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Murine Local Lymph Node Assay for Predictive Testing of Allergenicity: Two Irritants Caused Significant Proliferation

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The murine local lymph node assay is a method for predictive testing of contact allergenicity, but its ability to discriminate between allergens and irritants has been questioned. To explain some of the conflicting results with irritants, the proliferation induced by methyl salicylate and nonanoic acid, both considered to be non-sensitisers, was further investigated. Both substances showed a dose – response relationship and clearly positive results when tested at higher concentrations ($\geq$50%) and would thus be classified as potential sensitisers according to the present criteria for a positive assay result. In the case of methyl salicylate, the use of either dimethyl formamide or methyl ethyl ketone as vehicle did not significantly influence the results. The negative results obtained for methyl salicylate in some earlier reports were probably due to testing at too low concentrations. The proliferation induced by irritants such as methyl salicylate and nonanoic acid and inter alia sodium dodecyl sulfate, Triton X-100, oxalic acid, chloroform/methanol (2:1) must be better recognized and elucidated before the assay can be generally accepted as a predictive test method. ***Key words: contact allergens; LLNA; methyl salicylate; mouse; nonanoic acid; predictive testing.***

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The murine local lymph node assay (LLNA) is a predictive test for identifying contact allergens. It measures the proliferative response, as thymidine incorporation, in the draining, auricular lymph nodes in mice during the induction phase after epicutaneous application of a chemical to the ear (1). During the past 10 years the method has undergone extensive validation studies, including intra- and interlaboratory evaluations and comparison with standard guinea pig tests and human maximization test results (see (2) for review). The assay is recommended in OECD Test Guideline 406 (3) for predictive testing as a preliminary screening method in the assessment of skin sensitisation potential; in the case of a positive result a chemical may be designated as a potential sensitiser, while if a negative result is obtained, a guinea pig test must be conducted. However, the ability of this assay to discriminate between weak allergens and irritants has been questioned and an increasing number of substances, considered to be non-sensitisers, induce cell proliferation in the LLNA (4 – 9).

Methyl salicylate and nonanoic acid are both considered to be non-sensitisers and have been used in experimental studies on irritancy (10 – 15). Methyl salicylate has earlier been reported to elicit positive results in the LLNA (4, 8). These findings are in contrast to other studies, claiming methyl salicylate to be negative in the LLNA (5, 16 – 19). This difference in LLNA data has been suggested to be due to the vehicles used (18). Nonanoic acid has been reported to induce a positive result in the LLNA, but no details were given (4).

We therefore wished to further investigate the proliferative response induced by nonanoic acid and methyl salicylate in the LLNA. This study forms part of our evaluation of the LLNA as a predictive assay for contact sensitisation potential of chemicals.

MATERIALS AND METHODS

Animals

Inbred CBA/Ca strain female mice (7 – 10 weeks) obtained from B&K Universal AB (Sollentuna, Sweden) were used. The mice were allowed to acclimatise for at least 5 days prior to first exposure.

Chemicals

Nonanoic acid (Sigma, N-5502), 2-hydroxybenzoic acid methyl ester (Sigma, M-6752) (methyl salicylate), and phosphate buffered saline; pH = 7,4 (PBS) were purchased from the Sigma Chemical Co., St. Louis, MO, USA. [*methyl*-^{3}H] thymidine (specific activity 2.0 Ci/mmol) was purchased from Amersham International plc, Amersham, UK. All other chemicals used were of analytical grade and used as delivered.

Assay

The LLNA was carried out as recommended by Kimber & Basketter (1). Briefly, mice in groups of four received 25 µl of nonanoic acid, neat or dissolved in dimethylformamide (DMF), or methyl salicylate, neat or dissolved in DMF or methyl ethyl ketone (MEK), in the concentrations indicated (w/v). DMF and MEK were approximated to a density of d = 1.0. Treatments were carried out on the dorsum of both ears, once a day, for three consecutive days. Control mice were treated with 25 µl of DMF or MEK (neat). To compare the effect of vehicle treatment, a non-treated (naive) group was also included. Five days after the first treatment, all mice were injected intravenously through the tail vein with 20 µCi [^{3}H]thymidine in 250 µl of PBS. After 5 h, the mice were sacrificed and the draining auricular lymph nodes were excised, pooled for each group and the average lymph node weight was determined. A single-cell suspension of lymph node cells was prepared. After washing in PBS and precipitation with trichloroacetic acid, the incorporated thymidine was determined by β-scintillation counting as previously described (8). Results are expressed as mean decomposition/min (dpm)/lymph node for each experimental group. A stimulation index (SI), i.e. test group value/control group (vehicle-treated) value, was calculated for each concentration tested. Alternatively, the untreated (naive) group was used as control group to calculate SIs. A chemical is classified as a sensitiser if two criteria are fulfilled (1): (i) At least one concentration of the test chemical induces a stimulation index of a threefold or greater value than that of the vehicle control; (ii) the result must not be incompatible with a biological dose response.

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RESULTS

None of the exposed animals died; they showed little or no sign of irritancy (erythema, oedema) at the test sites.

Methyl salicylate

Methyl salicylate was tested in four experiments (experiments 1, 2, 3a and 3b) on three different occasions (Table I and Fig. 1). With test concentrations at or below 20–25%, the response was negative in most cases, i.e. SI < 3. However, higher test concentrations, 50 and 100%, with one exception (50% methyl salicylate in DMF in experiment 1), resulted in clearly positive responses with SIs well above 3. At 100%, the SI values were 4.0, 10.7, 7.1 and 9.4, respectively. Experiments 3a and 3b were conducted on the same occasion to test the influence of the vehicle on methyl-salicylate-induced proliferation. They showed that the use of DMF or MEK as vehicles had only marginal influence.

Nonanoic acid

Similarly, nonanoic acid showed a dose–response relationship and gave stimulation indices of 3.3 and 5.4 when tested at 50 and 100% concentration, respectively (Table II and Fig. 1).

DISCUSSION

Both methyl salicylate and nonanoic acid showed a dose-dependent increase in cell proliferation and gave positive results in the LLNA when tested at higher concentrations, i.e. 50 and 100% (Fig. 1). The increase in mean lymph node weight, after treatment with different concentrations of methyl salicylate or nonanoic acid, roughly follows the increase in thymidine incorporation (Tables I and II). However, the increase is less pronounced and thus a less sensitive indicator of lymph node activation, as shown earlier (8, 20). These results are well in agreement with our earlier published results for methyl salicylate (8) as well as with those of Robbins et al.

Table I. *Proliferation induced by methyl salicylate in the murine local lymph node assay*

Groups of mice ($n = 4$) received 25 μl of methyl salicylate, neat or dissolved in a vehicle dimethylformamide (DMF) or methyl ethyl ketone (MEK) in the concentrations indicated, on the dorsum of both ears daily for 3 consecutive days. Control animals were treated with the vehicle alone and a non-treated (naive) group was also included. All mice were injected intravenously 5 days after the first treatment with 250 μl of PBS containing 20 μCi of [^{3}H]thymidine. Five hours later, the draining auricular lymph nodes were excised and pooled for each group and a single-cell suspension of lymph node cells was prepared. The thymidine incorporation was measured with β-scintillation counting. The experiments were done on three occasions (experiments 1, 2 and 3a and b); experiments 3a and 3b were performed on the same occasion. For further experimental details see Materials and Methods.

Treatment	Concentration of methyl salicylate (w/v %)	Lymph node weight (mg/node)	[^{3}H]Thymidine incorporation (dpm/node)	SI[a]	SI[b]
Experiment 1					
Naive	0	2.4	132		–
DMF	0	3.0	368	–	2.8
Methyl salicylate	10	3.5	454	1.2	3.4
	20	4.0	575	1.6	4.4
	25	4.2	875	2.4	6.6
	50	4.6	945	2.6	7.2
	100	4.0	1464	4.0	11.1
Experiment 2					
Naive	0	2.7	98		–
MEK	0	2.8	148	–	1.5
Methyl salicylate	10	3.2	264	1.8	2.7
	25	4.0	782	5.3	8.0
	50	5.0	1572	10.7	16.0
Experiment 3a					
Naive	0	2.3	150		–
MEK	0	2.9	279	–	1.9
Methyl salicylate	12.5	3.1	405	1.5	2.7
	25	3.2	465	1.7	3.1
	50	5.0	1697	5.9	11.3
	100	4.8	1984	7.1	13.2
Experiment 3b					
Naive	0	2.3	150		–
DMF	0	2.3	211	–	1.4
Methyl salicylate	12.5	3.3	428	2.0	2.9
	25	3.9	509	2.4	3.4
	50	4.5	1607	7.6	10.7
	100	4.8	1984	9.4	13.2

[a]The increase in thymidine incorporation relative to vehicle-treated controls was derived for each experimental group and recorded as a stimulation index (SI), i.e., test group dpm/vehicle treated group dpm.
[b]The increase in thymidine incorporation relative to naive (untreated) controls was derived for each experimental group and vehicle-treated group and recorded as a stimulation index (SI), i.e. test or vehicle-treated group dpm/naive group dpm.

Table II. *Proliferation induced by nonanoic acid in the murine local lymph node assay*

Groups of mice ($n=4$) received 25 µl of nonanoic acid, neat or dissolved in dimethylformamide (DMF) in the concentrations indicated, on the dorsum of both ears daily for 3 consecutive days. Control animals were treated in the same way with the vehicle alone, and a non-treated (naive) group was also included. All mice were injected intravenously 5 days after the first treatment, with 250 µl of PBS containing 20 µCi of [^{3}H]thymidine. Five hours later, the draining auricular lymph nodes were excised and pooled for each group and a single-cell suspension of lymph node cells was prepared. The thymidine incorporation was measured with β-scintillation counting. For further experimental details see Materials and Methods.

Treatment	Concentration of nonanoic acid (w/v %)	Lymph node weight (mg/node)	[^{3}H]Thymidine incorporation (dpm/node)	SI[a]	SI[b]
Naive	0	2.6	144		–
DMF	0	2.5	178	–	1.2
Nonanoic acid	12.5	3.2	390	2.2	2.7
	25	3.5	481	2.7	3.3
	50	4.0	584	3.3	4.1
	100	4.4	968	5.4	6.7

[a]The increase in thymidine incorporation relative to vehicle-treated controls was derived for each experimental group and recorded as a stimulation index (SI), i.e. test group dpm/vehicle treated group dpm.
[b]The increase in thymidine incorporation relative to naive (untreated) controls was derived for each experimental group and vehicle-treated group and recorded as a stimulation index (SI), i.e. test or vehicle-treated group dpm/naive group dpm.

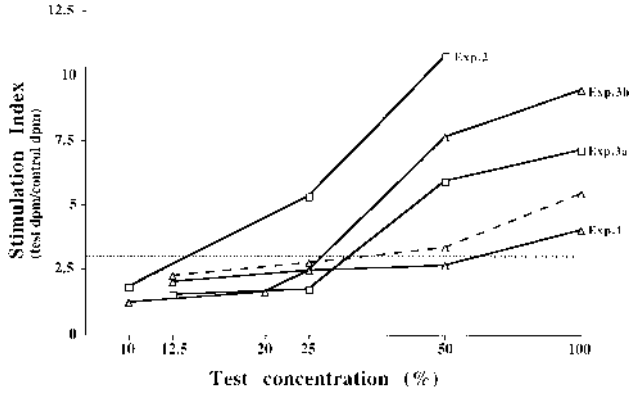

Fig. 1. Summary of results obtained with methyl salicylate (————) and nonanoic acid (- - - - - -) in the local lymph node assay. Vehicles used were methyl ethyl ketone (□) and dimethylformamide (} – t). The dotted horizontal line shows three times the control value (SI = 3). Exp. = experiment. Data from Tables I and II.

(4), who reported positive results for both methyl salicylate and nonanoic acid. However, they do not agree with other studies which reported negative results with methyl salicylate (5, 16 – 19). It has been suggested that these differences with methyl salicylate may be attributed to the vehicle used (18). Most studies which report negative results with methyl salicylate used acetone/olive oil (4:1) as vehicle (16 – 19). In our opinion acetone/olive oil is not a suitable vehicle, since acetone/olive oil treatment alone induces highly variable responses compared to untreated animals (9, 21) and olive oil possesses an inherent capacity to induce high proliferation in the LLNA (9). The mechanism is not settled. Nevertheless, the most obvious explanation for the discrepancy in classification of methyl salicylate in the LLNA is the use of low test concentrations; all studies with negative results for methyl salicylate have used test concentrations of 25% or less (5, 16 – 19). In our hands, 25% methyl salicylate only occasionally gave rise to SI ≥ 3 (Table I and ref. 8). In the present study the concentrations of methyl salicylate and nonanoic acid were chosen according to the recommendations for the LLNA, i.e. "… to provide the highest possible test concentration, while avoiding unacceptable dermal trauma or systemic toxicity" (1).

The reason for including a non-treated (naive) control group (Tables I and II) was to check the magnitude of vehicle-induced proliferation. In the present study all vehicle-treated controls gave 1.2 to 2.8 times higher thymidine incorporation compared to untreated animals. This agrees well with previously published values for DMF and acetone (9). In addition, the untreated control group may constitute a more accurate control when high test concentrations are used. Using the untreated control group to calculate the stimulation indices when methyl salicylate and nonanoic acid were tested neat resulted in 24% to 178% higher values (Tables I and II).

It has been proposed that the LLNA may be used to rank the relative skin-sensitizing potential of chemicals (1, 19, 22 – 24), i.e. relative potency is ranked as a function of the concentration required to induce a stimulation index of 3, and this concentration is expressed as an EC_3 value (estimated concentration for SI = 3) (22). In the present study, the EC_3 values were graphically read from Fig. 1, and were approximated to 15 – 65% (0.99 – 4.27 mol/dm^3 (M)) for methyl salicylate and 35% (2.21 M) for nonanoic acid (Table III). These values are to be compared with EC_3 values of allergens, e.g. 2,4-dinitrochlorobenzene, 0.0765% (0.00383 M) (24), eugenol, 5.8 – 14.5% (0.353 – 0.883 M) (22), penicillin G, 20% (0.561 M) (estimated from ref. 25), hexyl cinnamic aldehyde, 6.85 – 9.63% (0.317 – 0.445 M) (26), and hydroxycitronellal, 20% (1.28 M) (estimated from ref. 9) and with the irritant sodium dodecyl sulphate, 1.5 – 17.1% (0.052 – 0.593 M) (22). Methyl salicylate and nonanoic acid are thus only slightly less potent inducers of proliferation in the LLNA than the allergens eugenol, penicillin G, hexyl cinnamic aldehyde, and hydroxycitronellal.

The number of substances, regarded as non-sensitisers, that induce significant cell proliferation in the LLNA and would be classified as sensitisers is continuously increasing. The following have so far been reported: chloroform/methanol, Triton X-100, oxalic acid (8), sodium dodecyl sulfate (4, 6, 8, 22), methyl salicylate (4, 8 and present study), nonanoic acid (4

Table III. *Relative activity of methyl salicylate and nonanoic acid to induce proliferation in the local lymph node assay (LLNA)*

Substance	Experiment	EC_3 value[a b]	
		%	M
Methyl salicylate	1	65	4.27
	2	15	0.99
	3a	33	2.17
	3b	28	1.84
Nonanoic acid		35	2.21

[a]EC_3 value is defined as the concentration of test material required to elicit a stimulation index of 3 in the LLNA (22) and is given in % (w/v) and in mol/dm^3 (M).
[b]The EC_3 values were read graphically from Fig. 1.

and present study), mineral oil (7), benzalkonium chloride, salicylic acid (5). In addition, we have tested several other substances, regarded as non-sensitisers or with low sensitising potential, i.e. heavy and light mineral oil, monoolein, squalene, squalane, pristane, Aracel A, peanut oil, olive oil and Freund's complete and incomplete adjuvant; all of them giving positive results in the LLNA (9, 27, and unpublished observations). In our opinion, the proliferation induced by such substances in the LLNA must be better recognized and elucidated before the method can be generally accepted as a predictive test method for suspected contact allergens. In the present design of the assay, valuable chemicals or drugs might otherwise be inappropriately prevented from introduction and use. Substances with exclusively irritating properties could be falsely classified as allergens or, alternatively, the allergenicity of chemicals with both allergenic and irritant properties could be overestimated. This also has implications if the method is used for scientific purposes other than predictive testing, e.g. in structure – activity relationship models for contact allergens (25, 28), where results may be misinterpreted and conclusions erroneous.

Methyl salicylate and nonanoic acid induce dose-dependent cell proliferation in the LLNA with positive stimulation indices when tested at higher concentrations, i.e. 50 and 100%. Both these substances, while regarded as non-sensitisers, would thus according to the present criteria for a positive result be classified as contact allergens.

Earlier reported negative results in the LLNA with methyl salicylate are most probably due to testing with low concentrations. The use of DMF or MEK as vehicles does not seem to have any major influence on the results.

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