Introduction
Exposure to sensitizing chemicals may cause serious health problems, such as glutaraldehyde-induced allergic reactions among healthcare workers and metal allergy associated with ear piercing (Katsumura et al., 1996). The Guinea Pig Maximization Test (GPMT) has long been used to assess moderate sensitizers. To address this issue, we propose a modified LLNA in which mice are exposed to the test material four times, instead of three times, and adenosine triphosphate (ATP) content is used as a measure of proliferation of lymph node cells (LNCs). The chemicals tested were 2,4- dinitrochlorobenzene (DNCB), eugenol, and α-hexyl cinnamic aldehyde (HCA), which are classified as strong-to-moderate sensitizers in the LLNA. Methyl salicylate (MS) was used as an example of a non-sensitizer. Using an SI of 3 as a cut-off, as recommended in the standard LLNA, DNCB, eugenol and HCA were classified as positive, while MS was classified as negative using the ATP-based non-RI method. Furthermore, there were no marked differences in SI levels for 10% eugenol and 10% HCA (a moderate sensitizer) between the non-RI method and the standard LLNA. Thus, it is reasonable to conclude that the non-RI method proposed in the present study could be a valid alternative to the standard LLNA.

Key words: local lymph node assay, non-RI method, stimulation index, adenosine triphosphate (ATP), eugenol
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(IL-2) produced by stimulated lymphocytes (Suda et al., 2002). Assessment of both the CD4/CD8 ratio in lymph nodes and IL-2 production can be useful for differentiation of irritant and allergic contact sensitizers (Hariya et al., 1999). A recent study using BrdU incorporation instead of isotope incorporation by LNCs as a measure of LNC proliferation was, in principle, conducted according to the standard LLNA protocol (Takeyoshi et al., 2001). However, when used to assess certain types of allergens, including eugenol and HCA, non-RI methods tend to yield lower SIs than those obtained with the standard LLNA, and may be subject to false-negative results. Therefore, the purpose of the present study is to develop a non-RI method with greater sensitivity and accuracy than existing non-RI methods.

Materials and Methods

Animals

Female CBA/JN mice (Charles River Japan Inc., Kanagawa) of 7 to 11 weeks old were used in the experiments, and each test group or control group contained three mice. Temperature and humidity inside the animal holding rooms were maintained at 23±2°C and 55±15%, respectively, and each room was ventilated at a frequency of 8-10 cycles per hour. All procedures were performed according to the guidelines of the Japanese Association for Laboratory Animal Science.

Chemicals and Instruments

The chemical agents tested in the study included (i) 2,4-dinitrochlorobenzene (DNCB) (Wako Pure Chemical Industries, Ltd.), which was used as an example of a strong sensitizer, (ii) eugenol (Wako Pure Chemical Industries, Ltd.) and -hexyl cinnamic aldehyde (HCA) (Wako Pure Chemical Industries, Ltd.), which were included as moderate sensitizers, and (iii) methyl salicylate (MS) (Wako Pure Chemical Industries, Ltd.), which was used as an example of a non-sensitizer. Sodium lauryl sulfate (SLS) was used to pretreat the test materials prior to application of the agents (or vehicle) to mice. Suitable vehicles and concentrations for testing were determined based on previous reports (Hariya et al., 1999; Van Och et al., 2000). Eugenol, DNCB, HCA and MS were dissolved in 4:1 v/v acetone and olive oil (Yoshida Pharmaceutical Co. Ltd.) (AOO) solvent vehicle, and SLS was dissolved in distilled water. Phosphate-buffered saline (PBS pH7.2) was purchased from Invitron Corporation.

An AViaLightTM HS (Cambrex BioScience Walkersville Inc., formerly BioWhittaker Inc.) cytotoxicity detection kit was used to measure adenosine triphosphate (ATP) content in the diluted LNC suspension and to assess ATP-dependent enzyme reactions. Bioluminescent measurement of ATP was performed using a LumitesterTM C-100 (Kikkoman Corporation, Japan). A cell strainer (Falcon 35-2340, Becton Dickinson) was used to homogenize the LNCs.

Experiment 1

Determination of an Optimal Dosing Schedule

Two experiments were carried out to determine an optimal dosing schedule. In the first experiment, 0.25% DNCB, 10% eugenol and AOO vehicle were tested, with three groups of mice (Group 1-3) assigned to each test material, giving a total of nine groups of mice. The mice were treated by topical application of the agent to the dorsum of both ears once per day over three consecutive days. The draining auricular lymph nodes were collected from the mice four days (Group 1), five days (Group 2), and seven days (Group 3) post-application to calculate the lymph node weight (LNW) in individual mice. The specific dosing schedule is shown in Figure 1(A). In the second
experiment, four groups of mice were treated with 10% eugenol, according to the schedule specified in Figure 1(B), to evaluate whether application of the agent four times instead of three times is useful for increasing lymph node proliferation in the proposed non-RI method. Mice were divided into two groups: those that did and those that did not receive the fourth treatment six or seven days after the initiation of treatment. Nodes were collected on either the seventh or the eighth day, after euthanasia of mice by an overdose of ether. In both experiments, mice were pretreated an hour prior to ear challenge with application of a small amount of 1% SLS to each ear, which is known to help improve the detection sensitivity of the test (Van Och et al., 2000).

**Experiment 2**

**Measurement of ATP**

To calculate the LNW for each mouse, both auricular lymph nodes were collected after euthanasia of mice by an overdose of ether. LNCs were filtered through a cell strainer and suspended in 500 μl of PBS. An aliquot of this suspension was diluted 1:100 in PBS, and the ATP content in the diluted LNC suspension was measured using bioluminescent technology (ViaLight™ HS, Lumitester™ C-100). A mixture of 90μl of diluted cell suspension and 90μl of ATP extract was prepared and left to stand for five minutes at room temperature (23±2°C). ATP content (or intensity of light emitted) was measured with a luminometer (Lumitester™ C-100) immediately after addition of 20μl of luminescent reagent to the above mixture. Lymph nodes were excised from mice treated with 0.25% DNCB, 10% eugenol and AOO vehicle, respectively, according to protocol No. 3 in Figure 1(A).

**Experiment 3**

**Assessment of Intermediate Precision**

Six independent experiments were performed, each of which used a group of three mice treated with 10% eugenol, according to protocol No. 3 in Figure 1(A). LNC proliferation in the control group was measured based on the LNW and the ATP content, and expressed as the stimulation index (SI).

**Experiment 4**

**Assessment of Sensitivity and Specificity**

HCA and MS were both tested at concentrations of 5%, 10% and 25% to assess the sensitivity and specificity of the proposed non-RI method. HCA is recommended as a positive control for sensitization in the LLNA (Basketter et al., 2002), whereas MS is known to be a non-sensitizer (Kimber et al., 1995). Mice were treated with these agents at four times, and the LNC proliferation was measured based on the LNW and the ATP content, and expressed as the SI.

**Statistical analysis**

Results are expressed as means ± SD. An F-test indicated that the LNW results were normally distributed, and therefore the Student t test was used to compare data. P < 0.05 was considered to be significant.

**Results**

**Determination of the Experimental Schedule**

The LNW-based SI, or the ratio of the LNW of the test group to that of the vehicle control group, was calculated for each concentration of DNCB and eugenol (Table 1, Figure 2). The LNW increased with time in all the DNCB-challenged groups. The eugenol-challenged groups that received three applications of the agent ceased to show a statistically significant increase in LNW after four or five days.

<table>
<thead>
<tr>
<th>Group</th>
<th>Day</th>
<th>Lymph node weight (g)</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>4</td>
<td>0.0039</td>
<td>0.0006</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.0047</td>
<td>0.0011</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.0045</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>DNCB 0.25%</td>
<td>4</td>
<td>0.0072</td>
<td>0.0013</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.0114</td>
<td>0.0022</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.0180</td>
<td>0.0011</td>
<td></td>
</tr>
<tr>
<td>Eugenol 10%</td>
<td>4</td>
<td>0.0047</td>
<td>0.0002</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.0036</td>
<td>0.0017</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.0075</td>
<td>0.0012</td>
<td></td>
</tr>
</tbody>
</table>

Results represent mean values and standard deviation (SD) in three mice. The stimulation index (SI) was calculated by the mean value obtained in each experimental schedule group of agent by that of the vehicle control group.
Whereas those groups that received a fourth application of the agent on the sixth day of the experiment continued to show a statistically significant increase in LNW. The LNW-based SIs for DNCB and eugenol on the seventh day of the experiment were 3.97 and 1.75, respectively (Table 1). The LNW was larger for mice that received the fourth application of eugenol on the sixth day, compared to mice that only received three applications of the agent. However, there was no statistically significant difference in the LNW between groups that received the fourth application of eugenol on the sixth day and those that received it on the seventh day (Figure 3).

**Assessment of ATP Content**

The ATP content in the diluted LNC suspension obtained from auricular lymph nodes of mice exposed to DNCB or eugenol was measured to calculate the ATP-based SIs. The ratio of the ATP...
content in the LNC suspension obtained from test groups to that in the LNC suspension obtained from control groups was also calculated. The ATP-based SI was 11.36 for DNCB-challenged mice and 4.41 for eugenol-challenged mice, whereas the LNW-based SI was 4.21 for DNCB-challenged mice and 2.03 for eugenol-challenged mice (Table 2, Figure 4).

Assessment of Intermediate Precision
The results obtained from six independent experiments conducted in mice treated with eugenol are summarized in Figure 5. The ATP-based SIs for each experimental group were 3.39, 3.55, 3.72, 5.22, 4.25 and 3.60, respectively, and the LNW-based SIs were 2.26, 2.17, 2.56, 3.07, 2.16 and 2.54, respectively. The mean ATP-based SI, standard deviation (SD), and coefficient of variation (CV) were calculated as 4.0, 0.7 and 17.3%, respectively.

Assessment of Sensitivity and Specificity
While a concentration-dependent increase was observed in the SIs for the HCA-exposed groups, no increase was observed in the SIs for the MS-exposed groups (Figures 6 and 7, respectively). The ATP-based SIs for the groups exposed to HCA were 1.29, 2.58, and 6.47, respectively, and these values for groups exposed to MS were 0.72, 0.96 and 1.20, respectively.

Table 2
The SI values of lymph node weight (LNW) and ATP content (ATP) with DNCB and eugenol were used.

<table>
<thead>
<tr>
<th>Group</th>
<th>Animal</th>
<th>LNW(g)</th>
<th>ATP as bioluminescent (RLU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>mean</td>
<td>SD</td>
</tr>
<tr>
<td>control</td>
<td>1</td>
<td>0.0044</td>
<td>0.0011</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.0042</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.0062</td>
<td></td>
</tr>
<tr>
<td>DNCB 0.25%</td>
<td>1</td>
<td>0.0218</td>
<td>0.0035</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.0236</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.0169</td>
<td></td>
</tr>
<tr>
<td>Eugenol 10%</td>
<td>1</td>
<td>0.0106</td>
<td>0.0013</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.0110</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.0085</td>
<td></td>
</tr>
</tbody>
</table>

Results represent mean values and standard deviations (SD) of LNW or ATP content in three mice. The SI values were calculated by the mean value obtained in agent by that of the vehicle control group.

![Fig. 4 SI values of lymph node weight (LNW) and ATP content (ATP). The data are expressed as mean SI ± SD of LNW and the mean SI ± SD of ATP for each agent.](image-url)
Discussion

The LLNA has been developed as a screening test to detect the effects of skin sensitizers, and the OECD guideline (No. 429) for conducting the LLNA was adopted in 2002 after thorough evaluation and reviews of the test. Because the standard LLNA uses a radioisotope to measure lymphocyte proliferation, it requires special facilities for the safe handling of radioactive substances (Takeyoshi et al., 2001). Several attempts have been made to develop a modified LLNA that uses a non-RI method, including (i) measurement of cytokine production by LNCs (Derman et al., 1999), (ii) flow cytometric analysis of LNCs (Hatao et al., 1995), and (iii) assessment of BrdU incorporation into LNCs as a measure of lymphocyte proliferation (Takeyoshi et al., 2001). However, when used to assess moderate sensitizers (such as eugenol and HCA), non-RI methods tend to yield lower SIs than those obtained with the standard LLNA (Hatao et al., 1995, Takeyoshi et al., 2001). Thus, using several dosing schedules, we examined whether LNW-based SIs and ATP-based SIs are

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Fig. 5 SI values of ATP content (ATP) and lymph node weight (LNW). The local lymph nodes were excised on day 7 according to the Fig. 1(A) No. 3 procedure, and the ATP and LNW were measured. The data showed the mean SI ± SD of ATP and the mean SI ± SD of LNW in the six independent experiments.

Fig. 6 Proliferation of local lymph node in response to α-hexyl cinnamic aldehyde (HCA). The lymph nodes of the mice that were excised on day 8, which were exposed on day 1, 2 and day 3, day 7. The data are expressed as mean SI ± SD of LNW and the mean SI ± SD of ATP for each concentration of HCA.
valid measures of LNC proliferation, with the ultimate goal of developing a non-RI method that can yield SIs comparable to those obtained with the standard LLNA, without the need to use higher sample concentrations.

The results obtained from an experiment conducted in mice treated with 10% eugenol indicated that applying the agent four times was effective for improving the LNW-based SI (the standard LLNA protocol recommends that mice are exposed to the agent three times). However, the LNW-based SI for eugenol was only 1.75. The SI for eugenol is usually higher in the standard LLNA; in fact, SIs of 2.4 and 3.2 have been reported for eugenol tested in two different laboratories, according to the standard LLNA protocol (Loveless et al., 1996). To develop a better measure of LNC proliferation, the ATP content in the LNC suspension was measured. ATP bioluminescence has long been used as a measure of cell proliferation and cytotoxicity in studies of bacteria and cultured cells (Crouch et al., 1993; Dexter et al., 2003). Assuming that LNC proliferation is mostly due to lymphocyte proliferation, and that ATP content per cell is similar across LNCs, ATP content can be a useful measure of LNC proliferation. In the present study, the ATP-based SI was 4.41 for 10% eugenol and 11.36 for 0.25% DNCB, whereas the LNW-based SI was 2.03 and 4.21 respectively (Figure 4), suggesting that the ATP-based SI provides a more sensitive index of LNC proliferation than the LNW-based SI. The minimum concentration of eugenol required to yield a SI of 3 or higher in the standard LLNA is 10% (Basketter et al., 1996); thus, it is justifiable to conclude that, as far as eugenol is concerned, the sensitivity of the proposed non-RI method is comparable to that of the standard LLNA. On the other hand, while 0.25% DNCB induced an ATP-based SI of 11.36 in the present study, the standard LLNA yields an SI of 14.2 for DNCB tested at the same concentration (Kimber et al., 1989). To determine if this difference may affect the sensitivity of the proposed non-RI method, further studies are required using lower concentrations of DNCB.

Six independent experiments in mice treated with 10% eugenol were performed to assess the intermediate precision of the results obtained from the proposed non-RI method. The mean ATP-based SI, SD and CV were calculated as 4.0, 0.7 and 17.3%, respectively. In a previous study conducted in mice treated with HCA (Haneke et al., 2001), six experiments were carried out to assess the repeatability of the effective concentrations for elicitation of a 3-fold increase in [3H]thymidine incorporation (EC3). The EC3 values obtained from the six experiments were 7.9, 6.9, 9.6, 8.7, 4.0 and 9.2% and the mean EC3, SD and CV were calculated as 7.7, 2.1 and 26.7%, respectively.
We also performed an experiment in mice treated with HCA and MS to assess the sensitivity and specificity of the proposed non-RI method. HCA is known to induce stable responses over time in the LLNA, and has been tested in several modified LLNAs using non-RI methods, while MS is known to be a non-sensitizer. The two substances were tested at concentrations of 5%, 10% and 25%, and the ATP-based SIs for each concentration of HCA were 1.29, 2.58 and 6.47, respectively. A similar test of HCA at the same concentrations using the standard LLNA protocol yielded SIs of 1.1, 2.5 and 10.4, respectively (Gerberick et al., 1999). On the other hand, a modified LLNA in which HCA was tested at concentrations of 12.5%, 25% and 50%, using BrdU incorporation as a measure of LNC proliferation, yielded SIs of 1.58, 2.40 and 3.63, respectively (Takeyoshi et al., 2003), and a second modified LLNA protocol using IL-2 production as a measure of LNC proliferation yielded a SI of approximately 6 for an HCA concentration of 50% (Hariya et al., 1999). Thus, it can be concluded that the sensitivity of the proposed non-RI method is comparable, if not superior, to that of existing non-RI methods, with respect to assessment of the sensitizing potential of HCA. The ATP-based SIs for MS, a non-sensitizer, were all lower than 3, which is the usual cut-off value in the standard LLNA. Based on this criterion, the results obtained from the non-RI method proposed herein indicate that HCA is a sensitizer and MS is a non-sensitizer.

To summarize, the modified LLNA proposed in the present study has the potential to serve as an alternative to the standard LLNA. The modified approach is characterized by exposure of mice to the test agent four times, instead of three times, and by the use of an SI based on ATP content as a measure of LNC proliferation. Regarding the chemicals tested in the present study, the ATP-based SIs were close to those calculated based on $[^{3}H]$thymidine incorporation in the LLNA. Furthermore, the proposed non-RI method was proven to be as effective as the standard LLNA in detecting moderate sensitizers, suggesting that it is less likely to yield false negative results, compared to existing non-RI methods. To demonstrate the overall validity of the proposed method further experiments will be required, using other chemicals that have been tested in the standard LLNA.

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