Influence of application vehicle on skin sensitization to methylchloroisothiazolinone/methylisothiazolinone: an analysis using the local lymph node assay

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The murine local lymph node assay (LLNA) is a method for the identification of skin sensitizing chemicals in which activity is measured as a function of proliferative responses induced in draining lymph nodes following topical exposure of mice to the test material. More recently, the LLNA has also been used for the determination of relative skin sensitizing potency based upon the mathematical derivation of an EC3 value, this being the estimated concentration of test chemical necessary to provoke a 3-fold increase in lymph-node cell-proliferative activity compared with concurrent vehicle-treated controls. Here we describe the use of the LLNA to determine the influence of vehicle on the skin-sensitizing potency of methylchloroisothiazolinone/methylisothiazolinone (MCI/MI), the active ingredient of preservatives such as Kathon® CG. To this end, LLNA responses to MCI/MI were measured using the vehicles 4:1 acetone:olive oil (AOO), methyl ethyl ketone, dimethylsulfoxide, dimethylformamide, propylene glycol (PG) and acetone. It was found that the vehicle in which MCI/MI was applied had a substantial impact on activity, with derived EC3 values varying from 0.0049% with AOO to 0.048% with PG. With the other vehicles, EC3 values ranged from 0.0068 to 0.0076%. The skin sensitizing potency of MCI/MI as judged from LLNA responses is consistent with what is known of the requirements for sensitization in humans. It is proposed that the LLNA not only provides a method for determination of relative skin sensitizing potency, but is also appropriate for assessing the influence of vehicle matrix on sensitizing activity.

Key words: isothiazolinones; local lymph node assay; skin sensitization; vehicles; acetone:olive oil; methyl ethyl ketone; dimethylsulfoxide; dimethylformamide; propylene glycol; acetone.

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ingredient of the biocide Kathon®CG and a confirmed contact allergen (11), have been measured following formulation of the chemical in each of the following vehicles: acetone:olive oil (4:1), methyl ethyl ketone, dimethylsulfoxide, dimethylformamide, propylene glycol and acetone.

Materials and Methods

Mice
Young adult (8–12 weeks old) CBA/Ca strain female mice (Harlan Seralab, Oxon, UK) were used throughout these studies. Animals were housed 4 per cage on flushing metal racks. Food (SDS PCD pelleted diet; Special Diets Services Ltd., UK) and water were available ad libitum.

Chemicals
Methylchloroisothiazolinone/methylisothiazolinone (MCI/MI; 1.15% 5-chloro-2-methyl-4-isothiazolin-3-one and 0.35% 2-methyl-4-isothiazolin-3-one) was obtained from Chesham Chemicals Limited (Harrow, UK) as a formulation of Kathon®CG. Working concentrations of MCI/MI were derived from this formulation which was diluted in the appropriate vehicle. Immediately prior to dosing, solutions (0.00075%–0.075% MCI/MI %v/v) were prepared freshly in the vehicle of choice. The vehicles selected for these investigations comprised acetone:olive oil (AOO; v/v 4:1), methyl ethyl ketone (MEK), dimethylsulfoxide (DMSO), dimethylformamide (DMF), propylene glycol (PG) and acetone. MCI/MI was completely miscible with all these vehicles at all concentrations tested.

Local lymph node assay
The murine local lymph node assay was conducted as described previously (1), except that 5, rather than 3, separate dose groups were used. An independent experiment was performed using each of the vehicles listed above. Groups of mice (n=4) were exposed topically on the dorsum of both ears to 25 μl of various concentrations of MCI/MI, or to the same volume of vehicle alone, daily for 3 consecutive days. 5 days after the initiation of exposure, all mice were injected intravenously via the tail vein with 250 μl of phosphate buffered saline (PBS) containing 20 μCi of [3H] thymidine ([3H]TdT; specific activity 2Ci.mmol⁻¹; Amersham International, Amersham, UK). 5 h later, the mice were sacrificed and the draining auricular lymph nodes excised and pooled for each experimental group. Single cell suspensions of lymph node cells (LNC) were prepared by mechanical disaggregation though 200-mesh stainless steel gauze. Cells were washed twice with PBS and precipitated in 5% trichloroacetic acid (TCA) at 4°C overnight. Pellets were then resuspended in 1 ml of 5% TCA and transferred to 10 ml of scintillation fluid (Optiphase ‘Hisafe 3’, Wallac, Turku, Finland). Incorporation of [3H]TdT was measured by β-scintillation counting as disintegrations per minute (dpm) per node for each experimental group. In each case, a stimulation index (SI) relative to the concurrent vehicle-treated control was derived.

Mathematical analysis
The estimated concentration of chemical required to induce a stimulation index of 3 relative to concurrent vehicle-treated controls, or EC3 value, was derived by linear interpolation as described previously (12). The EC3 value was calculated by interpolating between 2 points on the SI axis, one immediately above, and the other immediately below, the SI value of 3. The vehicle-treated control value (SI=1) cannot be used for the latter. Where the data points lying immediately above and below the SI value of 3 have the co-ordinates (a,b) and (c,d) respectively, then the EC3 value may be calculated using the following eq. (12):

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

In addition, the dose-response curves for stimulation indices were compared across experiments using analysis of covariance as described previously (6). The analysis allowed for experimental differences in overall stimulation indices, linear and quadratic dose covariates to represent a quadratic regression between stimulation indices and dose and the interactions between experiment and dose to allow the form of quadratic regressions to be different in each experiment.

Results
The influence of vehicle on local lymph node assay (LLNA) responses to MCI/MI was examined. The 6 vehicles selected for these investigations were AOO, MEK, DMF, DMSO, acetone and PG. The LLNA dose response data obtained with each of these vehicles used for topical application of MCI/MI are recorded in detail in Table 1. As described previously, there was some variation in the levels of thymidine incorporation in LNC derived from vehicle-treated animals, ranging from 199 dpm node⁻¹ for mice treated with PG to 727 dpm node⁻¹ for those treated with DMSO. The somewhat higher backgrounds observed with DMSO are consistent with previous
Table 1. Influence of vehicle on local lymph node assay responses to methylchloroisothiazolinone/methylisothiazolinone (MCI/MI)\(^a\)

<table>
<thead>
<tr>
<th>MCI/MI conc. (%v/v)</th>
<th>AOO dpm node(^{-1}) (SI)</th>
<th>MEK dpm node(^{-1}) (SI)</th>
<th>DMF dpm node(^{-1}) (SI)</th>
<th>Acetone dpm node(^{-1}) (SI)</th>
<th>DMSO dpm node(^{-1}) (SI)</th>
<th>PG dpm node(^{-1}) (SI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>486 (1)</td>
<td>609 (1)</td>
<td>230 (1)</td>
<td>204 (1)</td>
<td>727 (1)</td>
<td>199 (1)</td>
</tr>
<tr>
<td>0.00075</td>
<td>453 (0.9)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>0.0015</td>
<td>586 (1.2)</td>
<td>517 (0.9)</td>
<td>335 (1.5)</td>
<td>243 (1.2)</td>
<td>709 (1.0)</td>
<td>406 (2.0)</td>
</tr>
<tr>
<td>0.0075</td>
<td>2145 (4.4)</td>
<td>1981 (3.3)</td>
<td>701 (3.0)</td>
<td>588 (2.9)</td>
<td>2155 (3.0)</td>
<td>160 (0.8)</td>
</tr>
<tr>
<td>0.015</td>
<td>4433 (9.1)</td>
<td>5139 (8.4)</td>
<td>1090 (4.7)</td>
<td>1895 (9.3)</td>
<td>6885 (9.5)</td>
<td>412 (2.1)</td>
</tr>
<tr>
<td>0.0375</td>
<td>4123 (8.5)</td>
<td>8534 (14.0)</td>
<td>2374 (10.3)</td>
<td>3619 (17.7)</td>
<td>4636 (6.4)</td>
<td>448 (2.3)</td>
</tr>
<tr>
<td>0.075</td>
<td>ND</td>
<td>10688 (17.6)</td>
<td>6438 (28.0)</td>
<td>4796 (23.5)</td>
<td>7450 (10.3)</td>
<td>924 (4.7)</td>
</tr>
</tbody>
</table>

\(^a\) Groups of mice received 25 \(\mu\)l of various concentrations of MCI/MI in vehicle, or vehicle alone, on the dorsum of both ears daily for 3 consecutive days. 5 days following the initiation of treatment, all mice were injected intravenously with 250 \(\mu\)Ci of \(\textsuperscript{3}H\)Tdr in PBS. 5 h later, draining auricular lymph nodes were excised and a single cell suspension prepared. The incorporation of \(\textsuperscript{3}H\)Tdr was measured by \(\beta\)-scintillation counting and is displayed above (dpm node\(^{-1}\) and stimulation index [SI]) for 6 independent experiments, 1 conducted for each different vehicle. AOO, 4:1, acetone:olive oil; MEK, methyl ethyl ketone; DMF, dimethylformamide; DMSO, dimethylsulfoxide; PG, propylene glycol. ND, not determined.

Observations using this vehicle (13, 14). Levels of thymidine incorporation recorded for mice treated with acetone, PG, DMF and AOO were comparable, extending over a relatively narrow range, from 199 dpm node\(^{-1}\) for PG to 486 dpm node\(^{-1}\) for AOO. Irrespective of vehicle, topical exposure of mice to MCI/MI caused a dose-dependent induction of LNC proliferation. Application of MCI/MI in DMF, acetone and MEK resulted in particularly vigorous maximal proliferative responses, with stimulation indices of 17.6 to 28.0 being achieved at the highest application concentration (0.075%) of MCI/MI. When exposed to MCI/MI in PG vehicle, however, considerably weaker responses were observed, with an SI of greater than 3 (4.7) provoked only at the highest concentration (0.075%) of MCI/MI applied (Table 1).

The characteristics of the dose responses (expressed as stimulation indices) induced by MCI/MI when applied in each of the 6 vehicles used are illustrated graphically in Fig. 1. The shapes of the dose-response curves obtained for MCI/MI were dependent upon the vehicle used for application. Thus, exposure to MCI/MI in DMF resulted in a linear dose response profile at all concentrations tested, with a maximal SI value of 28.0 reached at 0.075% MCI/MI. In contrast, treatment with MCI/MI in AOO or DMSO stimulated strong proliferative responses which increased linearly from 0.0015% to 0.015% and then reached a plateau with maximal stimulation indices of approximately 10 being achieved following application of 0.015% to 0.075% MCI/MI. Similar dose-response characteristics were observed when MCI/MI was applied in MEK or acetone, although the levels of thymidine incorporation were somewhat higher, with proliferation increasing linearly from 0.015% to 0.0375% of MCI/MI and maximal stimulation indices of approximately 15 to 20 being provoked at application concentrations of between 0.0375% and 0.075%. In comparison with all of the other vehicles, exposure to MCI/MI in PG resulted in relatively low levels of thymidine incorporation at all concentrations tested, with an SI of greater than 3 being achieved at the highest concentration (0.075%) only. Analysis of covariance was used to compare the statistical significance of differences in overall stimulation indices and differences between dose-response curves. Significant differences in the dose-response profiles (\(p<0.01\)) were seen, due largely to the shallow dose response observed

![Fig. 1.](image-url)

Fig. 1. The influence of vehicle on local lymph-node assay responses to methylchloroisothiazolinone/methylisothiazolinone: 4:1, acetone:olive oil; dimethylformamide; methyl ethyl ketone; acetone; propylene glycol; dimethylsulfoxide. Local lymph node assays were performed according to the legend to Table 1. Results are recorded as stimulation indices. The broken horizontal line represents a stimulation index of 3.
for MCI/MI in PG, and to a lesser extent those obtained following delivery of MCI/MI in AOO and DMSO.

The concentration range of MCI/MI was chosen to allow the mathematical estimation of EC3 values. The EC3 values for MCI/MI in the six different vehicles derived by linear interpolation are shown in Table 2. There was a greater than 10-fold difference in derived EC3 values; these ranging from 0.0049% when AOO was used as the vehicle for application to 0.048% when MCI/MI was formulated in PG. The derived EC3 values from assays conducted using MEK, acetone, DMSO and DMF were, however, very similar, ranging from 0.0068% for MEK to 0.0076% for acetone. Regardless of the vehicle used, the calculated EC3 values for MCI/MI were all below 0.05%.

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>EC3* (value %)</th>
</tr>
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<tbody>
<tr>
<td>AOO</td>
<td>0.0049</td>
</tr>
<tr>
<td>MEK</td>
<td>0.0068</td>
</tr>
<tr>
<td>DMF</td>
<td>0.0075</td>
</tr>
<tr>
<td>DMSO</td>
<td>0.0075</td>
</tr>
<tr>
<td>acetone</td>
<td>0.0076</td>
</tr>
<tr>
<td>PG</td>
<td>0.048</td>
</tr>
</tbody>
</table>

*EC3: Concentration of MCI/MI required to induce a stimulation index of 3, derived mathematically by linear interpolation.

Table 2. Derived EC3 values for MCI/MI

Discussion

The data reported here reveal that, irrespective of the application vehicle employed, MCI/MI induces vigorous responses in the LLNA. With all vehicles examined it was calculated that application concentrations of 0.048% MCI/MI or less were required to stimulate a 3-fold induction of LNC proliferation (EC3 values ranging from 0.0049% to 0.048% MCI/MI). Based upon comparisons with responses induced by other contact allergens in the LLNA, the derived EC3 values for MCI/MI would suggest a skin sensitizing potency similar to, for instance, 2,4-dinitrochlorobenzene (13). As such these data are consistent with what is known of the skin sensitizing activity of MCI/MI, when formulated as Kathon®CG, among exposed human populations (15–18). An indication of the skin sensitizing potency of MCI/MI in humans can be derived from the results of repeat insult patch test (RIPT) studies and from diagnostic patch tests. In an RIPT conducted with a 25 ppm aqueous solution of the active isothiazolinone mixture (equivalent to 0.0025% MCI/MI), 1 of 18 subjects exhibited sensitization. With higher concentrations (56 ppm; equivalent to 0.0056% MCI/MI) formulated in nonionic creams or anionic lotions, response rates of 20% and 8%, respectively, were observed (11). In single diagnostic patch tests, of 976 patients tested with 300 ppm (0.03%) of the isothiazolinone mixture, 8 demonstrated iatrogenic sensitization, and of 170 patients patch tested with 250 ppm (0.025%), 2 were similarly sensitized (16). Taken together, the available data indicate that concentrations of between 0.0025% and 0.03% MCI/MI are able to induce skin sensitization in a fraction of the exposed population. These figures are remarkably similar to the EC3 values for MCI/MI derived from the LLNA and reported here (values of between 0.0049% and 0.048% depending upon the application vehicle used). As such, these results provide additional support for the use of EC3 values in determining the relative-skin sensitizing potency of chemical allergens and as a basis for human risk assessments (3).

With respect to risk assessment, the other important issue addressed in this report is the influence of application vehicle on skin sensitizing activity. Assessment of contact sensitization risks are not uncommonly based upon consideration of results obtained from animal studies in which a vehicle unrelated to the end-use formulation has been used. This may not always be appropriate, however, as there is clear evidence from both human and experimental investigations that the form in which a chemical is encountered on the skin can have a significant impact on the effectiveness of sensitization and the vigour of allergen-induced responses (7–10). Such vehicle-dependent effects on sensitization may be attributable to one or more of several variables, including penetration of allergen into the viable epidermis, local cutaneous inflammation and the migration of antigen-bearing Langerhans cells from the skin to the draining lymph nodes (7, 9, 10). Although in the present investigations no attempt has been made to determine the basis for the differences observed, it is apparent that the vehicle in which MCI/MI is delivered has a substantial impact on the magnitude of LLNA responses. Thus, when formulated in propylene glycol, MCI/MI was some 10-fold less active in the LLNA when compared with a formulation in 4:1 acetone:olive oil. The interpretation is that, as there exists a correlation in mice between the vigour of proliferative responses induced in draining lymph nodes and the effectiveness with which skin sensitization will be induced (19), the vehicle-dependent variations in LLNA responses will translate into differing sensitizing potency. The corollary is that it is appropriate to base hazard assessments on studies in which the chemical allergen is delivered in a matrix relevant to the end-use conditions.

In conclusion, the data reported here demonstrate that the LLNA provides not only a basis for
determination of relative skin sensitizing potency, but also a means of assessing the impact that formulation is likely to have on that activity.

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**References**

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