

Use of the local lymph node assay in the evaluation of the sensitizing potential of pharmaceutical process intermediates

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The murine local lymph node assay (LLNA) has recently been developed to determine the contact sensitization potential of chemicals. Since its original development, the LLNA results have been the subject of extensive comparisons with guinea pig and human data. The investigations described here were designed to explore the ability of the LLNA to identify accurately, pharmaceutical process intermediates (PIs) known to cause contact allergy in humans. To that end, 16 PIs previously tested in the guinea-pig maximization test (GPMT) were tested in the LLNA. Another PI known to be a contact sensitizer in humans was tested only in the LLNA. Cases of contact sensitization in humans were reported only for PIs that were extreme sensitizers in the GPMT and had low EC₃ values (concentration of the test substance required to generate a threefold increase in lymph node cell proliferation) in the LLNA. These data provide additional evidence that the LLNA is able to discriminate skin sensitizers from chemicals that do not possess a significant skin sensitization potential and is thus a useful method for hazard identification. In addition, this method also offers important animal welfare benefits and may also be useful for risk assessment purposes.

Key words: contact allergy; guinea-pig maximization test; human data; local lymph node assay; potency estimation; process intermediates; risk assessment. © Blackwell Munksgaard, 2003.

Accepted for publication 30 September 2003

As part of worker safety evaluation, the potential of process intermediates to cause allergic contact dermatitis is important to evaluate. Traditionally, skin sensitization potential has been determined largely by the conduct of guinea-pig assays such as the guinea-pig maximization test (GPMT) (1). The guinea-pig test methods employ a biphasic protocol and are based on the visual assessment of erythema produced upon the elicitation of a contact response in previously sensitized animals. Although guinea-pig tests have proven useful to identify skin sensitization hazards, they are not well suited for the determination of relative skin sensitization potency. In contrast, in the murine local lymph node assay (LLNA), the standard endpoint measurement is an immunologically relevant quantitative dose response which allows the assessment of the relative sensitizing potency of chemicals. This test identifies skin-sensitizing chemicals as a function of events that are associated with the induction phase of contact sensitization (2), by measuring lymphocyte-proliferative responses in draining lymph nodes,

following epicutaneous application of the test material. In addition, this test causes less animal distress than guinea-pig tests and is of shorter duration. Since its original development, the LLNA has been the subject of extensive comparisons with guinea-pig tests (3–6) and/or with human data (7–10). Although comparisons of LLNA data with the results of guinea-pig tests have been presented for various classes of materials, including process intermediates, comparisons of animal with human data have not been reported for process intermediates. Results obtained with process intermediates in the LLNA and GPMT are complemented with human data.

Materials and Methods

Animals

Female CBA/Jico mice, purchased from Charles River/Iffa Credo (Saint-Germain l'Arbresle, France), were 8–11 weeks old at study initiation and weighed approximately 25 g. Animals were

gang housed (5/box/group) in plastic cages. Animals received UAR A04C-Certified Rodent Diet (UAR, Villemoisson-sur-Orge, France) and water *ad libitum*.

Albino female Dunkin Hartley guinea pigs, purchased from Charles River (Saint-Aubin-lès-Elbeuf, France), were individually housed in plastic cages (compounds B, C, D, F, G, H, I, L, M, N and Q tested in the same laboratory). Albino male Dunkin Hartley guinea pigs, purchased from D. Hall (Newchurch, Staffordshire, UK), were gang housed (5/box) in stainless steel cages (compounds E, J, K, O and P tested in another laboratory). Animals were 5–8-weeks old at study initiation and weighed approximately 350 g. Animals received UAR R14C-Certified Laboratory Diet or a vitamin C-enriched guinea-pig diet FD2 (Special Diets Services Limited) with hay and water *ad libitum*.

Animals were housed in environmentally controlled rooms with a 12-h light cycle. They were individually identified and allocated to groups or cages at random.

The animal facilities had animal care and use programs accredited by AAALAC International and operated in accordance with current standards or conformed with similar guidelines of the country where the studies were conducted. All studies were approved by the Institutional Animal Care and Use Committee of Merck Research Laboratories (West Point, PA, USA).

Chemicals

Materials tested were from various chemical classes, were pharmaceutical process intermediates (PIs) that had been tested in the GPMT over a period of several years and were of various allergenicity grades. Only one PI, known to be a contact sensitizer in humans, was not tested in the GPMT. All PIs (A–Q) were synthesized at Merck Research Laboratories (Rahway, NJ, USA) except test material C, which was supplied from Sigma Aldrich (Saint Quentin Fallavier, France). Trivial chemical names of the chemicals tested are listed in Table 1.

Vehicles used in the LLNA included *N,N*-dimethylformamide (DMF) and/or dimethyl sulfoxide (DMSO) from Merck (Darmstadt, Germany) or a mixture (4v/1v) of acetone/olive oil (AOO) from Prolabo (Fontenay-sous-Bois, France) (acetone) and from Cooperation Pharmaceutique Française (Melun, France) (olive oil).

Vehicles used in the GPMT included Freund's complete adjuvant (FCA) from Difco laboratories (Detroit, MI, USA), petrolatum (pet.) from Cooperation Pharmaceutique Française or

Table 1. Test chemicals

Code	Trivial chemical names
A	<i>p</i> -Nitrobenzyl bromide
B	Chloromethyl triazolinone
C	Dicyclohexylcarbodiimide
D	Chloromethylbenzofuran
E	Bromosulfone
F	CDT phosphate
G	TPAB HCL
H	Enol phosphate
I	Aniline intermediate
J	Iodoaniline
K	Echinocandin crude
L	Vinyl ether
M	Cis ester
N	Ketosulfone
O	Monoaldehyde
P	Backbone diol
Q	Titanocene dimer

acetone and Alembicol D, a product of coconut oil from Alembic Products (Saltney, Chester, England). Sodium lauryl sulfate (SLS) from Merck was used to enhance penetration.

The selected PIs, concentrations and vehicles used are given in detail in Tables 2 and 3.

Experimental designs and treatments

The local lymph node assay. The method used followed the recommendations of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) (11).

Preliminary study

Decisions regarding the choice of the vehicles were made on the basis of solubility of the test article in selected vehicles and suitability for topical application. All test articles were evaluated in DMF, one of the solvents in which the best solubility was obtained. Some of them were also evaluated in DMSO and AOO, either to evaluate the effects of these solvents on the outcome of the test or to evaluate when the solubility was low in DMF. The highest achievable concentration in an appropriate vehicle was determined and evaluated, unless concerns regarding local or systemic toxicity (mortality or decrease in body weight >10%) dictated a more conservative approach. This was not a concern within the group of compounds tested in this study.

Main study

Compounds were initially tested at several concentrations in one of the solvent (DMF or DMSO). If positive responses were observed at all tested concentrations, additional lower doses were tested. As no positive responses were

Table 2. Local lymph node assay (LLNA)/human results obtained with pharmaceutical process intermediates

Test material/GPMT classification	Human cases	Vehicle	Concentrations (% w/v)	Stimulation indices	EC3 _‡ (%)
A*	+	DMF	0.03, 0.1, 0.3, 1, 3	3.7, 28.7, 40.9, 38.2, 35.6	0.028 [§]
B/extreme	+	DMF	0.03, 0.1, 0.3 [†]	2.8, 18.9, 34.9	0.031
C/extreme	+	DMF	0.003, 0.01, 0.03, 0.1, 0.3	1.1, 1.1, 1.8, 12.3, 60.5	0.038
D/extreme	–	DMF	0.1, 0.3, 1, 3, 10	4.8, 28.3, 47.6, 55.5, 66.7	0.06 [§]
E/extreme	+	DMF	0.003, 0.01, 0.03, 0.1, 0.3 [†]	0.9, 1, 1.3, 5, 17.3	0.062
F/extreme	+	DMF	0.03, 0.1, 0.3, 1, 3 [†]	1.1, 1.5, 8.7, 13.4, 35	0.14
G/extreme	–	DMSO	5, 10, 20, 50	4, 4.9, 6.6, 7.7	NE
		DMF	0.1, 0.3, 1, 3, 10	1.5, 1.3, 2.1, 3.6, 5.8	2.2
H/extreme	–	DMF	2.5, 5, 10, 25, 50	1.8, 1.4, 2.2, 1.7, 3.3	45.3
I/extreme	–	DMSO	5, 10, 20, 30	1.1, 1.3, 2.2, 1.5	–
		DMF	2.5, 5, 10, 25, 35	1.6, 1.6, 1.7, 1.7, 1.7	–
J/moderate	–	DMF	60	1.1	–
K/moderate	–	DMF	35	1.5	–
L/moderate	–	DMF	1, 3, 10, 30, 50	1.7, 3.1, 1.3, 1.6, 1.2	–
		AOO	55	1.1	–
M/mild	–	DMF	1, 3, 10, 30, 60	1.1, 1.3, 1.4, 1.8, 2.5	–
N/negative	–	DMF	6	1.2	–
O/negative	–	DMF	1	1.3	–
P/negative	–	DMF	45	2.2	–
Q/negative	–	DMF	1, 3, 10, 30	0.9, 0.7, 0.9, 0.8	–

AOO = mixture (4v/1v) of acetone/olive oil; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; NE = not estimated; + = contact sensitization cases in humans; – = no contact sensitization cases in humans or negative in the LLNA.

*No results available in the guinea-pig maximization test (GPMT) but known contact sensitizer in humans.

[†]Positive responses observed at higher concentrations.

[‡]Concentration eliciting a stimulation index of 3.

[§]Estimated value.

observed at any concentrations in the LLNA for compounds up to moderate in the GPMT, further compounds (negative–moderate in the GPMT) were only tested at the maximum-achieved concentration. In all cases, groups of 5 mice were

exposed daily, for 3 consecutive days, to the topical application of 25 µl of the test chemicals in selected vehicles on the dorsum of both ears. Together with the relevant vehicle control, 3–5 concentrations were evaluated for most test

Table 3. Guinea-pig maximization test (GPMT)/human results obtained with pharmaceutical process intermediates

Test material/GPMT classification	Human cases	Concentrations (% w/v or w/w)	Percentage of affected animals	Mean score
A*	+	–	–	–
B/extreme	+	4/10/2	100	1.7
C/extreme	+	2/5/0.5	82	2.0
D/extreme	–	2/5/0.2	100	1.1
E/extreme	+	0.1/70/70	100	2.0
F/extreme	+	1/25/25	100	2.4
G/extreme	–	5/25/25	100	2.5
H/extreme	–	1/25/25	100	2.2
I/extreme	–	2/25/25	82	1.1
J/moderate	–	2.5/50/50	44	0.4
K/moderate	–	5/50/50	60	1.2
L/moderate	–	5/25/25	36	0.5
M/mild	–	5/25/10	10	0.1
N/negative	–	1/25/25	0	0
O/negative	–	7.5/50/50	0	0
P/negative	–	2.5/60/60	0	0
Q/negative	–	5/25/25	0	0

+ = contact sensitization cases in humans; – = no contact sensitization cases in humans or not tested in the GPMT. Concentrations are intradermal induction/epicutaneous induction/epicutaneous challenge. Mean score is ratio sum of scores in the treated group/total number of animals.

*No results available in the GPMT but known contact sensitizer in humans.

substances. For 5 test substances (2 moderate and 3 negative contact sensitizers in the GPMT), only 1 concentration of the test material was evaluated. In all cases, the highest achievable concentration was selected. 5 days following the initiation of exposure (2 days after the last topical application), all mice received an intravenous injection of [^3H]-methyl thymidine, 5 h after which they were killed. Draining auricular lymph nodes were excised and pooled for each individual mouse, and single cell suspensions were prepared. The incorporation of [^3H]-methyl thymidine was determined by β -scintillation counting, and the results were expressed as mean disintegrations per minute (DPM) for each experimental group. For each concentration of the test material, a stimulation index (SI), represented by the mean DPM in the treated group/mean DPM in the vehicle control group, was calculated. A SI of 3 was established to be a reliable discriminant between sensitizing and non-sensitizing chemicals as proposed by ICC-VAM. The concentration eliciting a SI of 3 is identified as the EC3 value and is calculated by linear interpolation of points on the dose-response curve, immediately above and below the threefold threshold having the co-ordinates (a , b) and (c , d), respectively, according to the equation (12):

$$\text{EC3} = c + \left[\frac{3 - d}{b - d} \right] \times (a - c)$$

This EC3 value can be used to determine the relative skin-sensitizing potency of chemicals (13).

The guinea-pig maximization test. The method used was the one developed by Magnusson and Kligman (1).

Preliminary study

Induction and challenge concentrations were first determined for every material as follows: the concentrations producing mild skin irritation and no systemic toxicity were used for induction and the maximal non-irritating concentration was used for challenge.

Main study

Guinea pigs were randomly distributed into groups of 5–10 animals (control group) or 10–11 animals (treated group). The treatment procedure was as described below. Compounds B, C, D, F, G, H, I, L, M, N and Q: for treated guinea pigs, on day (D) 1, induction on the shaved scapular area was performed by 2 intradermal injections.

These consisted of 0.1 ml of the compound in a 50/50 (v/v) dilution of FCA with distilled water. Then, on D7, 10% (w/w) SLS in pet. was applied on the day before topical induction, when 400 mg of a mixture of the compound in pet. was applied in an occluded patch on the shaved scapular area for 48 h. On D22, topical challenge was performed on both shaved flanks by applying 200 mg of a mixture of the compound in pet. for 24 h.

Compounds E, J, K, O and P: for treated guinea pigs, on D1, induction on the shaved scapular area was performed by 3×2 intradermal injections. These consisted of 2 injections of 0.1 ml of a 50/50 (v/v) dilution of FCA with distilled water, 2 injections of 0.1 ml of the compound in 5% (v/v) acetone in Alembicol D and 2 injections of 0.1 ml of the compound in a 50/50 (v/v) dilution of FCA with 5% (v/v) acetone in Alembicol D. Then, on D7, 10% (w/w) SLS in pet. was applied on the day before topical induction, when 0.4 ml of a mixture of the compound in acetone was applied in an occluded patch on the shaved scapular area for 48 h. On D22, topical challenge was performed on both shaved flanks by applying 0.2 ml of a mixture of the compound in acetone for 24 h.

Control guinea pigs received the same treatment as treated animals, except that on D1 and D8, the test substances were not administered.

For all compounds, application sites were evaluated on D24 and D25. Sensitizing potential was based on the percentage of guinea pigs showing positive responses. The sensitizing potential classification used was the one developed by Magnusson and Kligman (1).

Human exposure conditions. Cases of contact sensitization in humans were reported when there was a potential for more than incidental contact, i.e. for exposures higher than that which would be expected using good chemical-handling practices. The exposure conditions to the test materials in laboratories or pilot plants were consistent with good chemical-handling practices. Occupational exposure limits and classification of the test materials were established from preclinical and/or clinical data, and handling was performed accordingly. Industrial hygiene-monitoring data (e.g. wipe test results) were not available to characterize exposures associated with the anecdotal reports of skin sensitization.

Results

Evaluation of process intermediates in the LLNA

The vehicles, concentrations, stimulation indices and EC3s are listed in Table 2. All process inter-

mediates, but one, which were extreme sensitizers in guinea pigs or a known contact sensitizer in humans were found positive ($SI \geq 3$) in the LLNA. The EC3 values varied from 0.028 to 45.3%. All other compounds, including mild and moderate sensitizers in the GPMT, were found negative in this test.

Evaluation of process intermediates in the GPMT

The induction and challenge concentrations, the percentage of affected animals, the mean score and the classification for each material are listed in Table 3. Amongst 16 process intermediates tested in the GPMT, 8, 3 and 1 were extreme, moderate and mild sensitizers, respectively, and 4 were negative in this test.

Evaluation of GPMT and LLNA data versus human data

References to contact sensitization cases in humans are summarized in Tables 2 and 3. Although the frequency of cases of skin sensitization in humans was not assessed objectively in this study, several observations are noteworthy.

Test material A, which is known to be a contact sensitizer in humans and not tested in the GPMT, was found positive in the LLNA with an EC3 value of 0.028%. Test materials B, C, E and F that were associated with anecdotal reports of skin sensitization were considered extreme sensitizers in the GPMT and were positive in the LLNA with EC3 values of 0.031, 0.038, 0.062 and 0.14%, respectively.

There were no reported contact sensitization cases in humans for test materials D, G and H which were considered extreme sensitizers in the GPMT. Test material D was positive in the LLNA with an EC3 value of 0.06%, similar to the ones that cause contact sensitization in humans. However, because of early discontinuation of the synthesis process, there had been limited opportunity for human exposure to this compound. Compounds G and H were positive in the LLNA with EC3 values of 2.2 and 45.3%, respectively, which were higher than the compounds with EC3 values that were associated with contact sensitization in humans.

There were no reported contact sensitization cases in humans for test material I which was an extreme sensitizer in the GPMT as well as for test materials J, K, L, M, N, O, P and Q which were moderate or mild sensitizers or negative in the GPMT. All were negative in the LLNA.

As to test materials B, L, M and Q (process intermediates of the same final product), which were extreme, moderate, mild and negative con-

tact sensitizers, respectively, in the GPMT, contact sensitization cases in humans were observed only with compound B which had an EC3 value of 0.031% in the LLNA. As for test materials F and N (process intermediates of another final product), which were extreme and negative contact sensitizers, respectively, in the GPMT, contact sensitization cases in humans were observed only with F which had an EC3 value of 0.14% in the LLNA.

Thus, only the compounds that had the lowest EC3 values in the LLNA were shown to be extreme sensitizers in the GPMT and/or caused skin sensitization in humans.

Discussion

All process intermediates, but one, which were extreme sensitizers in the GPMT were found to be positive ($SI \geq 3$) in the LLNA. All other test materials including mild and moderate sensitizers in the GPMT were found to be negative in this test. However, all test materials reported to be contact sensitizers in humans were extreme sensitizers in the GPMT and had low EC3 values in the LLNA.

In studies that investigated the correlation between GPMT and LLNA results, where both were conducted in parallel, it was shown that the LLNA assay was capable of detecting chemicals that exhibited a strong sensitization potential in the GPMT. The moderate sensitizers were generally positive or gave equivocal results in the LLNA, whereas the weak sensitizers were not detected (5, 7).

The discrepancies between the 2 tests may be due to the methodological features of the GPMT (1, 14). The GPMT maximizes the exposure by using not only epicutaneous induction under an occluded patch but also intradermal induction with FCA and therefore increases the probability of a positive response. Moreover, its outcome is based on the percentage of animals which display dermal reactions following challenge to sometimes high concentrations and does not take into account the intensity of the sensitization response. In contrast, the LLNA uses epicutaneous induction only, and the outcome of the test is based on the intensity of the response for an objective, quantitative endpoint.

In the present study, amongst the 5 test substances which were positive in the GPMT and negative in the LLNA:

- (1) Test material I, which was classified as an extreme sensitizer in the GPMT, produced an 82% rate of sensitization at very high induction concentrations, with a mean

score of 1.1 out of a maximum achievable score of 3; this was the lowest mean score obtained with test substances classified as extreme sensitizers in the GPMT

- (2) Test materials J, K and L, which were classified as moderate sensitizers in the GPMT, produced 44, 60 and 36% rates of sensitization, respectively, at very high induction concentrations. The mean scores were 0.4, 1.2 and 0.5, respectively
- (3) Test material M, which was classified as a mild sensitizer in the GPMT, tested at the maximum achievable induction concentrations, produced a 10% rate of sensitization with a mean score of 0.1.

European legislation requires a minimum incidence of 30% of positive responses in the GPMT before a chemical can formally be classified as a skin sensitizer (15). According to European legislation, test articles I, J, K and L are classified as skin sensitizers, whereas compound M is not classified as a skin sensitizer. The EU now prefers the use of the LLNA as a stand-alone test.

From the analysis generated during the review process by ICCVAM (11), when comparing the LLNA results for 74 test materials with the current recommended guinea-pig methods (GPMT and Buehler assay) for 57 test articles, it was concluded that the LLNA provided an equivalent prediction of the risk of human contact sensitization. Test materials such as benzocaine, nickel salts and neomycin sulfate, known as weak sensitizers in humans, yielded negative or equivocal results in the LLNA. Furthermore, recent comparisons between LLNA results and human sensitization data have shown that the LLNA was able to discriminate between skin sensitizers and chemicals which do not possess a significant skin sensitization potential in humans (10). The quantitative measure of allergen potency, the EC₃ value obtained in the LLNA, correlated with human data/clinical evidence (16, 17, 18) and with human potency classes (19). The LLNA is considered to be the most suitable test to provide an estimation on the relative sensitization potency in the context of risk assessment (16).

It has been reported that, for some substances, the vehicle could have a substantial effect on the observed response of an allergen, with a greater than 10-fold vehicle-dependent change being observed (20). In the present study, the vehicles had no effect on lymph node proliferative responses for 3 test materials G, I and L which were each tested in 2 different solvents (DMF and/or AOO or DMSO).

Furthermore, the investigations described here were designed to explore the ability of the LLNA to identify accurately those chemicals that cause allergic contact dermatitis in humans compared to the GPMT. Anecdotal reports of skin sensitization were observed only amongst process intermediates, which were extreme skin sensitizers in the GPMT and positive in the LLNA with low EC₃ values. The data obtained from this present study provides further evidence that the LLNA is a reliable method for identifying chemicals that are classified as human skin sensitizers of practical significance in the workplace.

The use of a tiered (screening) approach to evaluate process intermediates, which we intend to employ, will trigger a full dose-response assessment only when a significant response is observed at the maximum achievable concentration. This further reduces the number of animals used in the LLNA, which is already considered a significant refinement to existing skin-sensitization tests.

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