REVIEW

Local Lymph Node Assay: Validation Assessment for Regulatory Purposes

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For the prediction of skin sensitization potential of substances, the murine local lymph node assay (LLNA) is an alternative to the widely used guinea pig tests. For more than 10 years, this method has undergone extensive development, evaluation, and validation. In this review, the validation status of the LLNA is considered, specifically with regard to its use for regulatory identification of skin sensitization hazards. The LLNA is a method for the predictive identification of chemicals that have a potential to cause skin sensitization. Activity is measured as a function of lymph node cell proliferative responses stimulated by topical application of test chemicals. The LLNA has successfully passed all reasonable validation stages. It provides a reliable and relevant source of predictive skin sensitization data, which unlike results from guinea pig tests, are reproducible from laboratory to laboratory. In summary, the LLNA is now ready for acceptance as a viable and complete alternative to traditional methods, offering a substantial reduction in animal numbers and refinement opportunities without compromising the standards for the identification of important skin sensitizers.

ALLERGIC CONTACT DERMATITIS (ACD) is a frequent occupational health problem and, in common with other forms of allergic disease, develops in 2 phases. The first, or induction, phase is initiated when a susceptible individual's skin encounters sufficient amounts of an inducing chemical allergen to stimulate a primary cutaneous immune response. This results in allergic sensitization. If the now-sensitized individual is exposed subsequently to the same allergen, at the same or a different skin site, an accelerated and more aggressive secondary immune response will be provoked at the site of contact. Allergen-responsive T lymphocytes are activated in the skin at the site of contact and release cytokines and other inflammatory mediators that cause the accumulation of mononuclear cells and the inflammatory reaction that is recognized clinically as ACD. For many years, the species of choice for the identification of contact allergens was the guinea pig. A variety of guinea pig test methods has been described, and although these vary in detail, the principles of the assays are the same in each case, sensitizing activity being measured as a function of challenge-induced erythematous and edematous reactions in previously sensitized animals. There is no doubt that at least some of these guinea pig methods have served toxicologists well. Nevertheless, it is clear that such assays are subject to some important limitations, including the fact that the endpoints are subjective and may be difficult to measure and interpret if colored or irritant chemicals are evaluated. Moreover, some of the more sensitive guinea pig methods demand the use of an adjuvant. These limitations encouraged consideration of alternative approaches.

More than 10 years ago, the local lymph node assay (LLNA) was described,[1,2] a standard protocol prepared,[3] and the data produced subsequently reviewed.[4,5] This method was founded on the belief that an increasingly sophisticated appreciation of the immune system would facilitate the design of alternative methods for the identification of chemical allergens that cause adverse effects through the stimulation of specific immune responses. The LLNA uses mice, the experimental species for which the most detailed information is available about the induction and regulation of immunologi-

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cal responses. In contrast to guinea pig test methods, the LLNA identifies potential skin-sensitizing chemicals as a function of events associated with the induction, rather than elicitation, phase of skin sensitization. The induction phase of skin sensitization is characterized by the stimulation of an allergen-specific immune response in lymph nodes draining the site of exposure. After the skin encounters a chemical allergen, cutaneous dendritic cells, and Langerhans cells in particular, are mobilized and stimulated to migrate from the skin to the draining lymph nodes. A proportion of these cells bears high levels of antigen. The dendritic cells that accumulate in the lymph nodes are immunologically active and are able effectively to present the inducing allergen to responsive T lymphocytes. Antigen-driven T lymphocyte activation results in division and differentiation and is characterized by increases in lymph node weight and cellularity. The division of activated T lymphocytes provides an increase in the number of allergen-reactive T lymphocytes; this clonal expansion represents the cellular basis of immunological memory and skin sensitization. If the now-sensitized subject is exposed subsequently, at the same or a different skin site, to the inducing allergen, then an elicitation reaction will be provoked. The expanded population of specific T lymphocytes recognizes the allergen in the skin at the site of challenge; they become activated and, through the release of cytokines and chemokines, cause the influx of other cells and initiate the inflammatory response that is recognized clinically as ACD. It is now clear that the induction phase of skin sensitization is associated with the activation of both CD4 (T helper) and CD8 (T cytotoxic) cells, and that both of these populations contribute to the subsequent elicitation of dermal hypersensitivity reactions.[6] The importance of the clonal expansion of T lymphocytes is reflected by the fact that the vigor of proliferative responses induced by chemical allergens in draining lymph nodes correlates closely with the extent to which sensitization will develop.[7,8] It is on the measurement of this response that the LLNA is based.

In initial investigations, several parameters of draining lymph node activation were measured after topical exposure of mice to contact allergens and to nonsensitizing chemicals. These comprised changes in lymph node weight and cellularity and lymphocyte proliferation measured as a function of radiolabeled thymidine incorporation during culture of lymph node cells.[1,9,10] The marker that proved to be the most sensitive and selective correlate of skin-sensitizing activity was the induction of lymph node cell proliferation, and subsequent investigations focused upon it. Another change introduced after these preliminary investigations was to measure the proliferative activity in situ, by intravenous injection of tritiated thymidine, rather than after culture of isolated lymph node cells.[2,11] This version of the method has been evaluated extensively in the context of national and international collaborative trials and has been the subject of detailed comparisons with guinea pig tests and human data.

LLNA Method

The standard protocol, described previously by Kimber and Basketter,[3] uses young adult (6 to 16 wk-old) female CBA/Ca strain mice. In strain comparisons, CBA/Ca mice were found to show a more marked response to contact allergens than the other strains examined.[9] However, female CBA/J and CBA/JHsd strain mice, which were used in several interlaboratory validation studies, respond in a comparable way to CBA/Ca strain mice and are also acceptable for the assay.[12,13] The method is depicted in Figure 1. Groups of mice (n = 4 or 5) are treated by topical application, on the dorsum of both ears, with 25 μL of 1 of several concentrations of test material, or with an equal volume of the relevant vehicle alone. They are treated daily for 3 consecutive days, followed by a 2-day rest period before analysis. On the sixth day (5 days after initiation of treatment), the mice are injected intravenously, by the tail vein, with 250 μL of sterile phosphate-buffered saline (PBS) containing 20 μCi of [^{3}H] methyl thymidine. Five hours later, the mice are killed, and the draining auricular lymph nodes are excised and pooled for each experimental group or for each individual animal. Single cell suspensions of lymph node cells are prepared. Lymph node cells are washed twice with an excess of PBS and precipitated with 5% trichloroacetic acid (TCA) at 4°C. The samples, pelleted by centrifugation, are resuspended 12 to 18 hours later in 1 mL of 5% TCA and transferred to 10 mL of scintillation cocktail. Incorporation of tritium-labeled thymidine [^{3}H-TdR] is measured by β-scintillation counting and expressed as disintegrations per minute (dpm). The use of iododeoxyuridine 125 (^{125}IUdR) rather than thymidine-labeled

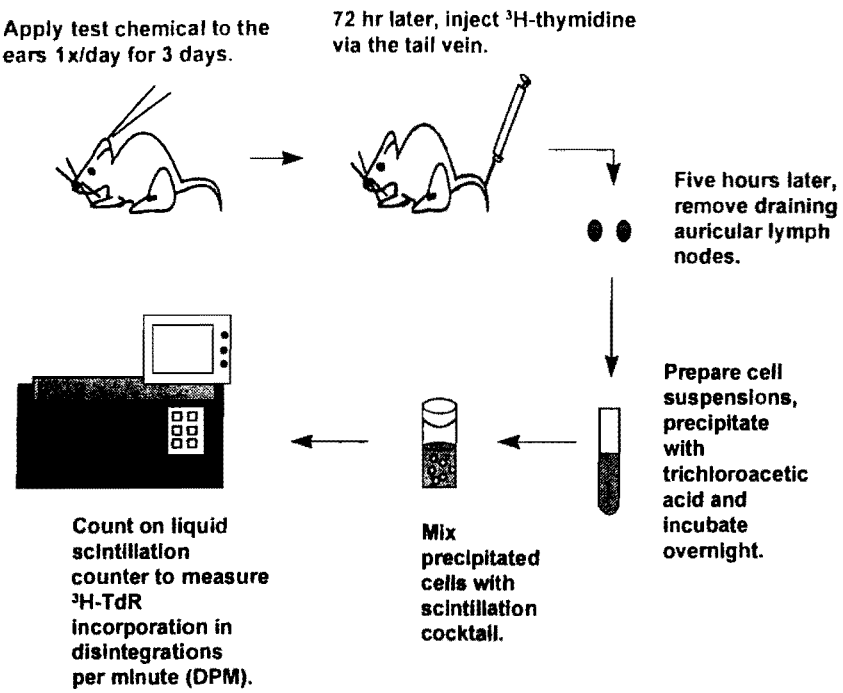

Figure 1. The local lymph node assay method. Reprinted from Kimber I, Gerberick GF: Toxicological aspects of allergic contact dermatitis: Report on Selected Proceedings of the Society of Toxicology meeting, New Orleans, LA, USA, March 14-18, 1999. Am J Contact Dermat 10:245-248,1999 with permission.

^{3}H-TdR) as the isotope has been shown to be comparably robust in the LLNA,[12-14] although the standard protocol uses ^{3}H-TdR.

Dose selection

No additional animals are used for dose range finding. The current practice is to select at least 3 consecutive concentrations from the following range: 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, 0.25%, and 0.1% (weight/volume). The selection is made to provide the highest possible test concentration, limited by compatibility with the vehicle chosen (and the suitability of the resultant preparation for unoccluded dermal application), while avoiding dermal trauma or systemic toxicity. The test chemical is dissolved or suspended in an appropriate vehicle. Vehicle selection is important, and a variety of organic solvents is suitable. The following are recommended, in order of preference: acetone-olive oil (4:1) (AOO), acetone, dimethylformamide, methyl ethyl ketone, propylene glycol, and dimethylsulphoxide.[3] Whereas aqueous vehicles are not recommended, aqueous and aqueous-organic mixtures such as 3 to 1 acetone to water concentration have been used successfully.

Control Materials

The current Organization for Economic Cooperation and Development (OECD) positive control sensitizers hexyl cinnamic aldehyde, (HCA) 2-mercaptobenzothiazole, and benzocaine each have been evaluated in the LLNA. Results with these positive controls in the LLNA met the standards set by the OECD.[15] The strong sensitizer, 2,4-dinitrochlorobenzene (DNCB), also may be used as a positive control as it has produced consistent responses in the LLNA, including when tested in 2 recent international interlaboratory trials.[12,14] The recommended positive control material, hexyl cinnamic aldehyde, was tested independently by 5 laboratories over a dose range of 2.5%, 5.0%, 10.0%, 25.0%, and 50% (weight/volume) in AOO vehicle.[14] All 5 laboratories correctly identified HCA as a contact allergen, with comparable sensitivity. Recently, the stability with time of responses induced in the local lymph node assay by HCA has been evaluated in a single laboratory. Over a 10-month period, HCA elicited very similar results in the LLNA.[16]

Currently, there are no recommended negative controls neither for the LLNA or for the reference guinea pig methods. However, methyl salicylate, tested at 1%, 2.5%, 5%, 10%, and 20% (weight/volume) in AOO[12,17] and *p*-aminobenzoic acid tested at 0.5%, 1%, 2.5%, 5%, and 10% (weight/volume) in AOO[14] have been used successfully as negative control chemicals in interlaboratory validation studies. In common with other skin sensiti-

zation tests, a control substance for irritation has not been defined for the LLNA.

Data Collection and Analysis

In vivo ^{3}H-TdR incorporation into lymph node cell DNA associated with proliferation induced by application of a contact sensitizer (measured by liquid scintillation counting) is an objective and quantitative response. The data are expressed as mean dpm for each experimental group, and the stimulation indices (SIs) for each experimental group are determined as the increase in ^{3}H-TdR incorporation relative to concurrent vehicle-treated controls. A test material that, at 1 or more concentrations, causes an SI of 3 or greater is considered to have skin-sensitizing activity. Thus, whether the draining auricular lymph nodes are excised and pooled for each experimental group or for each individual animal, the three-fold or greater increase in proliferative activity compared with concurrent vehicle-treated control animals is the sole criterion for a classification of skin-sensitizing activity.

In cases in which individual mice are used for determining the mean dpm value for an experimental group, statistical analysis may be performed. The value of statistical analyses, either alone or in conjunction with the three-fold SI, has not yet been established and is still the subject of investigations. Whereas isotope incorporation is determined for individual mice, a mean dpm value ± standard error of the mean (SEM) is calculated for each experimental group. An SI is derived for each experimental group by dividing the mean dpm of that group by the mean dpm of the vehicle-control group. One approach to the development of statistical methods that may prove of value in the LLNA is as follows: The mean dpm values for each treatment group and the vehicle control group are normalized initially by obtaining their log value. Bartlett's test[18] is then used to examine the data for homogeneity of the variance within chemical treatment. When analysis of variance reveals significant differences in parametric data, experimental groups are compared with vehicle-treated controls by using Dunnett's *t*-test.[19] For nonparametric data, a Kurskal-Wallis test is used,[20] followed by Dunn's multiple comparison procedure.[21] Groups differing from vehicle-treated controls at the level of $P \leq .05$ are considered to be significantly different. Alternatively, if Bartlett's test for homogeneity of variance is not significant, comparisons with the control group (and other specific, pair-wise comparisons of groups) are based on the least significant difference criterion. If Bartlett's test is significant, these comparisons are based on Wilcoxon's rank sum test.

In addition, an estimate of the test material concentration required to produce a SI of 3 (EC_3) can be calculated. An advantage of the EC_3 determination is that data from the entire dose response curve are used to produce a single value of intrinsic potency.[14] The EC_3 value can be used to rank the relative skin-sensitizing potential of chemicals. Stronger sensitizers, such as DNCB and oxazolone, have lower EC_3 values than more moderate sensitizers such as hexyl cinnamic aldehyde and eugenol.[14] Dose-response analyses in the local lymph node assay, combined with the mathematical derivation of the lowest test concentration of a chemical required for a defined SI, such as the EC_3, provide a convenient, reliable, and realistic approach to evaluation of relative potency.[22]

An examination of the application of statistical analyses to the LLNA is continuing. At present, it is not clear whether, or in what way, an evaluation of statistical significance would add value to the interpretation of the LLNA. This, together with consideration of EC_3 values for measurement of relative potency, are areas of investigation that may pay dividends in the future, but are not currently part of the standard protocol.

Prevalidation of LLNA

Reproducibility

There are extensive data available on the intralaboratory reproducibility of the LLNA, some of which has been published[17,23] and some of which is based on unpublished individual laboratory experience. Table 1 summarizes some information regarding the reproducibility of LLNA results.

Reference Data

A variety of guinea pig tests has been developed for evaluation of the skin-sensitizing potential of chemicals. Among those most widely applied are the guinea pig maximization test (GPMT)[24,25] and the occluded patch test of Buehler (BT).[26-28] These 2 assays are the preferred guinea pig sensitization tests outlined in the current OECD 406 guideline for skin sensitization.[29]

The GPMT used for comparisons with LLNA results is based on, and similar to, that described

Table 1. Reproducibility of LLNA Quantitative Data

Chemical	*Test 1*	*Test 2*	*Test 3*	*Test 4*	*Test 5*	*Test 6*
DNCB—Laboratory 1	0.05*	0.03	ND†	ND	ND	ND
DNCB—Laboratory 2	0.06	0.05	ND	ND	ND	ND
DNCB—Laboratory 3	0.04	0.06	ND	ND	ND	ND
DNCB—Laboratory 4	0.06	0.09	ND	ND	ND	ND
DNCB—Laboratory 5	0.03	0.06	ND	ND	ND	ND
Isoeugenol	0.3	0.4	0.4	0.4	0.6	ND
Hexyl cinnamic aldehyde	7.9	6.9	9.6	8.7	4.0	9.2
Hexyl cinnamic aldehyde	7.6	7.2	8.8	9.5	10.0	11.9
Eugenol	5.1	6.1	10.5	11.9	14.5	ND
Methyl salicylate	NS‡	NS	NS	NS	ND	ND
Benzocaine	NS	NS	?§	NS	NS	NS

*Percent concentration required to give a stimulation index of 3.
†ND, Not done.
‡NS, Not a sensitizer.
§Not possible to determine an EC_3 value from the dose response data.

by Magnusson and Kligman,[25] which uses Freund's adjuvant. Albino Dunkin-Hartley guinea pigs, weighing approximately 350g at the start of each study, are used. Preliminary irritation tests are conducted to determine the concentrations of test substances suitable for induction of sensitization and for subsequent challenge. Guinea pigs are treated by a series of 6 intradermal injections in the shoulder region to induce sensitization. After 6 to 8 days, sensitization is boosted by a 48-hour occluded patch placed over the injection site. Twelve to 14 days later, the animals are challenged on 1 flank by a 24-hour occluded patch at the maximum nonirritant concentration. Challenge sites are scored for erythema (scale, 0-3) and edema 24 hours and 48 hours after removal of the patches. The European Commission (EC) guidelines state that a material is positive if 30% or more of the test animals have an erythema score of 1 or greater.[30]

The standard BT protocol uses an occluded topical patch technique for the induction and elicitation of contact sensitization.[26-28] The procedure requires 20 animals in the test (sensitized) group, 10 naive (control) animals for challenge, and 10 separate naive control animals for rechallenge. For induction, a single dorsal site is used for three 6-hour induction patches (applied occluded once per week to the same preshaven induction site on the dorsal surface of the test animals). After a 2-week rest period, the test and noninduced control animals receive 6-hour challenge patches at a naive skin site for the primary challenge. The same test animals and additional new control animals can be rechallenged by this procedure 7 to 15 days after primary challenge at any remaining naive skin sites. Reactions are graded for erythema 24 hours and 48 hours after patch removal, according to a 5-point grading scale. The grades "1," "2," and "3" denote increasing severity of erythema with grades of 1 or greater considered positive. The EC guidelines state that a material is positive if the incidence is 15% or greater.[30]

In addition to comparison of the LLNA with guinea pig sensitization test data, the LLNA also has been compared with human data.[23,31] Specifically, the LLNA has been compared with the human maximization test (HMT).[32-34] This method was designed specifically to provide a rigorous assessment of the skin sensitization potential of chemicals in humans. In principle, a group of 25 subjects receive 48-hour occlusive patch treatments with as high a concentration of test chemical as possible. This treatment is repeated 5 times over a 2-week period. If the substance is not sufficiently irritating, the irritancy is enhanced by prior treatment of the site for 24 hours with sodium lauryl sulphate before each 48-hour patch. The extent of sensitization in the panel is assessed by 48-hour treatments on a slightly irritated skin site with the maximum nonirritant concentration of the test substance. The challenge sites are scored at 48 hours and 96 hours after application. In essence, this procedure can provide a stringent assessment of intrinsic sensitization hazard and relative potency.

To define the role of the LLNA in predictive testing, results from the assay have been compared with predictions from both guinea pig and human tests. In some instances, the LLNA results

and the reference results (guinea pig or human) are presented together. In other cases, LLNA studies have been conducted with chemicals for which sensitization potential, or the lack thereof, is well known. Basketter and Scholes[35] investigated the correlation between results in the LLNA and those derived from the GPMT for materials that covered a range of chemical types and levels of skin-sensitization potency. Kimber et al[36] reported comparative analyses in which 24 chemicals of previously unknown contact sensitizing potential were evaluated in both the LLNA and the BT. The data reported show that the LLNA successfully identified those chemicals that were classified as moderate or strong skin sensitizers in the BT. Basketter et al[37] evaluated the performance of the LLNA with 25 chemicals for which GPMT or BT data were available. The 25 chemicals included preservatives, perfume ingredients, surfactants, plastics/resin chemicals, and oil additives. A high level of agreement between the results of the LLNA and guinea pig test data was found.

As stated, an essential point of comparison for the LLNA is with human data. Basketter et al[23,31] compared HMT results with those obtained with the LLNA for the same 38 chemicals, the former being a rigorous assessment of the sensitization potential of chemicals in humans. It was found that the LLNA identifies those chemicals that are significant human contact allergens and that the specificity of the assay is good.

LLNA Prediction Model

For the purposes of developing a criterion for identification of contact allergens, an EC_3 relative to background cell turnover measured in concurrent vehicle-treated controls was proposed as an empirical arbiter. This value was chosen on the basis of previous experience with the LLNA and a high level of discrimination between contact allergens and nonsensitizing chemicals. Since that proposal was first adopted in 1990, a number of independent laboratories have gained greater experience with the method and in excess of 100 additional chemicals have been tested. The accumulated data reveal that the use of an EC_3 continues to provide an accurate and reliable criterion for the identification of skin-sensitizing chemicals. However, as discussed in a review article published in 1992,[3] although the three-fold SI provides a very useful criterion for judging sensitizing activity, in practice, a dose-related increase in proliferative activity that approaches, but does not reach, EC_3 may trigger a repeat analysis with higher application concentrations and/or an alternative application vehicle.[38] In this context, the potential utility of a higher or lower SI for the identification of sensitizing activity has been considered, but there is no evidence that this would enhance further the specificity or selectivity of the method.

Validation of LLNA

The first collaborative validation trial of the LLNA involved 4 independent laboratories in the United Kingdom, each of which evaluated 8 chemicals by using the same protocol, vehicles, and test concentrations. Each laboratory identified DNCB, formaldehyde, eugenol, isoeugenol, paraphenylenediamine, and potassium dichromate as positive, with benzocaine and methyl salicylate as negatives. With the exception of isoeugenol, no significant differences between the laboratories were found in regard to the characteristics of dose-response curves.[39]

The same 4 laboratories participated in a more extensive evaluation of 25 chemicals.[37] Of the chemicals tested, equivalent predictions of sensitizing potential were made for 18 chemicals by all laboratories. An additional 5 chemicals were identified as potential sensitizers in the LLNA by 2 or 3 laboratories. Three of these chemicals subsequently gave a positive response in laboratories that initially failed to detect them when retested under identical or altered conditions (e.g., higher concentration, different vehicle). It should be noted that these investigations were conducted before publication of the definitive LLNA protocol. For the final phase of this national collaboration, 9 chemicals were evaluated, and each laboratory independently selected the test concentrations and vehicles.[40] This study employed what is now the standard LLNA protocol: applying chemicals topically for 3 consecutive days and then terminating the experiment 5 days after the initiation of exposure. Chemicals were evaluated at 3 concentrations that were chosen independently by each laboratory in regard to potential toxicity and solubility. The choice of vehicle was based on solubility and viscosity. For 8 chemicals, equivalent predictions were made by all laboratories and by 3 of the 4 laboratories for the remaining chemical. Identical vehicles and concentrations were se-

lected independently by all laboratories for 2 chemicals and by 3 laboratories for 6 chemicals. In those cases where different concentrations or vehicles were chosen, equivalent predictions (positive or negative LLNA results) were nevertheless made. It is interesting to compare these results with those from unblinded interlaboratory studies of the GPMT and the BT.[28,41] In these instances, relatively poor interlaboratory reproducibility was achieved, which is in sharp contrast to experience with the LLNA.

To determine what effect, if any, minor protocol modifications would have on the predictive value of the test, the LLNA was evaluated in an international collaborative study by 5 independent laboratories, 2 of which had participated in the United Kingdom national validation exercise. Modifications to the standard protocol included exposure of mice for 4, rather than 3 consecutive days; removal of auricular lymph nodes 4 rather than 5 days after study initiation; and the use of an alternative isotope and analysis of lymph nodes pooled from individual mice rather than from experimental groups to allow for statistical evaluation (reviewed in references 42 and 132).

In the first phase of this international evaluation, 2 skin sensitizers, DNCB and potassium dichromate, and 1 nonsensitizer, methyl salicylate, were examined.[12] For the purpose of this investigation, in the laboratories analyzing nodes from individual mice, a positive result, in addition to an SI of 3 or higher, was also defined as treatment groups differing from vehicle-treated controls at a predetermined level of statistical significance ($P < .05$ or $P < .01$, depending upon the statistical method employed). By either criterion, and regardless of the protocol used, all 5 laboratories identified the 2 known sensitizers as being positive in the LLNA. Mathematically derived estimates of the test concentration required to yield an EC_3 value were very similar for all laboratories for both chemicals. Using the SI criterion, all laboratories reported a negative finding for methyl salicylate at all concentrations tested. Two of the 3 laboratories evaluating nodes from individual mice did detect a statistically significant increase in radioisotope incorporation at the highest of the 5 concentrations of methyl salicylate tested (20%).

In the second phase of the international collaborative trial, the sensitivity and selectivity of the assay were examined further by analysis of 6 additional chemicals: HCA, oxazolone, isoeugenol, eugenol, sodium lauryl sulphate (SLS), and *p*-aminobenzoic acid (PABA).[14] The last 2 are considered to be nonsensitizing chemicals, whereas the others exhibit skin-sensitizing potential to varying extents, with HCA being 1 of 3 chemicals recommended by the OECD for use as positive controls in skin sensitization studies.[29] All laboratories retested DNCB under the same conditions employed in phase I of the trial[12] to provide information on the temporal stability of the assay. All 5 laboratories identified as positive the 5 moderate-to-strong sensitizers (DNCB, HCA, oxazolone, isoeugenol, and eugenol). SLS, considered to be a nonsensitizing skin irritant, also induced weak positive responses in the assay. PABA, a nonsensitizing chemical, was negative in each laboratory. Results with HCA, eugenol, isoeugenol, and PABA were similar to previously published LLNA data.[15,31,35]

The results of Phases I and II provide strong support that the incorporation of minor procedural modifications did not affect the performance of the LLNA. An important modification assessed during Phases I and II of this international validation study was the analysis of proliferation within lymph nodes of individual mice as opposed to lymph nodes pooled for each experimental group. In the majority of cases, the lowest concentration yielding a positive response was identical by either method of analysis. One objective of Phase II was to examine interexperimental variability by re-evaluating DNCB. Of the 5 laboratories, 3 obtained identical results to those obtained in the first study.[12] Depending on which of the criteria was used, the other 2 participating laboratories had either identical interexperimental results or were within 1 adjacent concentration level. Therefore, the intralaboratory interexperimental variability was very low.

The overall conclusion from this phase and the previous phases of the evaluation studies[12] is that 5 independent laboratories, despite the use of minor procedural modifications and different methods for data analysis, successfully and consistently reached identical conclusions on the sensitizing potential of 9 chemicals with the LLNA.

The most recent interlaboratory validation study involved the same 5 laboratories, working in collaboration with the United States Food and Drug Administration. In this study,[17] a small series of chemicals used in topical drug products was examined. Again, there was very close agreement be-

tween laboratories, with all 5 correctly classifying benzoyl peroxide, hydroquinone, penicillin G, and methyl salicylate. Streptomycin sulfate induced equivocal responses, insofar as this material provoked a positive LLNA response in only 1 of the 5 laboratories, and only at the highest concentration tested. Ethylenediamine dihydrochloride response was uniformly negative. Collectively, these data serve to confirm that the LLNA is sufficiently robust to yield equivalent results when performed independently in separate laboratories. The data indicate also that the LLNA is of value in assessing the skin-sensitization potential of topical medicaments.

A total of 7 laboratories has been involved in interlaboratory validations of the LLNA. The results of the work have appeared in the several associated publications.[12,14,17,37,39,40] This work has involved the investigation of more than 40 different chemicals.

Good Laboratory Practice (GLP) Compliance

GLP is intended to assure the quality and integrity of data. When data are intended for submission to a regulatory agency, studies are conducted with strict adherence to GLP as dictated by the agency involved. Because the majority of the validation work for the LLNA used test materials with known sensitization potential, as determined by other test methods or by documentation as human contact allergens, no submissions were intended. Therefore, the studies did not adhere strictly to some regulatory GLP requirements. However, the LLNA validation studies did observe the basic principles that constitute GLP: The studies followed a sound protocol, were conducted by qualified personnel, were controlled by written and understood standard operating procedures, were conducted in proper and adequate facilities using calibrated and fully maintained equipment, were thoroughly documented, and have fully retrievable raw data.

Discussion

It is concluded that the LLNA provides a viable alternative method for use in the identification of skin-sensitizing chemicals and for confirming that chemicals lack a significant potential to cause skin sensitization. This does not necessarily imply that the LLNA should be used in place of guinea pig tests in all instances, but rather that the assay is of equal merit and utility and may be employed as a full alternative in which positive and negative results require no further confirmation.

The LLNA is not an in vitro method and, as a consequence, will not eliminate the use of animals in the assessment of contact-sensitizing activity. However it will permit a reduction in the number of animals required for this purpose. It has been estimated that, in practice, half the number of animals (on average) required for a standard guinea pig test is needed to conduct an LLNA. Moreover, the LLNA does offer a substantial refinement of the way in which animals are used for contact sensitization testing. One important point is that, unlike some of the guinea pig methods such as the GPMT, the LLNA does not require the use of an adjuvant. Furthermore, the LLNA is based on consideration of immunobiological events stimulated by chemicals during the induction phase of sensitization. Therefore, unlike guinea pig tests, the LLNA does not require that challenged-induced dermal hypersensitivity reactions be elicited.

Because of the fact that the LLNA requires far fewer animals than is needed for standard guinea pig tests, it can be conducted for approximately half the cost. The time taken for conduct of an LLNA is some 8 times less than that needed for a standard guinea pig method.

The predictive power of the LLNA, in comparison with standard guinea pig methods, is provided in Table 2. This type of information has been reviewed in detail in a recent article.[23] Although the LLNA may not be quite as sensitive as the GPMT, it is of similar or greater sensitivity than the BT. It is important to note that this comparison is accurate only when the guinea pig tests have been conducted to the very highest standards. In terms of predictive identification of important skin

Table 2. Comparison of LLNA and Guinea Pig Classifications

	Guinea Pig Positive	*Guinea Pig Negative*	*Unclear*	*Total*
LLNA classification				
LLNA Positive	83	6	0	89
LLNA Negative	9	28	0	37
Total	92	34	0	126

NOTE. Guinea pig classifications are based on GPMT or Buehler results; some of the results are derived from nonstandard GPMT guinea pig tests. Sensitivity, 90%; prevalence, 2.71; specificity, 82%; positive predictivity, 93%; negative predictivity, 76%; accuracy, 88%; χ^2 test, 59.58 ($P < .001$).

sensitizers, the LLNA is at least as sensitive as, and much more reliable than, current guinea pig tests. Of the 126 chemicals tested in the reference guinea pig tests, 88% gave identical results in the LLNA and the guinea pig assays.

Table 2 allows comparison of the in vivo classifications of skin sensitization in the guinea pig test with the in vivo predictions obtained in the LLNA. This procedure is a standard means of assessing data from validation studies.[43] However, it is important to point out that not all the guinea pig results are based on data generated by a standard protocol. Moreover, the guinea pig classifications are derived from both GPMT and BT studies. With these limitations in mind, the accuracy of the prediction of the LLNA amounts to 88%, with a sensitivity of 90% and a specificity of 82%. The test is characterized by a high positive predictivity of 93% and by a negative predictivity of 76%. Obviously, the LLNA does an excellent job of correctly identifying chemicals that are classified as skin sensitizers in the guinea pig tests. The high χ^2 value confirms that the classification of test chemicals by the LLNA is significant ($P < .001$) in comparison with the guinea pig tests. Overall, the results reveal a high level of concordance between the LLNA and guinea pig data in the determination of the skin-sensitization potential of a wide range of chemicals.

There are a few chemicals for which there is discord in results between the LLNA and guinea pig or human test methods. It is important, however, to emphasize that comparisons between LLNA data and the results of guinea pig tests should be viewed with caution. Guinea pig test data cannot be regarded as representing the gold standard in skin sensitization testing. Thus, it should not be concluded, for instance, that the failure of the LLNA to identify as a contact allergen a chemical that is known to elicit a positive response in a guinea pig test necessarily suggests a false negative in the former method. A case in point is sulphanilic acid, a chemical that is positive in the GPMT but fails to provoke a response in the LLNA. There is compelling evidence that sulphanilic acid fails to induce ACD in humans despite extensive occupational exposure.[44] In contrast to the case of sulphanilic acid, ammonium thioglycollate, an important occupational contact allergen, notably among hairdressers, was positive in the LLNA but was found not to give a significant response in the GPMT. This particular chemical would be expected to test positive in a predictive assay. Ethylene glycol dimethyacrylate (EGDMA) produced a positive LLNA response but produced a negative response in GPMT. Acrylate allergy is a complex subject, with many acrylate derivatives being suspected of causing at least some degree of clinical disease. In the case of EGDMA, the LLNA result may be the more accurate reflection of the true importance of this substance as a potential human contact allergen; however, the clinical evidence is lacking.

Guinea pig or mouse data may not always mirror precisely and quantitatively the extent of hazard to humans. Benzocaine, a substance selected as an OECD positive control for skin sensitization,[29] has proven to be notoriously difficult with respect to obtaining reliable/reproducible positive results in either the LLNA or the GPMT.[15] Although it is well known as a human skin sensitizer, 1 of its most common presentations arises from its use in pruritus ani. In this situation, it is the repeated semiocclusive exposure to inflamed mucosal tissue that renders a rather weak allergen positive. In contrast to the previously mentioned situation with ammonium thioglycollate is the preservative propyl paraben. It is negative in both the LLNA and GPMT.[35] This is not altogether surprising, because, except for behaving as a medicament allergen, notably in stasis ulcers, it is a very rare skin sensitizer, despite extensive skin exposure (e.g., from cosmetics). Therefore, it is unreasonable to expect a normal predictive skin sensitization test to identify this substance as an allergen. Neither nickel chloride nor nickel sulphate produced clear positive results in the standard LLNA. In contrast, although nickel has been documented as a difficult allergen in predictive tests per se,[45] positive results can be obtained in the GPMT. Although nickel is a common allergen, it is not a strong allergen, because it is the extensive and intimate exposure of humans, such as in pierced ears, that results in the high incidence of allergy. Thus, the conclusion is that the failure of the LLNA to identify nickel salts as allergens is as unsurprising as it is unimportant.

Comparison of skin sensitization data from predictive tests such as the GPMT and the LLNA with human clinical information is far from simple. Clinical data are complicated by the varying nature and extent of exposure to which individuals may have been subjected together with their individual sensitivities. Thus, it is easy to confuse a

strong allergen with a common one such as nickel or to expect that the parabens esters or lanolin should be positive in predictive tests because clinicians often refer to these as allergens. In this latter case, skin allergies are observed, but most commonly in specialized groups of patients (stasis eczema/medicament allergy) who present dermatologists with particular problems. However, it is evident from the large list of chemicals in Table 3 that the LLNA is capable of detecting essentially all of the major human contact allergens. It is worth repeating here what has been said elsewhere about metals: The precise mechanisms of metal allergy are probably rather different than those for organic chemicals. In addition, it is known which metals are allergens and which are not, and given that new metals are not being invented, the ability of the LLNA, or indeed any other predictive sensitization assay, to detect metal allergens is irrelevant to the main need, which is the identification of new organic chemical skin sensitizers.

From our current knowledge of the mechanism of skin sensitization to organic chemicals, and what is known of the immunology of guinea pigs, mice, and man, it is not expected that the LLNA will face special problems. Little is known of the impact of interspecies differences in skin metabolism of prohaptens and its importance in predictive testing. What limited information exists has suggested that there may be species differences,[46] but examination of the concordance in the identification of skin sensitizers implies that these may not be of major practical importance.

One question commonly asked about skin sensitization tests concerns their ability to discriminate allergens from irritants. This question has been posed for the LLNA[47] as it has for the guinea pig maximization test.[48,49] In practice, all guinea pig skin sensitization tests may have such difficulties, and strategies for dealing with them are available.[48,50] The LLNA deals well with irritancy; it is not a confounding factor for dose selection, and the majority of irritants are negative in the assay. Strategies for dealing with potential false positives in the LLNA and other predictive skin sensitization tests have been reviewed recently.[51]

If the LLNA is considered to be an acceptable alternative, then this assay will continue to be used ever more widely as the first-choice method for assessment of the skin sensitization potential of an unknown chemical. The limitations of the assay are minor compared with its advantages. They comprise the inability to evaluate the elicitation response and to test for cross-challenge reactions. This latter item is of some use in research but rarely forms part of testing for regulatory purposes, which is the reason for this assay validation.

The LLNA is already mentioned in detail in the main internationally accepted regulatory guideline describing test methods, namely, by the OECD,[29] where it is presented currently as a screening method. It is also similarly represented in European Union (EU) guidelines.[52] If the result is positive, then the chemical can be defined as a contact allergen. On the basis of this OECD update to the skin sensitization test guideline, the EC adopted the LLNA as a screening method acceptable for the identification of skin sensitizers, which, in its view, should be formally classified and labeled as such.[30] Chemicals so classified would carry the R43 risk phrase, "May cause sensitization by skin contact." However, both the OECD and EC guidelines currently state that when the result of the LLNA is negative, it is necessary to conduct a confirmatory guinea pig test with a standard protocol. It is important to point out that these guidelines were crafted before much of the LLNA validation work had been completed. In fact, the references cited in the OECD 406 guidelines dated from 1989 and 1990.

It is our view is that the LLNA should be employed as a stand-alone method for reaching decisions about the skin-sensitizing potential of chemicals. There would be no added value in using instead a battery of methods that included with the LLNA, for instance, analyses of skin penetration or identification of structural alerts by using structure-activity relationships. The LLNA provides a holistic, mechanistically based assessment of the ability of a test chemical to provoke the cutaneous immune response necessary for the induction of contact sensitization. If the tested chemical fails to gain access through the skin, or is unable to interact with protein to form an immunogenic hapten-macromolecular complex, then immune activation will not be initiated, and sensitization will fail to develop.

Recently, it was stated that the LLNA has been extensively and rigorously validated against both animal and human data and that the assay should be adopted by the OECD and accepted by the EU as a suitable method for classification purposes for skin sensitization.[53] In the light of advancing knowl-

Table 3. Comparison of LLNA to Standard Guinea Pig and Human Test Methods

Chemical Name	*CAS Number*	*LLNA*	*GPMT/BT**	*HMT*
Abietic acid	514-10-3	+	+	
2-Acetamidofluorene	53-96-3	−		
2-(*N*-acetoxy-acetamido)fluorene		+		
3-Acetylphenyl benzoate		+	+	
4-Acetylphenyl benzoate	1523-18-8	−		
C_{16}-1,3-alkene sultone		+	+†	
4-Allylanisole	140-67-0	+	+	
4-Aminobenzoic acid	150-13-0	−	−	−
2-Aminophenol	95-55-6	+	+†	
3-Aminophenol	591-275	+	+†	
Ammonium tetrachloroplatinate	13820-41-2	+	+	
Ammonium thioglycollate	5421-46-5	+	−	
Aniline	62-53-3	−	+	+
β-Propiolactone	57-57-8	+		
Benzalkonium chloride	8001-54-5	−	−	
3-(Benzenesulphonyloxymethyl)-5,5-dimethyldihydro-2(3H)-furanone		−		
Benzene-1,3,4-tricarboxylic anhydride		+	+	
1,2-Benzisothiazolin-3-one		+	+	
Benzo[a]pyrene	50-32-8	+		
Benzoquinone	106-51-4	+	+	
Benzoyl chloride	98-88-4	+	+	
Benzoyloxy-3,5 benzene dicarboxylic acid		−	+†	
Benzoyl peroxide	94-36-0	+	+	
Benzyl bromide	100-39-0	+		
Beryllium sulphate	7787-56-6	+	+	+
1-Bromobutane	109-65-9	−		
1-Bromododecane	143-15-7	+	+†	
12-Bromododecanoic acid	73367-80-3	+		
12-Bromo-1-dodecanol	3344-77-2	+		
1-Bromohexadecane	112-82-3	+	+	
1-Bromohexane	111-25-1	+	+†	
3-Bromomethyl-3-dimethyldihydrofuranone		+	+	
1-Bromopentadecane	629-72-1	+		
7-Bromotetradecane		+		
2-Bromotetradecanoic acid	10520-81-7	+		
2,3-Butanedione	431-03-8	+		
Butyl glycidyl ether	2426-08-6	+	+	+
$C_{12,13}$-β branched primary alcohol sulphate		+		
Camphorquinone	465-29-2	+		
Chloramine T	10599-90-3	+	+	
4-Chloroaniline	106-47-8	+	+	
Chlorobenzene	108-90-7	−	−	
3-(Chlorobenzenesulphonyloxymethyl)-5,5-dimethyl dihydro-2(3H)-furanone		−		
2-Chloroethanol	107-07-3	−		
2-Chloromethylfluorene		+		
(Chloro)methylisothiazolinone	55965-84-9	+	+	
5-Chloro-2-methyl-4-isothioazolin-3-one	26172-55-4	+	+	
1-Chloromethylpyrene	1086-00-6	+		
1-Chlorononane	2473-01-0	+		
1-Chlorooctadecane	3386-33-2	+		
1-Chlorotetradecane	2425-54-9	+		
Chlorpromazine	69-09-0	+	+†	+
Cinnamic aldehyde	104-55-2	+	+	+
Citral	5392-40-5	+	+	+
Clotrimazole	23593-75-1	+		

Table 3. Comparison of LLNA to Standard Guinea Pig and Human Test Methods (Cont'd)

Chemical Name	*CAS Number*	*LLNA*	*GPMT/BT**	*HMT*
Cobalt chloride	7646-79-9	+	+	+
Cocoamidopropyl betaine	59141-98-9	+	+	
Copper chloride	7758-89-6	+	−	
Dextran	9004-54-0	−	−	
1,2-Dibromo-2,4-dicyanobutane	35691-65-7	+	+	
2,4-Dichloronitrobenzene	611-06-3	−	−	
Diethylenetriamine	111-40-0	+	+	+
Diethyl sulphate	64-67-5	+		
Di-2-furanylethanedione	492-94-4	−		
3,4-Dihydrocoumarin	119-84-6	+		
Dihydroeugenol	2785-87-7	+	+	
3-Dimethylaminopropylamine	109-55-7	+	+	
7,12-Dimethylbenz[a]anthracene	57-97-6	+		
Dimethyl isophthalate	1459-93-4	−	−	
5,5-Dimethyl-3-(mesyloxymethyl)dihydro-2(3H)-furanone		−	+†	
5,5-Dimethyl-3-(methoxybenzenesulphonyloxymethyl) dihydro-2(3H)-furanone		−	+†	
5,5-Dimethyl-3-methylenedihydro-2(3H)-furanone		+	−†	
5,5-Dimethyl-3-(nitrobenzenesulphonyloxymethyl) dihydro-2(3H)-furanone		−	+†	
Dimethyl sulphate	77-78-1	+		
5,5-Dimethyl-3-(thiocyanatomethyl)dihydro-2(3H)-furanone		+	+†	
5,5-Dimethyl-3-(tosyloxymethyl)dihydro-2(3H)-furanone		−	−†	
2,4-Dinitrochlorobenzene	97-00-7	+	+	
2,4-Dinitrofluorobenzene	70-34-8	+		
2,4-Dinitrothiocyanobenzene	1594-56-5	+	+	
Diphenylmethane-4-4′diisocyanate	101-68-8	+	+	
Disodium benzoyloxy-3,5-benzenedicarboxylate		−	−	
Disodium 1,2-diheptanoyloxy-3,5-benzenedisulphonate		+	+†	
Ditallowdihydropropenetrimethyl ammonium		−	−	
Dodecyl methanesulphonate	51323-71-8	+	+†	
Dodecyl thiosulphonate		+	+	
Ellipticine	519-23-3	+		
Ethylenediamine	107-15-3	+	+	
Ethylene glycol dimethacrylate	97-90-5	+	−	
Ethyl methanesulphonate	62-50-0	−		
1-Ethyl-3-nitro-1-nitrosoguanidine		+		
N-Ethyl-*N*-nitrosourea	759-73-9	+		
Eugenol	97-53-0	+	+	
Fluorescein isothiocyanate	25168-13-2	+		
Formaldehyde	50-0-0	+	+	+
Geraniol	106-24-1	−	−	−
Glycerol	56-81-5	−	−	
Glyoxal	107-22-2	+	+	+
Gold chloride	16903-35-8	+		+
Hexadecanoyl chloride	112-67-4	+		
Hexane	110-54-3	−		−
Hexyl cinnamic aldehyde	101-86-0	+	+	
Hydrocortisone	50-23-7	−		+
Hydroquinone	123-31-9	+	+	
4-Hydroxybenzoic acid	99-96-7	−	−	
Hydroxycitronellal	107-75-5	+	+	+
2-Hydroxyethyl acrylate	818-61-1	+	+	
2-Hydroxypropylmethacrylate	923-26-2	−	−	
Imidazolidinyl urea	39236-46-9	+	+	
1-Iodohexadecane	544-77-4	+		
1-Iodohexane		+		

Table 3. Comparison of LLNA to Standard Guinea Pig and Human Test Methods (Cont'd)

Chemical Name	*CAS Number*	*LLNA*	*GPMT/BT**	*HMT*
1-Iodononane	4282-42-2	+		
1-Iodooctadecane		+		
1-Iodotetradecane	192-94-1	+		
Isoeugenol	97-54-1	+	+	
Isononanoyloxybenzene sulphonate		+	+	
Isophorone diisocyanate	4098-71-9	+	+	
Isopropanol	67-63-0	−	−	
Isopropylisoeugenol	29653-00-7	+	+	
Kanamycin	25389-94-0	−	−†	+
Lactic acid	50-21-5	−	−	
Lanolin	8006-54-0	−	−	
Lead acetate	15347-57-6	−		
2-Mercaptobenzothiazole	149-30-4	+	+	+
Mercuric chloride	7487-94-7	+	+	+
2 Methoxy-4-methyl phenol	5635-98-3	+	+	
3-Methoxyphenylbenzoate	5554-24-5	+		
4-Methylaminophenol sulphate	55-55-0	+	+	
3-Methylcatechol	488-17-5	+		
4-Methylcatechol	452-86-8	+	+	
6-Methylcoumarin	92-48-8	−	−	−
N'-(4-Methylcyclohexyl)-*N*-(2-chloroethyl)-*N*-nitros ourea		−		
Methyl dodecane sulphonate		+	+	
3-Methyleugenol		+		
5-Methyleugenol		+		
6-Methyleugenol		+		
Methyl hexadecane sulphonate		+	+†	
3-Methyl isoeugenol		+	+†	
Methyl methane sulphonate	66-27-3	+		
1-Methyl-3-nitro-1-nitrosoguanidine	70-25-7	+		
N-Methyl-*N*-nitrosourea	684-93-5	+		
Methyl salicylate	119-36-8	−	−	−
Methyl(2-sulphomethyl)octadecanoate		+		
2-Methyl-4,5-trimethylene-4-isothiazolin-3-one		+	+	
Musk ambrette	83-66-9	+	−	
α-Naphthoflavone	604-59-1	+		
β-Naphthoflavone	6051-87-2	+		
Neomycin sulphate	1405-10-3	−	−	
Nickel chloride	7718-54-9	−	+	
Nickel sulphate	10101-98-1	−	+	+
4-Nitrobenzyl bromide	100-11-8	+	+†	
4-Nitrobenzyl chloride	100-14-1	+	+†	
2-Nitrofluorene	607-57-8	−		
4-Nitroso-*N*,*N*-dimethylaniline	138-89-6	+	+	
Nonanoyl chloride	764-85-2	+		
Octadecanoyl chloride	112-76-5	+		
Octadecyl methane sulphonate	31081-59-1	−	+†	
Octyl gallate	1034-01-1	+		
Oxazolone	15646-46-5	+	+	
Penicillin G	61-33-6	+	+	+
Pentachlorophenol	87-86-5	+		+
Phenol	108-95-2	−		−
Phenyl benzoate	93-99-2	+	+	
3-Phenylenediamine	108-45-2	+	+†	
4-Phenylenediamine	106-50-3	+	+	+
Phthalic acid diethyl ester	84-66-2	−		
Phthalic anhydride	85-44-9	+	+	
Picryl chloride	88-88-0	+	+	

Table 3. Comparison of LLNA to Standard Guinea Pig and Human Test Methods (Cont'd)

Chemical Name	*CAS Number*	*LLNA*	*GPMT/BT**	*HMT*
Polyhexamethylene biguanide		+	+	+
Potassium dichromate	7778-50-9	+	+	+
β-Propiolactone	57-57-8	+		
Propylene glycol	57-55-6	−	−	
Propylgallate	121-79-9	+	+	
1-Propyl-3-nitro-1-nitrosoguanidine		+		
Propylparaben	94-13-3	−	−	+/−
Pyridine	110-86-1	+		+/−
Resorcinol	108-46-3	−	−	−
Salicylic acid	69-72-7	−	−	−
Sodium benzoyloxy-2-methoxy-5-benzene sulphonate		+	+†	
Sodium 4-(2-ethylhexyloxycarboxy)benzene sulphonate		+	+	
Sodium lauryl sulphate	151-21-3	+	−	−
Sodium norbornanacetoxy-4-benzene sulphonate		+	+†	
Sodium 4-sulphophenyl acetate		+	+†	
Streptozotocin	18883-66-4	−		
Sulphanilamide	63-74-1	−	−	+
Sulphanilic acid	121-57-3	−	+	
Tartaric acid	87-69-4	−	−†	
Tetrachlorosalicylanilide	7426-07-5	+	+	+
Tetradecyl iodide	19218-94-1	+		
Tetramethyl thiuram disulphide	137-26-8	+	+†	+
1-Thioglycerol	96-27-5	+	+	+
Tixocortol pivalate	55560-96-8	−		
Toluene diamine bismaleimide		+	+	
Toluene sulphonamide formaldehyde resin		−	−	
2,4,5-Trichlorophenol	95-95-4	+		
2,4,6-Trichloro-1,3,5-triazine	87-90-1	+		
Trimethylammonium-3-tolyl-ϵ-caprolactimide chloride		−		
α-Trimethylammonium-4-tolyloxy-4-benzene sulphonate		+	+†	
3,5,5-Trimethylhexanoyl chloride	36727-29-4	+	+	
Tween 80	9005-65-6	−	−	
Vinyl pyridine	1337-81-1	+		
Xylene	1330-20-7	+		−
Zinc sulphate	7733-02-0	+		

*Positive results based on EC classification threshold.
†Result obtained in a nonstandard guinea pig test.

edge and experience, and given animal welfare considerations, it is our opinion that the LLNA is now fully validated as a method for the identification of significant skin sensitizers and should therefore be adopted formally as an alternative skin sensitization test and incorporated fully into OECD guideline 406. Additionally, the LLNA is used successfully in contact sensitization risk assessment and has been reviewed in detail elsewhere.[22,54]

For practical purposes, the following recommendations are made for use of the LLNA: (1) a chemical that, at one or more test concentrations, elicits a three-fold or greater increase in proliferative activity compared with concurrent vehicle-treated controls should be classified as being a contact allergen and handled and labeled accordingly, and (2) chemicals that fail at all test concentrations to elicit a positive response in the LLNA should be classified as lacking significant skin-sensitizing potential and should be handled and labeled accordingly. No further confirmation of negative results is required.

The LLNA has undergone extensive development, evaluation, and validation. It has demonstrated its value as a reliable and robust method for assessment of the contact sensitization potential of chemicals. The LLNA should be now formally adopted as a stand-alone method for regulatory purposes.

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