

The local lymph node assay and skin sensitization: a cut-down screen to reduce animal requirements?

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The local lymph node assay (LLNA), an alternative approach to skin-sensitizing testing, has made a significant contribution to animal welfare by permitting a reduction and refinement of animal use. Although there is clearly an aspiration to eliminate the use of animals in such tests, it is appropriate also to consider other opportunities for refinement and reduction of animal use. We have therefore explored the use of a modified version of the LLNA for screening purposes when there is a need to evaluate the sensitizing activity of a large number of chemicals, as will be the case under the auspices of registration, evaluation and authorization of chemicals (REACH). Using an existing LLNA database of 211 chemicals, we have examined whether a cut-down assay comprising a single high-dose group and a concurrent vehicle control would provide a realistic approach for screening chemicals for sensitizing potential. The analyses reported here suggest this is the case. We speculate that the animal welfare benefits may be enhanced further by reducing the number of animals per experimental group. However, a detailed evaluation will be necessary to provide reassurance that a reduction in group size would provide adequate sensitivity across a range of skin sensitization potencies.

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Allergic contact dermatitis is a common occupational and environmental health issue, and many hundreds of chemicals have been implicated as skin sensitizers (1). There is therefore an important need to identify chemicals that have the potential to cause the acquisition of skin sensitization and to estimate accurately the likelihood of health risks in humans. Traditionally, hazard identification was addressed using one or other of several guinea pig methods, usually either the Guinea Pig Maximization Test or the Occluded Patch Test of Buehler (2, 3). More recently, however, an alternative approach in mice, the local lymph node assay (LLNA), has found favour. This method is based on measurement of skin-sensitizing potential as a function of lymph node cell proliferative responses induced in mice following repeated topical exposure to the test chemical.

The LLNA has been the subject of rigorous evaluations and comparisons and has now been formally adopted as a validated method that

provides both scientific and animal welfare advantages compared with guinea pig tests (4–6). Although the LLNA has been shown to reduce the requirement for animal numbers in providing a robust assessment of hazard, and to provide a reduction in the trauma to which animals are potentially subject (6, 7), there remains an important aspiration to reduce further, or eliminate completely, the need for experimental animals in skin sensitization safety assessments.

In this context, there is currently a significant investment in the exploration of alternative (*in vitro* or *in silico*) methods for skin sensitization testing that would obviate the need for experimental animals (8–12). Although some progress has been made, and undoubtedly will continue to be made, with the design of such approaches, it would be prudent to continue to look for opportunities to refine existing animal approaches such as the LLNA. For this reason, and in parallel with a continued and vigorous commitment to the development of non-animal methods, we

have explored here whether it would be possible to consider a further significant reduction in the number of animals needed for skin sensitization testing using a modification of the LLNA.

Specifically, the question we have addressed here is whether there exist opportunities to identify accurately chemicals with the potential for skin sensitization based on the evaluation of the responses provoked by a single dose (rather than several doses) of the test chemical.

Currently, in the standard LLNA, 3 concentrations of the test material, plus the relevant vehicle alone, are used – in each, case responses being measured in groups each comprising 4 or 5 mice (6). The additional question that is worth posing, but which has not been directly addressed here, is whether decisions could be reached regarding likely sensitizing activity if, instead of the standard assay design, only the top dose of chemical and the relevant vehicle were used, and then in groups of 2, rather than 4, mice.

The approach used makes use of an extensive database comprising comprehensive information on the behaviour of 211 chemicals, of very differing sensitizing potency, in the LLNA (13). The ability to reach conclusions about skin sensitization potential based only on measurement of responses by the top dose of the test chemical has been investigated retrospectively. The data suggest that a cut-down version of the LLNA can be used for screening for skin-sensitizing activity, with potentially very significant reductions in the requirement for test animals. However, for this to be a robust and practical way forward, the limitations of this approach, and the need for robust experimental technique and judicious interpretation of data, must be understood and acknowledged. These issues are considered in detail in the *Discussion*.

Materials and Methods

LLNA protocol

Groups of CBA female mice (7–12 weeks of age) were exposed topically on the dorsum of both ears to 25 μ l of test material or to an equal volume of the vehicle alone. Treatment was performed daily for 3 consecutive days. 5 days following the initiation of exposure, all mice were injected via the tail vein with 250 μ l of phosphate-buffered saline (PBS) containing 20 μ Ci of tritiated thymidine. Mice were sacrificed 5 hr later and the draining lymph nodes excised. The incorporation of tritiated thymidine measured by β -scintillation counting was reported in disintegrations per minute (dpm). A stimulation index (SI) was calculated for each chemical as the ratio

of the dpm of the treated group over the dpm of the concurrent vehicle control group (4).

Data analysis

Published data on the LLNA results for 211 substances (13) were examined retrospectively to assess whether in each case the highest concentration tested would provide an indication of whether the substance should be classified as a skin sensitizer (14). Thus, where the SI at the highest concentration was equal to or greater than 3, the substance was regarded as a skin sensitizer; where the SI was less than 3 at this concentration, then the substance was categorized as being non-sensitizing (strictly speaking 'not classified'). However, an important criterion for deriving such a conclusion from the single highest concentration data was that if the result was negative, and the highest tested concentration was less than 10%, then the result was considered invalid. The value of 10% was based on the practical considerations discussed elsewhere (15).

Results

In a recent report (13), the results of standard LLNAs conducted previously with 211 chemicals (of which 169 were identified as sensitizers) have been collated as a comprehensive database. It is the information contained within this database that has been used here for the purposes of examining whether it would be possible to make accurate predictions of sensitizing activity based on consideration only of responses elicited with a single dose of the test chemical and for determining what difficulties might be associated with such an approach.

In most instances, accurate identification of sensitizers in the cut-down assay was found not to represent a problem. Thus, irrespective of the dose range selected in the standard LLNA, if the top dose was found to elicit an SI of 3 or greater, then a prediction of skin-sensitizing activity could be made with confidence. The only difficulty encountered in this respect can be illustrated by reference to one of the chemicals examined as part of this comparison. In the standard LLNA, 2-methyl-2H-isothiazol-3-one was found to provoke an SI of 3.8 at a test concentration of 2.5% and an SI of 2.5 at the highest concentration tested of 5%. On the basis of these data from a conventional LLNA, the chemical was accurately classified as being a skin sensitizer. However, in the reduced LLNA, in which reference would be made only to data deriving from a single dose of the chemical (in this instance 5%), then a

negative result might be assumed inappropriately. The two other chemicals that were found to be sensitizers in standard LLNAs conducted previously, and which would be missed in a reduced assay if the same top doses were chosen again, were C₁₉-azlactone and camphorquinone. In both cases, predictions of sensitizing activity from the standard LLNA derived from results obtained with the mid-dose group in experiments where the top dose elicited an SI of less than 3. Taken together, then the overall prediction rate in the reduced assay for skin sensitizers identified in the standard assay was 166 of 169, the 3 exceptions being the chemicals discussed above.

In the light of these observations, and with the aim of providing a screening tool of maximum utility and with the minimum number of false negatives, it was concluded that it would be appropriate to interpret potential negative responses in the context of the dose of chemical with which the data were derived. Specifically, the recommendation is that for the purposes of judging a chemical to lack skin-sensitizing activity, it would be appropriate to ensure that the test had been conducted with a defined minimum concentration of the test material, the concentration selected for the purposes of these analyses being 10%.

To illustrate by example the proposed approach, Table 1 summarizes some typical outcomes of the reduced LLNA-screening assay, with results compared with those derived from the standard method, the latter data being derived from a published dataset (13). Potent sensitizers such as oxazolone and lauryl gallate are identified clearly as positives (in the case of oxazolone, even at very low concentrations). This contrasts with a clear negative result obtained with isopropanol that was tested at a concentration of 50%, but which failed to stimulate activity in the LLNA. Very occasionally, test materials

were found to yield responses close to the threshold value in the standard prediction model. For example, as summarized here in Table 1, clotrimazole elicited an SI of 3 at the highest concentration tested (10%). Clearly, regarding this material as a potential skin sensitizer is correct, although it must be acknowledged that repeat testing at the same concentration would not necessarily always yield a positive outcome. Finally, and as described above, the Table includes data obtained with 2-methyl-2H-isothiazolinone, a chemical that in the standard assay was classified as positive based on the elicitation of an SI of greater than 3 with a test concentration other than the highest used (5%). In the reduced assay, this would potentially represent a false negative with the top dose selected giving an SI of less than 3. However, applying the cautionary approach to the identification of chemicals as being non-sensitizers articulated above, then because the dose considered was less than 10%, it would be inappropriate to regard this as a secure negative response. As a consequence, for the purposes of these analyses, the result obtained with 2-methyl-2H-isothiazolinone would be regarded as not interpretable and therefore invalid. These and other issues are discussed further below.

Discussion

The data reviewed here suggest that, in principle at least, it might be appropriate to consider the use of a cut-down version of the LLNA for some applications and for some purposes. Clearly, it is very important to acknowledge that such a modification of the LLNA confers no scientific advantages and that the reason for considering seriously this option is that there may, in some specific circumstances, be opportunities for substantial reductions in the number of animals

Table 1. Illustrative examples of the reduced LLNA in practice

Chemical	Vehicle	Highest test concentration	Stimulation index	Screen LLNA	Full* LLNA
Oxazolone	Acetone	0.05%	33.0	Positive	Positive
Lauryl gallate	DMSO†	50%	36.0	Positive	Positive
Isoeugenol	AOO‡	5%	12.4	Positive	Positive
Abietic acid	AOO	25%	5.2	Positive	Positive
Clotrimazole	AOO	10%	3.0	Positive	Positive
2-Methyl-2H-isothiazolinone	AOO	5%	2.5	Invalid	Positive
Camphorquinone	AOO	25%	1.7	Negative	Positive
Lactic acid	DMSO	25%	2.2	Negative	Negative
Isopropanol	AOO	50%	1.0	Negative	Negative

*Results of the standard LLNA were taken from a comprehensive dataset published elsewhere (Gerberick *et al.* 2005), and were selected to provide illustrative examples of how data might be interpreted in a cut down version of the assay.

†Dimethylsulfoxide.

‡Acetone/olive oil, 4 : 1, v/v.

required for skin sensitization testing. Indeed, it is necessary to appreciate that a modified assay of the type described here would inevitably be associated with reduced scientific rigour and, as a result, increased uncertainty. As a consequence, therefore, it will be necessary to weigh the potential animal welfare benefits that might accrue from such an approach, with an increased level of uncertainty. It is the latter that is addressed in more detail below.

The results described above illustrate that, in the vast majority of circumstances, it is possible (with a properly conducted and controlled LLNA) to identify skin sensitization potential – or indeed the absence of skin-sensitizing activity – based solely on the evaluation of responses provoked by the top dose of the chemical tested. In some cases, such as for instance when a high stimulation index results from exposure to a comparatively low dose of chemical, then identification of the material as a skin sensitizer is without difficulty. Similarly, there would be confidence in characterization of a chemical, as a non-sensitizer in circumstances where a high dose of the test material was examined in an appropriate vehicle (yielding a good solution and application characteristics) failed to provoke a stimulation index of 3 or greater. More problematic would be confident interpretation of an SI approaching the threshold value of 3 in circumstances where the chemical had been tested at a relatively low concentration (of say less than 10%) and/or in cases where an adequate solution has not been achieved. This particular point was addressed in the approach to data analysis that we adopted here; negative results where the tested concentration was less than 10% were considered invalid in this context. We have, for the purposes of this article, adopted a 10% application concentration as a threshold of confidence for categorization of a chemical as being negative. However, this figure should not be regarded as inviolable; there may be a case to be made for a higher value of (for instance) either 15% or 20% providing greater certainty of the absence of sensitizing activity.

Associated with the above issue are considerations of dose selection for screening purposes. It is normal and sound practice in the standard LLNA, for the purposes of sensitivity, to maximize test concentrations consistent with acceptable solubility. However, this naturally has to be reconciled with animal welfare considerations. In the UK, for instance, the selection of doses of 10% or greater for use in the LLNA demands an understanding that unacceptable local or systemic toxicity is unlikely to result from the concentrations(s) proposed. In the absence of such

information, then a preliminary sighting study would normally be required. However, this would be the case whether a standard or a cut-down LLNA were being conducted, and in the latter, instance would not impact adversely on the opportunity to reduce animal numbers.

Although the analyses summarized above suggest that such problematic interpretations would not be of high frequency, even if they were found to occur more commonly than predicted from analyses to date, it would not invalidate this strategy when the requirement was for screening large numbers of chemicals. The approach with chemicals of uncertain categorization would undoubtedly be to conduct a standard LLNA.

Another potential approach to deliver additional animal welfare benefits is to consider reduction not only of the number experimental groups used but also of the number of mice within each group. A reduction from 4 mice to 2 per group would result in a significant saving of animals required, particularly in circumstances where the sensitizing potential of a number of chemicals was to be screened concurrently using a single vehicle control group. Attractive though this proposal is in reducing the need for experimental animals, there is again a price to pay in terms of scientific rigour and increased uncertainty. Reliance on results from just 2 mice in each experimental group would demand a high level of technical competence, for example in ensuring that each animal received exposure to radio-labelled thymidine via intravenous injection. In this context, a case could possibly be made to increase the confidence in measurement of immune activation, perhaps for instance by including evaluation of lymph node weight in tandem with lymph node cell proliferative responses. As with the suggestion of reducing the number of animals per group, the value of adding additional parameters in a cut-down version of the LLNA is uncertain and unproven, and this option is raised here not as a recommendation but as a subject that might warrant further consideration.

The proposed reconfiguration of the LLNA for the purposes of screening as described here appears counter-intuitive in the context of a common goal among toxicologists of striving for increased scientific rigour and more accurate assessment of hazards and risks. However, in the interests of animal welfare, and in recognition of the challenges associated with the implementation of REACH, it behoves practicing toxicologists to think imaginatively about how best we can refine experimental systems to improve animal welfare without compromising our

responsibilities to ensure human and environmental safety and to respond positively to changing legislative requirements. It is against this background, and with these practical considerations in mind, that the proposed modification of the LLNA for screening purposes should be viewed.

It is important to recognize also that the possible application of a cut-down LLNA would provide one strand of what in reality is likely to be a co-ordinated approach to maximizing animal welfare in the context of skin sensitization tests. Other initiatives, such as for instance assessment of sensitizing activity as a function of cellular responses to chemical allergens, the exploitation of structural alerts and/or reactivity *in vitro* with defined peptides are also legitimate considerations in the development of a coherent safety assessment strategy (10–12).

Finally, it is important to emphasize here that the approach that has been explored is in response to a specific need, and it is not our recommendation that the standard LLNA should be modified for the normal purposes of hazard identification and hazard assessment.

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