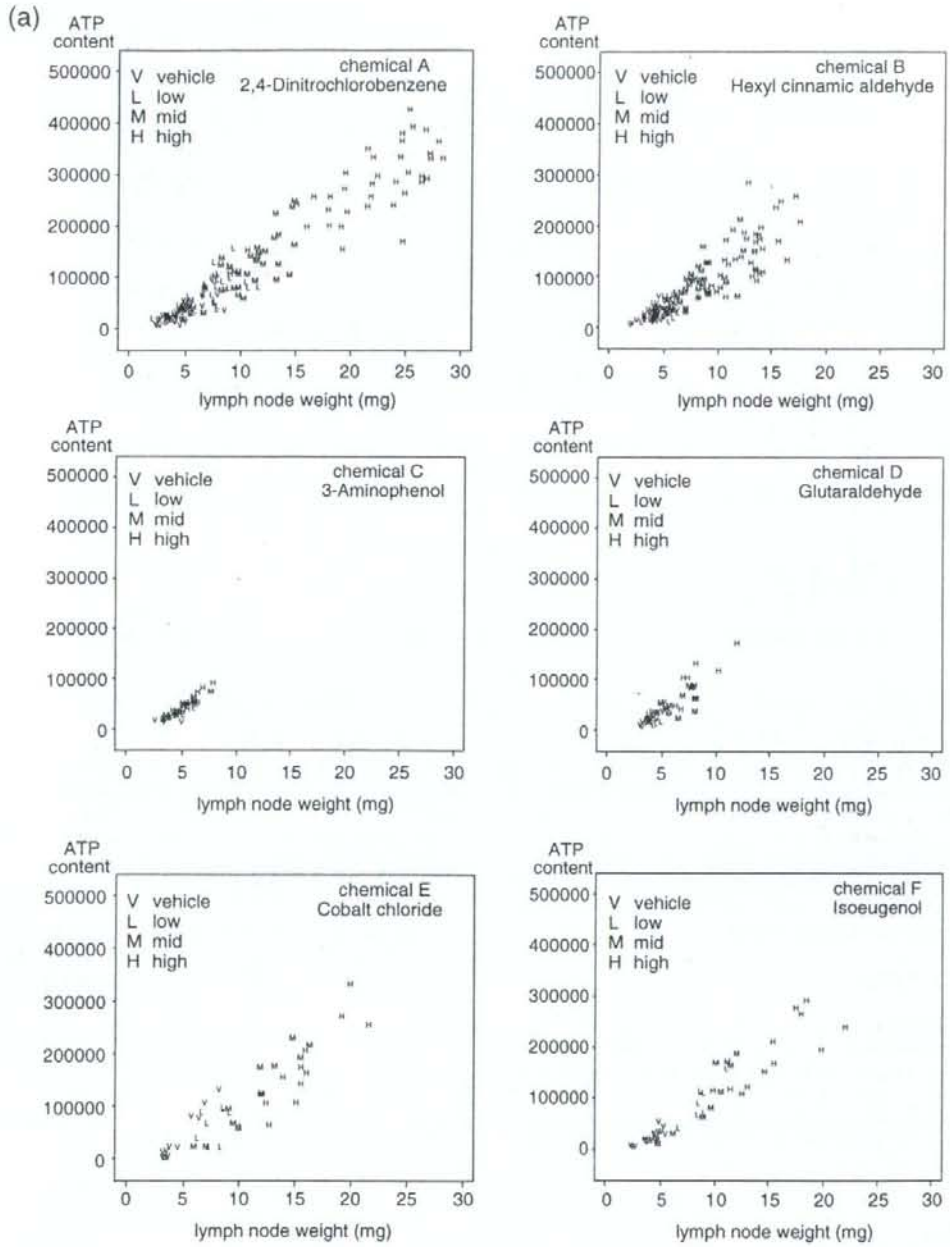


**Fig. 2.** (a). Scatter plots indicating the ATP content with the LNW (mg) recorded for the vehicle (V), low-dose (L), middle-dose (M), and high-dose groups (H) for each chemical in the first study. (b). Scatter plots indicating the ATP content with the LNW (mg) recorded for the vehicle (V), low-dose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

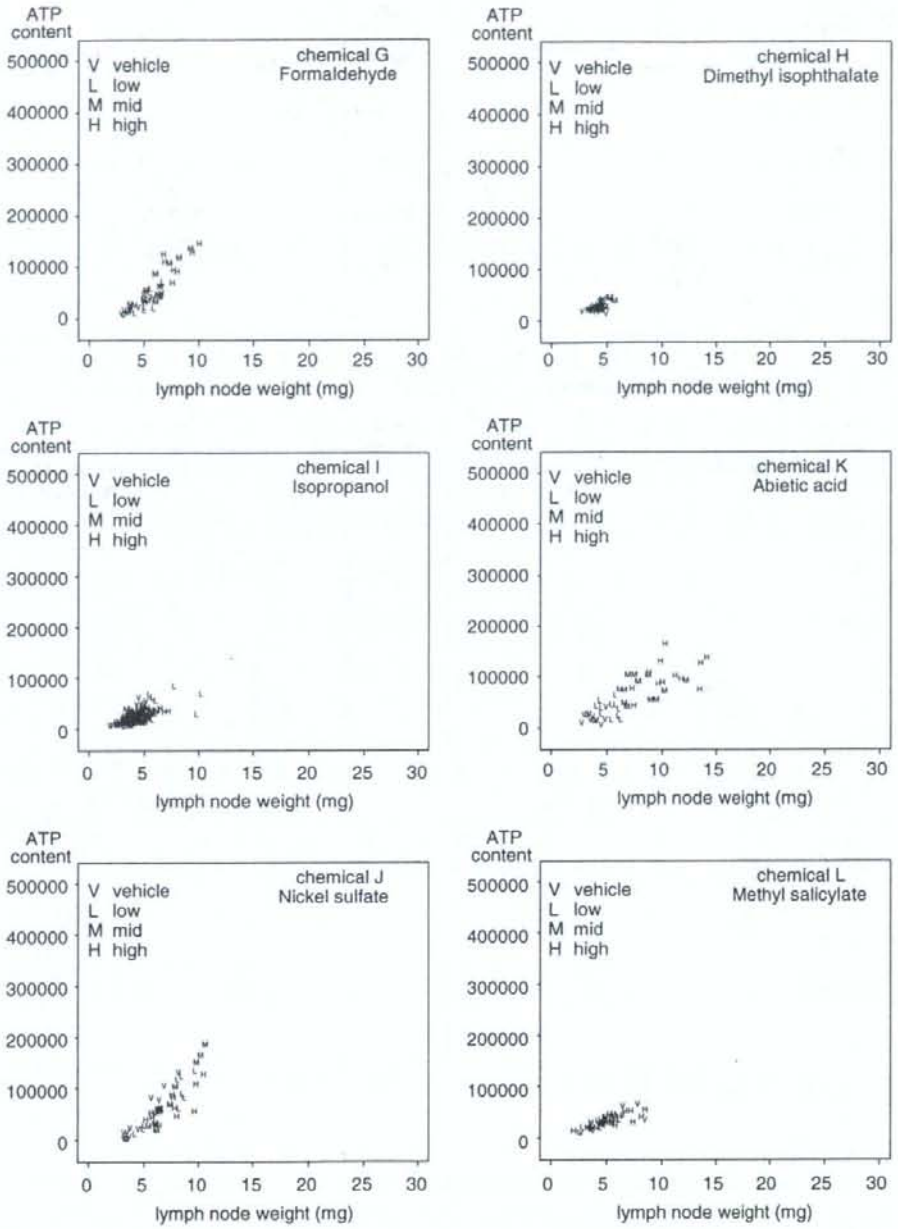


**Fig. 2** (*continued*).

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.

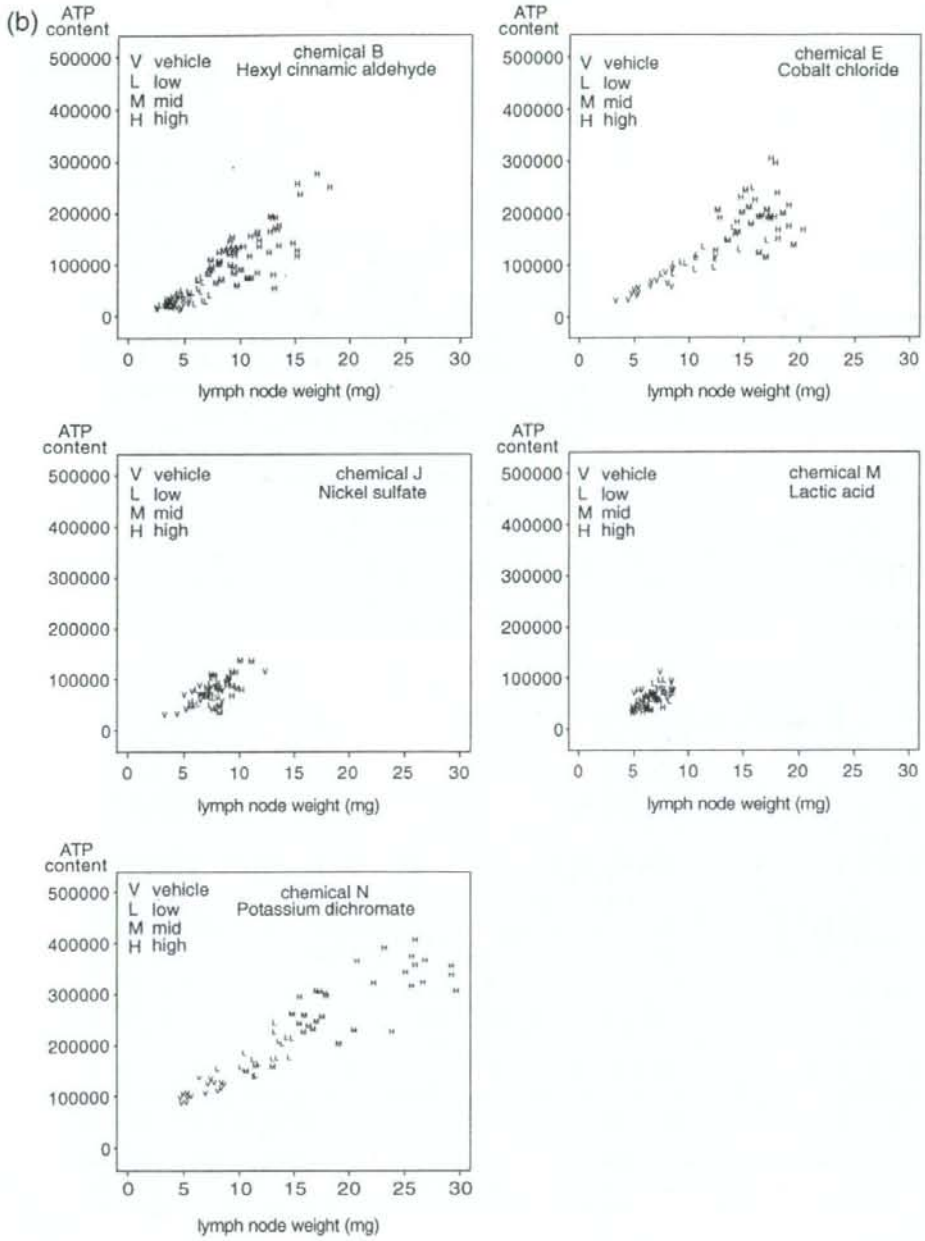


**Fig. 2** (*continued*).

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.

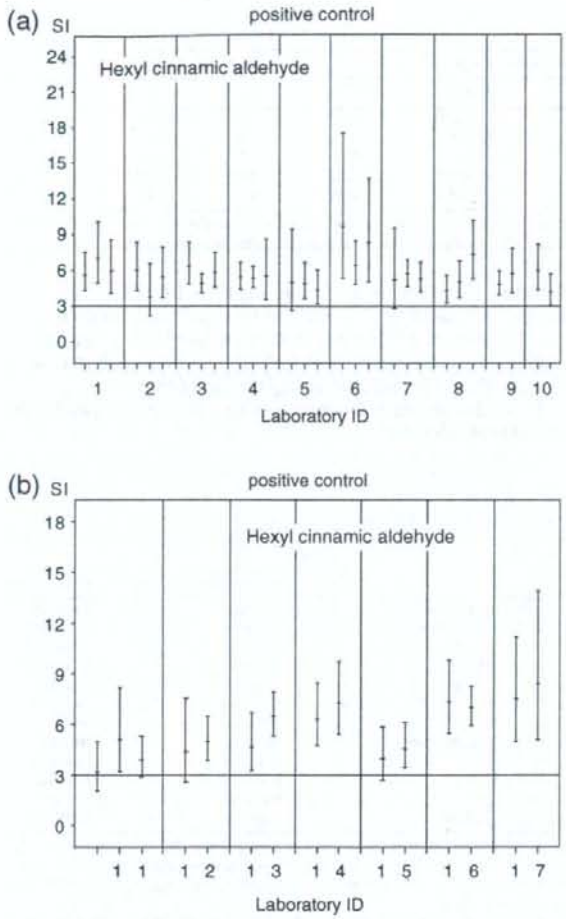


**Fig. 3.** (a). SI values with 95% confidence intervals obtained for the positive control (25% hexyl cinnamic aldehyde) groups in all the laboratories in the first study. (b). SI values with 95% confidence intervals obtained for the positive control (25% hexyl cinnamic aldehyde) groups in all the laboratories 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, $\tau^2$, in the first study

| Chemical | Low-dose group | | | Middle-dose group | | | High-dose group | | |
|---|---|---|---|---|---|---|---|---|---|
| | SI | 95%CI | $\tau^2$ | SI | 95%CI | $\tau^2$ | SI | 95% CI | $\tau^2$ |
| 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 $\tau^2$ 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 $\tau^2$ 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,($\tau^2$, in the second study

| Chemical | Low-dose group | | | Middle-dose group | | | High-dose group | | |
|---|---|---|---|---|---|---|---|---|---|
| | SI | 95%CI | $\tau^2$ | SI | 95%CI | $\tau^2$ | SI | 95% CI | $\tau^2$ |
| 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 $\tau^2$ 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 $\tau^2$ 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.

## Acknowledgment

This study was partially supported by a grant from the Ministry of Health, Labour and Welfare (principal investigator: Yasuo Ohno) and Japanese Society for Alternatives to Animal Experiments.

We would like to express our appreciation to the editors and the reviewers of the original manuscript, who made insightful suggestions that improved the clarity of the presentation.

## References

Basketter, D. A., Blaikie, L., Dearman, R. J., Kimber, I., Ryan, C. A., Gerberick, G. F., et al. (2000). Use of the local lymph node assay for the estimation of relative contact allergenic potency. *Contact Dermatitis, 42*, 344–348.

Basketter, D. A., Casati, S., Gerberick, G. F., Griem, P., Philips, B., & Worth, A. (2005). Skin sensitisation. *Alternatives To Laboratory Animals, 33*(Supplement 1), 83–103.

Basketter, D. A., Evans, P., Fielder, R. J., Gerberick, G. F., Dearman, R. J., & Kimber, I. (2002). Local lymph node assay: Validation, conduct and use in practice. *Food and Chemical Toxicology, 40*, 593–598.

Basketter, D. A., Gerberick, G. F., & Kimber, I. (1998). Strategies for identifying false positive responses in predictive skin sensitization tests. *Food and Chemical Toxicology, 36*, 327–333.

Basketter, D. A., Gerberick, G. F., Kimber, I., & Loveless, S. E. (1996). The local lymph node assay: A viable alternative to currently accepted skin sensitization tests. *Food and Chemical Toxicology, 34*, 985–997.

Basketter, D. A., Lea, L. J., Cooper, K. J., Ryan, C. A., Gerberick, G. F., Dearman, R. J., et al. (1999). Identification of metal allergens in the local lymph node assay. *American Journal of Contact Dermatitis, 10*, 207–212.

Basketter, D. A., Lea, L. J., Dickens, A., Briggs, D., Pate, I., Dearman, R. J., et al. (1999). A comparison of statistical approaches to the derivation of EC3 values from local lymph node assay dose responses. *Journal of Applied Toxicology, 19*, 261–266.

Basketter, D. A., & Scholes, E. W. (1992). Comparison of the local lymph node assay with the guinea-pig maximization test for the detection of a range of contact allergens. *Food and Chemical Toxicology, 30*, 65–69.

Basketter, D. A., Scholes, E. W., Kimber, I., Botham, P. A., Hilton, J., Miller, K., et al. (1991). Interlaboratory evaluation of the local lymph node assay with 25 chemicals and comparison with guinea pig test data. *Toxicology Methods, 1*, 30–43.

Dean, J. H., Twerdok, L. E., Tice, R. R., Sailstad, D. M., Hattan, D. G., & Stokes, W. S. (2001). ICCVAM evaluation of the murine local lymph node assay. II. Conclusions and recommendations of an independent scientific peer review panel. *Regulatory Toxicology and Pharmacology, 34*, 258–273.

Dearman, R. J., Hilton, J., Basketter, D. A., & Kimber, I. (1999). Cytokine endpoints for the local lymph node assay: Consideration of interferon-g and interleukin 12. *Journal of Applied Toxicology, 19*, 149–155.

Durand, G., De Burlet, G., Virat, M., & Nauman, B. D. (2003). Use of the local lymph node assay in the evaluation of the sensitizing potential of pharmaceutical process intermediates. *Contact Dermatitis, 49*, 148–154.

Ehling, G., Hecht, M., Heusener, A., Huesler, J., Gamer, A. O., van Loveren, H., et al. (2005). An European inter-laboratory validation of alternative endpoints of the murine local lymph node assay: First round. *Toxicology, 212*, 60–68.

Ehling, G., Hecht, M., Heusener, A., Huesler, J., Gamer, A. O., van Loveren, H., et al. (2005). An European inter-laboratory validation of alternative endpoints of the murine local lymph node assay: 2nd round. *Toxicology, 212*, 69–79.

FDA (2002). *Guidance for industry—immunotoxicology evaluation of investigational new drugs.*

Gerberick, G. F., Ryan, C. A., Kern, P. S., Dearman, R. J., Kimber, I., Patlewicz, G. Y., et al. (2004). A chemical dataset for evaluation of alternative approaches to skin-sensitization testing. *Contact Dermatitis, 50*, 274–288.

Gerberick, G. F., Ryan, C. A., Kimber, I., Dearman, R. J., & Basketter, D. A. (2000). Local lymph node assay: Validation assessment for regulatory purposes. *Toxicology, 11*, 3–18.

Haneke, K. E., Tice, R. R., Carson, B. L., Margolin, B. H., & Stokes, W. S. (2001). ICCVAM evaluation of the murine local lymph node assay. III. Data analyses completed by the national toxicology program interagency center for the evaluation of alternative toxicological methods. *Regulatory Toxicology and Pharmacology, 34*, 274–286.

Hastings, K. L. (2001). Pre-clinical methods for detecting the hypersensitivity potential of pharmaceuticals: Regulatory considerations. *Toxicology, 158*, 85–89.

Hatao, M., Hariya, T., Katsumura, Y., & Kato, S. (1995). A modification of the local lymph node assay for contact allergenicity screening: Measurement of interleukin-2 as an alternative to radioisotope-dependent proliferation assay. *Toxicology, 98*, 15–22.

Idehara, K., Yamagishi, G., Yamashita, K., & Ito, M. (2008). Characterization and evaluation of a modified local lymph node assay using ATP content as a non-radio isotopic endpoint. *Journal of Pharmacological and Toxicological Methods, 58*, 1–10 (this issue).

Kimber, I. (2001). The local lymph node assay and potential application to the identification of drug allergens. *Toxicology, 158*, 59–64.

Kimber, I., Hilton, J., Botham, P. A., Basketter, D. A., Scholes, E. W., Miller, K., et al. (1991). The murine local lymph node assay: Results of an inter-laboratory trial. *Toxicology Letters, 55*, 203–213.

Kimber, I., Hilton, J., Dearman, R. J., Gerberick, G. F., Ryan, C. A., Basketter, D. A., et al. (1998). Assessment of the skin sensitization potential of topical medicaments using the local lymph node assay: An interlaboratory evaluation. *Journal of Toxicology and Environmental Health, Part A, 53*, 563–579.

Kimber, I., Hilton, J., Dearman, R. J., Gerberick, G. F., Ryan, C. A., Basketter, D. A., et al. (1995). An international evaluation of the murine local lymph node assay and comparison of modified procedures. *Toxicology, 103*, 63–73.

Lee, J. K., Park, J. H., Park, S. H., Kim, H. S., & Oh, H. Y. (2002). A nonradioisotopic endpoint for measurement of lymph node cell proliferation in a murine allergic contact dermatitis model, using bromodeoxyuridine immunohistochemistry. *Journal of Pharmacological and Toxicological Methods, 48*, 53–61.

Loveless, S. E., Ladics, G. S., Gerberick, G. F., Ryan, C. A., Basketter, D. A., Scholes, E. W., et al. (1996). Further evaluation of the local lymph node assay in the final phase of an international collaborative trial. *Toxicology, 108*, 141–152.

Normand, S. L. T. (1999). Meta-analysis: Formulating, evaluating, combining, and reporting. *Statistics in Medicine, 18*, 321–359.

OECD (1992). Organization for Economic Co-operation and Development—OECD guidelines for testing of chemicals. *No. 406: Skin sensitization.*

OECD (2002). Organization for Economic Co-operation and Development—OECD guidelines for testing of chemicals. *No. 429: Skin sensitization: Local lymph node assay.*

OECD (2005). Organization for Economic Co-operation and Development—OECD series on testing and assessment. *No. 34: Guidance document on the validation and international acceptance of new or updated test methods for hazard assessment.*

Omori, T., Ikarashi, Y., Kanazawa, Y., Idehara, K., Kojima, H., Sozu, T., et al.(2008). Validation studies on an alternative endpoint for the local lymph node assay (LLNA-DA): Importance of study management. *Alternatives to Animal Testing and Experimentation*, 14, Special Issue, 429–432.

Omori, T., & Sozu, T. (2007). Variance of the stimulation index for the local lymph node assay. *Alternatives to Animal Testing and Experimentation, 12*, 321–359.

Sailstad, D. M., Hattan, D., Hill, R. N., & Stokes, W. S. (2001). ICCVAM evaluation of the murine local lymph node assay. I. The ICCVAM review process. *Regulatory Toxicology and Pharmacology, 34*, 249–257.

Scholes, E. W., Basketter, D. A., Sarll, A. E., Kimber, I., Evans, C. D., Miller, K., et al. (1992). The local lymph node assay: Results of a final inter-laboratory validation under field conditions. *Journal of Applied Toxicology, 12*, 217–222.

Takeyoshi, M., Yamasaki, K., Yakabe, Y., Takatsuki, M., & Kimber, I. (2001). Development of non-radio isotopic endpoint of murine local lymph node assay based on 5-bromo-2'-deoxyuridine (BrdU) incorporation. *Toxicology Letters, 119*, 203–208.

Wright, Z. M., Basketter, D. A., Blaikie, L., Cooper, K. J., Warbrick, E. V., Dearman, R. J., et al. (2001). Vehicle effects on skin sensitizing potency of four chemicals: Assessment using the local lymph node assay. *International Journal of Cosmetic Science, 23*, 75–83.

Yamashita, K., Idehara, K., Fukuda, N., Yamagishi, G., & Kawada, N. (2005). Development of a modified local lymph node assay using ATP measurement as an endpoint. *Alternatives to Animal Testing and Experimentation, 11*, 136–144.

ORIGINAL ARTICLE

# An *in Vitro* Evaluation Method to Test Ocular Irritation using a Human Corneal Epithelium Model

**Shoko Arai[1], Naoki Yamamoto[2], Masakazu Katoh[3] and Hajime Kojima[1]**

*[1]Japanese Center for the Validation of Alternative Methods(JaCVAM), National Institute of Health Sciences, Tokyo, Japan; [2]Fujita Health University, Aichi, Japan; [3]Japan Tissue Engineering Co., Ltd., Aichi, Japan*

**Abstract**

Recently, safety evaluation tests that do not involve animal experiments have been prosperously developing. However, the optimal evaluation materials and methods for assessing ocular irritancy have not been well investigated. In this study, we determined the optimal evaluation method for testing ocular irritation using a human cultured corneal epithelium model (corneal model). In order to assess adequate treatment conditions for the corneal model, we used cetylpyridinium chloride (CPC), which has been recognized as an irritant chemical by the Draize eye test. The irritancy elicited by multiple concentrations of CPC was evaluated by a cytotoxicity assay under nine treatment conditions and compared to the Draize score. The treatment conditions that included a 5-second exposure period followed by a 24-hour post-incubation period (hereafter called protocol "5-sec+24-h") showed a significant correlation between cytotoxicity and the Draize score. Furthermore, the dose-dependent cytotoxicity of six test chemicals was assessed by protocol "5-sec+24-h" and found to correlate with the Draize eye test results.

***Key words:*** *ocular irritation, corneal model, Draize eye test, cytotoxicity assay, dose-dependency*

## Introduction

A classical and reliable method to evaluate ocular irritation is an eye mucosal irritation assay (Draize eye test) devised by J. H. Draize (Draize *et al.*, 1944). According to guideline 405 of the OECD (Organization for Economic Cooperation and Development), the Draize eye test should be performed by observing the cornea, conjunctiva, and iris for three days after applying 100 μL of liquid test chemicals with an eye dropper or 100 mg of powder test chemicals into one eye of a rabbit (OECD, 2002). The damage level of each eye section is used to assign a Draize score (110 total points). Since the development of the Draize eye test, five irritancy groups (none, mild, moderate, severe or very severe) were classified in accordance with the Draize score and used to assess the irritancy levels of various chemicals (Kay *et al.*, 1962). However, these animal experiments often involve the suffering of laboratory animals and are against with 3R's concept (Reduction, Refinement and Replacement). Therefore, an *in vitro* evaluation method for assessing ocular irritancy is greatly needed.

To date, a number of *in vitro* assays have been developed as alternative methods to the Draize eye test. Above all, it was shown that the bovine corneal opacity and permeability (BCOP) test and isolated chicken eye (ICE) test lead the correlative results with the Draize score when severe irritants or corrosive test chemicals were adopted by the NICEATM (The NTP Interagency Center for the

Evaluation of Alternative Toxicological Methods) and the ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM, 2006a, 2006b, Merrill *et al.*, 2006)). Furthermore, the international validation studies were performed successfully and these two tests were accepted by the ESAC, scientific advisory committee of ECVAM (European Centre for the Validation of Alternative Methods) (ECVAM, 2007). However, these tests are not considered complete *in vitro* assays because animal-derived tissues are still used. Recently, a rabbit corneal epithelial cell line was reported as a useful alternative when evaluated by a cytotoxicity assay (Ohno *et al.*, 1999). Although this assay is easy to perform and moderate in price, there are some disadvantages; insoluble and powder chemicals and stock solutions are often not testable because they must be dissolved in culture medium.

To correct these problems, we focused on a human cultured corneal epithelium model. In this study, we determined the optimal treatment conditions to assess cytotoxicity using a human cultured corneal epithelium model (corneal model) and demonstrated that dose-dependent ocular irritancy could be detected by our evaluation method.

## Materials and Methods

### *1. Human cultured corneal epithelium model (the corneal model)*

The corneal model was supplied as a kit by Japan Tissue Engineering Co., Ltd. Corneal epidermal cells were prepared from the normal human corneal epidermis which was provided by the Human & Animal Bridging Research Organization (Tokyo, Japan). The corneal epidermal cells were proliferated by co-culturing them with mouse 3T3-J2 cells as a feeder. The corneal model consisting of the proliferated corneal epidermal cells was cultivated on a cell culture inert (surface 0.3cm$^2$, BD Biosciences, CA, USA) in an optimized medium containing 5% fetal bovine serum, and kept at the air-liquid interface for 4 days. The corneal model was embedded in an agar gel containing nutrient solution and shipped at around 18℃. In order to eliminate the stress that follows shipment, the corneal models were incubated in sterile 24-well culture plates containing pre-warmed assay medium for 2 hours before starting the assay. During the experiments, the corneal models were maintained under the standard incubation conditions at 37°C with 5% $CO_2$ in humidified atmosphere. It is recommended that the corneal models be preserved at 4°C and used within 24 hours of arrival.

### *2. Test chemicals*

The chemicals used in this study were previously evaluated by the Draize eye test (Ohno *et al.*, 1999). The irritancy level of each test chemical was identified using at least two or more concentrations. The test chemicals were purchased from the following suppliers: cetylpyridinium chloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan: Wako), benzyl alcohol (Acros Organics, Morris Plains, NJ, USA), calcium thioglycolate (Wako), m-phenylenediamine (Wako), sodium salicylate (Wako), lactic acid (Wako), and ethanol (Wako). Distilled water was used as a solvent or negative control.

### *3. Reagents and antibodies*

MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was purchased from Sigma-Aldrich, Milwaukee, USA, Dulbecco's phosphate-buffered saline (D-PBS) from Gibco Laboratories, Grand Island, NY, USA and isopropanol from Wako. Antibodies used for immunohistochemical staining were purchased from the following suppliers: anti-human cytokeratin-3 mouse monoclonal antibody (BIOMOL Research Laboratories Inc., Plymouth Meeting, PA, USA) and anti-human mucin mouse monoclonal antibody (MUC-1, Novus Biologicals Inc., Littleton, Co., USA). These antibodies were diluted with D-PBS containing 1% bovine serum albumin (Takara Shuzo Co., Ltd., Kyoto, Japan) at 1:100 or 1:50, respectively.

### *4. Immunohistochemical staining*

The corneal models were fixed with two different fixative solutions overnight at room temperature. The bottom and upper layers were treated simultaneously with 10% formalin neutral buffer solution (Mildform, Wako) and an original fixative solution of SUPER FIX (KURABO Industries, LTD., Osaka, Japan), respectively. After fixation, the corneal models were paraffin-embedded as previously described (Nishi *et al.*, 2007; Yamamoto *et al.*, 2008). The human corneal tissue was provided by an unbiased volunteer, and 3-micron paraffin sections of corneal tissue were prepared using a sliding microtome (REM-700, Yamato Kohki Industrial Co., Ltd., Saitama, Japan) and mounted on silanized glass slides (DAKO Japan Co., Ltd., Kyoto, Japan). After the dewaxing process, the sections were blocked with a non-specific staining

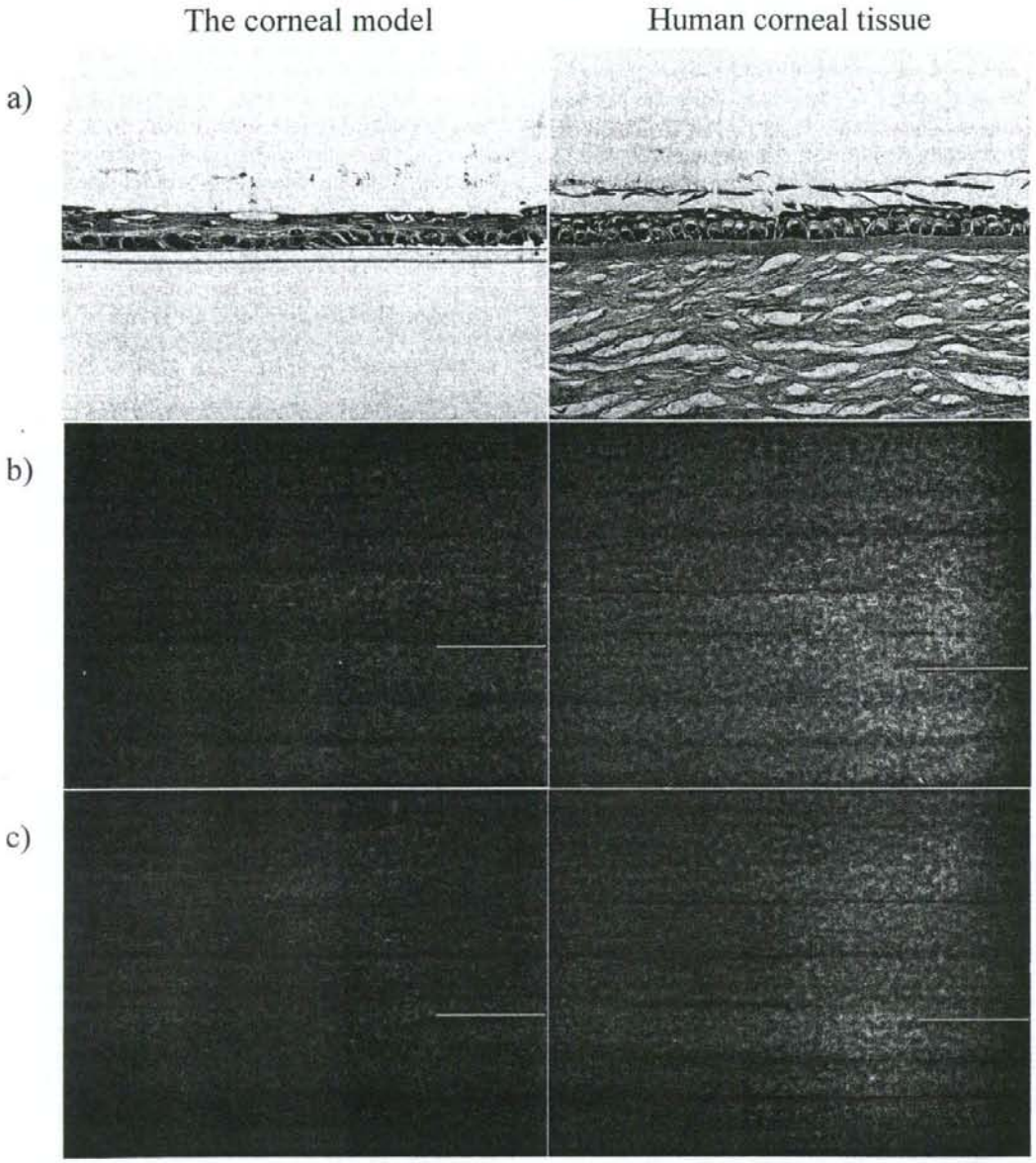


**Fig. 1** Comparison between the corneal model and human corneal tissue
a) H&E staining. b, c) Immunohistochemical staining by human cytokeratin-3 or MUC-1 antibodies.
The scale bars represent 100 μm.

regent (DAKO Japan Co., Ltd.) and then probed with primary antibody for one hour at 37°C. After washing with D-PBS, immunofluorescence staining was performed using Alexa Fluor 488 conjugated anti-mouse IgG antibody (Invitrogen Corporation, Carlsbad, CA, USA). Each sample was counterstained with DAPI (VECTASHIELD, Vector Laboratories Inc., Burlingame, CA, USA), and images were acquired on an Olympus BX41 inverted microscope with a fluorescent attachment (Olympus Japan Co., Ltd., Tokyo, Japan) and Photonic Science CCD camera (DP50, Olympus Japan) controlled by DP controller software (Olympus Japan).

### *5. Treatment with test chemicals*

Thirty μL of liquid or 10 mg of powder test chemicals were applied directly onto the corneal models. Liquid test chemicals were dropped by a micropipette and powder test chemicals were sprinkled with a microspatula onto the corneal models. At the end of each exposure period, the corneal models were washed gently with D-PBS and incubated in fresh assay medium during the post-incubation period.

### *6. Cytotoxicity assay*

Cytotoxicity was quantified using an MTT assay. The corneal models were placed in a 24-well plate containing 500 μL of MTT working solution (0.5 mg/mL in assay medium) and incubated for 3 hours at 37°C protected from light. At the end of the incubation period, a 6-mm diameter punch out was obtained by biopsy trepan (Kai Industries Co., Ltd., Gifu, Japan) together with membrane filters. To extract the intracellular formazan crystals, samples were incubated with 300 μL of isopropanol overnight at 4°C.

Two hundred μL of each extraction solution was dispensed into 96-well microplates (MICROTEST Flat Bottom, BD Falcon, San Jose, CA, USA) for colorimetric analysis. The optical density was read at 570 nm using a spectrophotometer (SoftMax Pro, Molecular Devices, Sunnyvale, CA, USA), and cell viability was calculated.

## Results

### *1. The corneal model expresses human corneal epithelium-specific molecular markers*

It is well established that human corneal epithelium is constructed of four to six cell layers and expresses several specific molecular markers (Inatomi *et al.*, 1995; Chen *et al.*, 2004; Vascotto *et al.*, 2006). Our histopathological findings suggested that the corneal model has a layered structure similar to human corneal epithelium (Fig. 1-a). The corneal model expresses cytokeratin-3, a specific molecular marker of human corneal epithelium (Fig. 1-b) and MUC-1, a mucosal glycoprotein secreted by human corneal epithelium cells that helps keep the cornea surface humid (Fig. 1-c). Intriguingly, the histological localization of cytokeratin-3 and MUC-1 in the corneal model was consistent with human corneal epithelium.

### *2. The optimal treatment condition is protocol "5-sec+24-h"*

To determine the optimal treatment conditions for the corneal model, cetylpyridinium chloride (CPC) was used as a test chemical in the cytotoxicity assay. As shown in Table 1, the corneal damage elicited by multiple concentrations of CPC was previously scored using the Draize eye test (Ohno *et al.*, 1999). A high score indicates severe irritancy, and the maximum score was defined as 80. Based on the Draize eye data, CPC is considered to induce dose-dependent corneal damage. In order to identify adequate treatment conditions for the corneal model, the dose-dependent cytotoxicity elicited by CPC was examined under nine different

**Table 1** Test chemicals and Draize scores

| Chemical Name | Chemical Number | Conc. | Draize Score (cornea) |
|---|---|---|---|
| Cetylpyridinium chloride | 1- 0.1 | 0.1% | 0 |
| | 1- 1 | 1% | 21.7 |
| | 1- 10 | 10% | 80 |
| Benzyl alcohol | 2- 1 | 1% | 0 |
| | 2- 10 | 10% | 23 |
| | 2- 100 | as is | 31 |
| Calcium thioglycolate | 3- 10 | 10% | 10 |
| | 3- 100 | as is | 60 |
| Sodium salicylate | 4- 10 | 10% | 0 |
| | 4- 100 | as is | 66.7 |
| m-Phenylene diamine | 5- 10 | 10% | 1.7 |
| | 5- 100 | as is | 66.7 |
| Lactic acid | 6- 10 | 10% | 5 |
| | 6- 100 | as is | 80 |
| Ethanol | 7- 10 | 10% | 0 |
| | 7- 100 | as is | 26.7 |

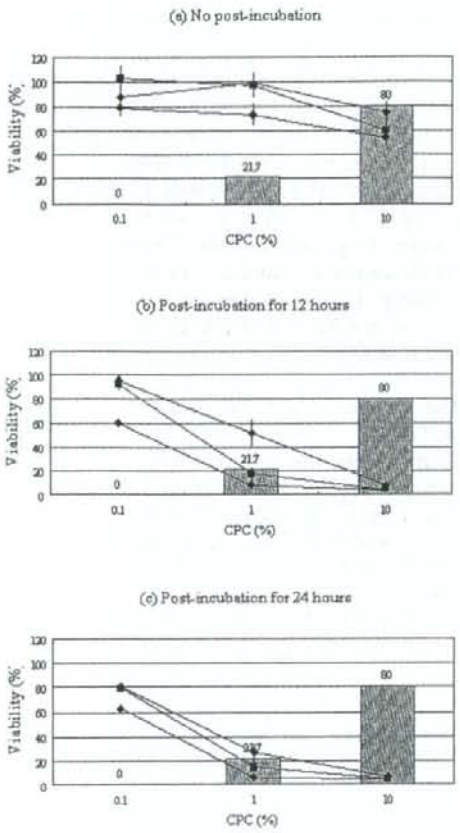


**Fig. 2** Cytotoxicity assay results with nine treatment conditions
a) Post-incubation for an hour. b) Post-incubation for 12 hours. c) Post-incubation for 24 hours.
The exposure period was fixed as follows: ♦=5-sec, ■=30-sec, ●=120-sec. The results are presented as the means ± SD for three samples from two independent experiments per chemical. The bar graph indicates the Draize score.

treatment conditions (Fig. 2). The exposure period was fixed at 5, 30 or 120 seconds, followed by a post-incubation period that was fixed at 1, 12 or 24 hours. The dose-dependent cytotoxicity was detectable under conditions where the post- incubation period was fixed at 12 or 24 hours. In particular, protocol "5-sec+12-h", protocol "30-sec+12-h" and protocol "5-sec+24-h" gave a significant correlation between cytotoxicity and the Draize score. After accounting for the general-purpose of experimental procedures, it was concluded that protocol "5-sec+24-h" provided optimal treatment conditions for the corneal model.

### *3. Dose-dependent cytotoxicity is evaluable for six test chemicals by protocol "5-sec+24-h"*

To clarify the utility of the above-mentioned test protocol, six additional test chemicals (Table 1) were examined for dose-dependent

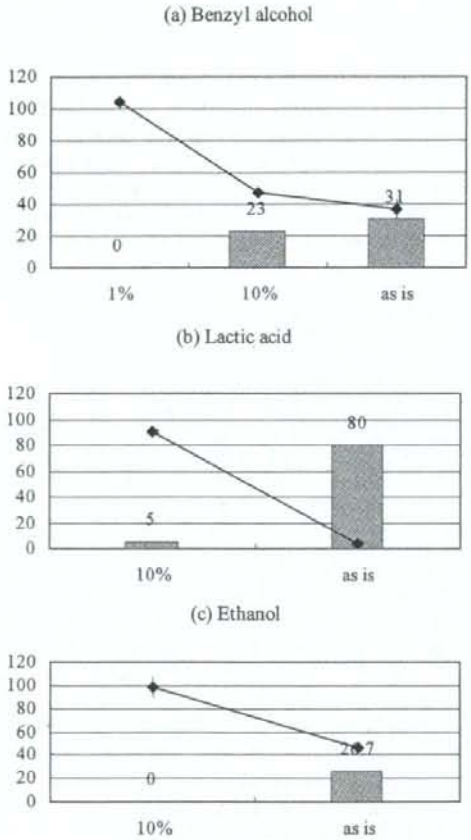


**Fig. 3** Evaluation of dose-dependent ocular cytotoxicity for three liquid test chemicals
Test chemicals were applied directly to the corneal models. The results are presented as the means ± SD for three samples from two independent experiments per chemical. The bar graph indicates the Draize score.

cytotoxicity. Dose-dependent cytotoxicity was detected for benzyl alcohol, lactic acid, and ethanol (Fig. 3-a, b and c). However, calcium thioglycolate, sodium salicylate, and m- phenylenediamine failed to show cytotoxicity when tested in bulk (Fig. 4-a, b and c). The difference between these successful and unsuccessful test chemicals was whether their bulk physical state was a liquid or powder. Therefore, a treatment procedure for powder test chemicals was reconsidered. In the last analysis, one of our trials revealed that a suitable treatment procedure for powder test chemicals was to cover the corneal models with filter paper after applying the powder test chemicals. During this analysis, it was recommended that a 6-mm diameter filter paper (2.5-mm thick) be incubated at 37°C in D-PBS for 3 hours before use. Just after applying the powder test chemicals, the filter paper was mounted on the surface of the corneal models for 5 seconds, a time equivalent to the exposure period for liquid test chemicals. At the end of the mounting period, the filter paper was removed with forceps after the addition of D-PBS. Using this process, the filter paper floated to the liquid level and the corneal models were protected from extrinsic damages such as friction or pressure induced by direct removal with forceps. As a result, no cytotoxicity was detected after filter paper application alone (data not shown). Following this revised procedure, the cytotoxicity of powder test chemicals could also be detected (Fig. 4-d, e and f).

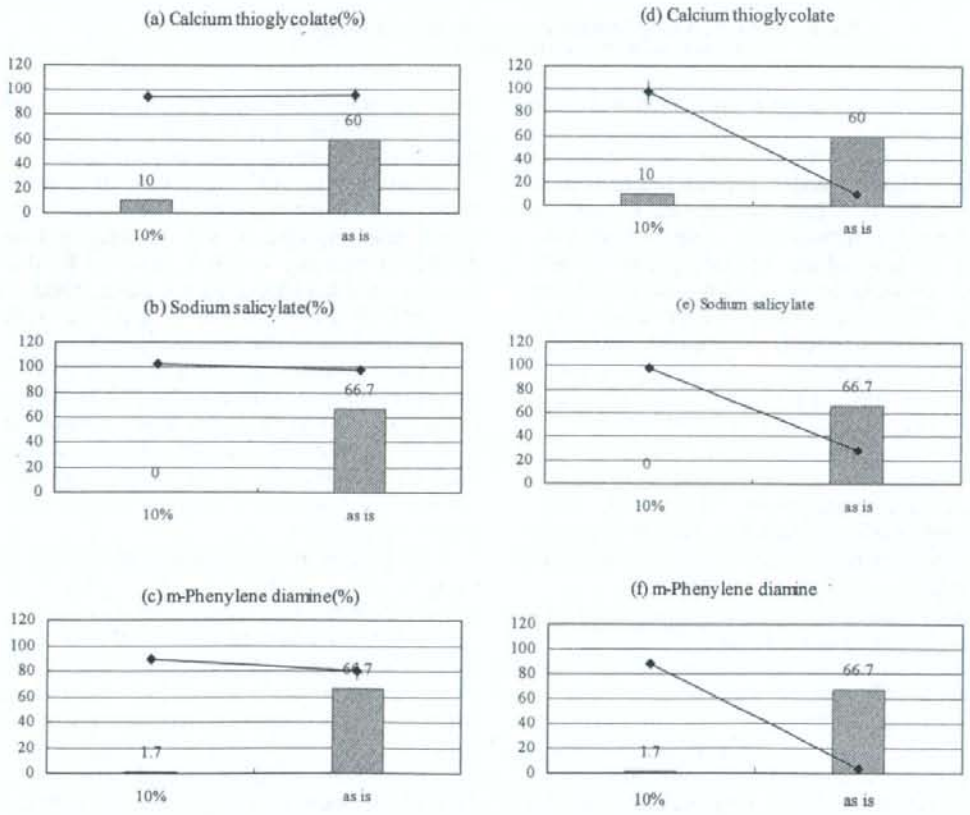


**Fig. 4** Evaluation of dose-dependent ocular cytotoxicity for three powder test chemicals
a, b, c) Test chemicals were applied directly to the corneal models. d, e, f) Filter paper was mounted on the corneal models after applying the powder test chemicals. The results are presented as the means ± SD for three samples from two independent experiments per chemical. The bar graph indicates the Draize score.