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Research Section

Threshold for Classification as a Skin Sensitizer in the Local Lymph Node Assay: a Statistical Evaluation

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Abstract—For more than 15 years, the murine local lymph node assay (LLNA) has undergone development, evaluation and validation as an alternative approach to the predictive identification of skin sensitizing chemicals. The criteria by which sensitizing chemicals are distinguished from those without significant skin sensitising hazard were developed empirically and were based on experience rather than a mathematical formula or statistical method. The current practice is to classify, as skin sensitizers, those chemicals which at one or more test concentrations stimulate a threefold or greater increase in the proliferative activity in draining lymph node cells. Despite the apparent confirmation of the utility of this approach from the extensive data available, there has not previously been any attempt to substantiate the accuracy of this criterion. In this present investigations, data from 134 chemicals tested in the LLNA and in the guinea pig and/or for which there exists clear evidence relating to human skin sensitization potential, have been subjected to a rigorous statistical evaluation using Receiver Operating Characteristic (ROC) curves. Whether the analysis is based on a comparison with guinea pig or human data, the results indicate that the empirically derived threefold threshold is an acceptable practical value for hazard identification.

Keywords: local lymph node assay; skin sensitization; threshold; classification.

Abbreviations: LLNA = local nymph node assay; LNC = lymph node cell; ROC = receiver operating characteristic; SI = stimulation index.

INTRODUCTION

The murine local lymph node assay (LLNA) has been developed (Basketter *et al*., 1996; Chamberlain and Basketter, 1996; Gerberick et al, 1999; Kimber and Weisenberger, 1989) as a mechanistically based alternative to the commonly used guinea pig methods (Andersen and Maibach, 1985; Botham *et al*., 1991) for the prospective identification of contact sensitizing chemicals. The LLNA offers important animal welfare advantages as well as scientific benefits, without compromising the standards required for the regulatory identification of skin sensitizers (Basketter *et al*., 1995; Gerberick *et al*., 1999). Advantages include an objective and quantitative endpoint derived using the relevant route of exposure, together with avoidance of the use of adjuvant, intradermal injection of the test substance or the need for clipping and shaving of test sites.

The scientific basis for the LLNA is that topical exposure to a contact allergen stimulates immune activation in draining lymph nodes. The important immunological events associated with the induction of cutaneous immune responses have been reviewed elsewhere (Kimber and Dearman, 1997). A requirement for skin sensitization is that the inducing allergen is delivered in an immunogenic form to draining lymph nodes. There, allergen responsive T lymphocytes are activated and stimulated to divide and differentiate, the induced cell division resulting in the clonal expansion of allergen reactive T cells.

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Table 1. Catalogue of LLNA data and the guinea pig, human and EU "classifications"

Chemical	LLNA	GPMT/BT[#]	Human	EU classification
Abietic acid	+	+	+	+
3-Acetylphenylbenzoate	+	+		+
4-Allylanisole	+	+		+
2-Aminophenol	+	+*	+	+
3-Aminophenol	+	+*	+	+
Ammonium tetrachloroplatinate	+	+	+	+
Ammonium thioglycolate	+	–	+	+
1,2-Benzisothiazolin-3-one	+	+	+	+
Benzo[*a*]pyrene	+		+	+
Benzoquinone	+	+	+	+
Benzoyl chloride	+	+	+	+
Benzoyl peroxide	+	+	+	+
Benzyl bromide	+		+	+
Beryllium sulfate	+	+	+	+
1-Bromododecane	+	+*		+
1-Bromohexadecane	+	+		+
1-Bromohexane	+	+*		+
3-Bromomethyl-3-dimethyldihydrofuranone	+	+		+
Butylglycidyl ether	+	+	+	+
$C_{12\text{-}13}$-β branched primary alcohol sulfate	+		–	–
C_{16}-1,3-alkene sultone	+	+*	+	+
Chloramine T	+	+	+	+
4-Chloroaniline	+	+	+	+
(Chloro)methylisothiazolinone	+	+	+	+
Chlorpromazine	+	+*	+	+
Cinnamic aldehyde	+	+	+	+
Citral	+	+	+	+
Cobalt chloride	+	+	+	+
Cocoamidopropyl betaine	+	+	+	+
Copper chloride	+	–	–	–
Dibromodicyanobutane	+	+	+	+
Diethylenetriamine	+	+	+	+
Dihydroeugenol	+	+		+
3-Dimethylaminopropylamine	+	+	+	+
5,5-Dimethyl-3-methylenedihydro-2(3*H*)-furanone	+	+		+
5,5-Dimethyl-3-(thiocyanatomethyl)dihydro-2(3*H*)-furanone	+	–*		+
2,4-Dinitrochlorobenzene	+	+*	+	+
2,4-Dinitrothiocyanobenzene	+	+	+	+
Disodium 2-diheptanoyloxy-3,5-benzenedisulfonate	+	+		+
Dodecylmethanesulfonate	+	+*		+
Dodecylthiosulfonate	+	+*		+
Ethylene diamine	+	+	+	+
Ethylene glycol dimethacrylate	+	–	+	+
Eugenol	+	+	+	+
Formaldehyde	+	+	+	+
Glyoxal	+	+	+	+
Gold chloride	+		+	+
Hexyl cinnamic aldehyde	+	+	+	+
Hydroquinone	+	+	+	+
Hydroxycitronellal	+	+	+	+
2-Hydroxyethyl acrylate	+	+	+	+
Imidazolidinyl urea	+	+	+	+
Isoeugenol	+	+	+	+
Isopropylisoeugenol	+	+		+
Isononanoyloxybenzene sulfonate	+	+	+	+
2-Mercaptobenzothiazole	+	+	+	+
Mercuric chloride	+	+	+	+
2-Methoxy-4-methyl phenol	+	+	+	+
4-Methylaminosulfate	+	+	+	+
4-Methylcatechol	+	+		+
Methyl dodecane sulfonate	+	+		+
Methyl hexadecane sulfonate	+	+*		+
3-Methyl isoeugenol	+	+*		+
2-Methyl-4,5-trimethylene-4-isothiazolin-3-one	+	+	+	+
4-Nitrobenzyl bromide	+	+*		+
4-Nitrobenzyl chloride	+	+*		+
4-Nitroso-*N*,*N*-dimethylaniline	+	+		+
Octyl gallate	+	+	+	+
Oxazolone	+	+	+	+
Penicillin G	+	+	+	+
Pentachlorophenol	+		+	+
Phenyl benzoate	+	+		+
3-Phenylenediamine	+	+*		+
4-Phenylenediamine	+	+	+	+
Picryl chloride	+	+	+	+
Polyhexamethylene biguanide	+	+	+	+
Potassium dichromate	+	+	+	+
Propylgallate	+	+	+	+

Pyridine	+		–	–
Sodium lauryl sulfate	+	–	–	–
Sodium 4-(2-ethylhexyloxycarboxy)benzene sulfonate	+	+*		+
Sodium norbornanacetoxy-4-benzene sulfonate	+	+*		+
Sodium 4-sulfophenyl acetate	+	+		+
Tetrachlorosalicyloanilide	+	+	+	+
Tetramethyl thiuram disulfide	+	+*	+	+
1-Thioglycerol	+	+	+	+
Tin chloride	+		+	+
Toluene diamine bismaleimide	+	+		+
α-Trimethylammonium-4-tolyloxy-4-benzene sulfonate	+	+*		+
3,5,5-Trimethylhexanoyl chloride	+	+		+
Xylene	+		–	–
Aluminium chloride	–		–	–
4-Aminobenzoic acid	–	–	–	–
Aniline	–	+	+	–
Benzalkonium chloride	–	–	–	–
Benzoyloxy-3,5 benzene dicarboxylic acid	–	+*		–
Chlorobenzene	–	–		–
Dextran	–	–	–	–
2,4-Dichloronitrobenzene	–	–		–
5,5-Dimethyl-3-(mesyloxymethyl)dihydro-2(3*H*)-furanone	–	–		–
5,5-Dimethyl-3-(methoxybenzenesulphonyloxymethyl) dihydro-2(3*H*)-furanone	–	+*		+
5,5-Dimethyl-3-(nitrobenzenesulphonyloxymethyl)dihydro -2(3*H*)-furanone	–	+*		+
Dimethylisophthalate	–	–	–	–
5,5-Dimethyl-3-(tosyloxymethyl)dihydro-2(3*H*)-furanone	–	–*		–
Disodium benzoyloxy-3,5-benzenedicarboxylase	–	–		–
Ditallowdihydroxypropenetrimethyl ammonium	–	–	–	–
Geraniol	–	–	–	–
Glycerol	–	–	–	–
Hexane	–		–	–
Hydrocortisone	–		–	–
4-Hydroxybenzoic acid	–	–		–
2-Hydroxypropylmethacrylate	–	–		–
Isopropanol	–	–	–	–
Kanamycin	–	–*	+	–
Lactic acid	–	–	–	–
Lead acetate	–		–	–
Manganese chloride	–		–	–
6-Methylcoumarin	–	–	–	–
Methyl salicylate	–	–	–	–
Neomycin	–	–		–
Nickel chloride	–	+	+	+
Nickel sulfate	–	+	+	+
Octadecylmethane sulfonate	–	+*		+
Phenol	–		–	–
Propylparaben	–	–	–	–
Propylene glycol	–	–	–	–
Resorcinol	–	–	–	–
Salicylic acid	–	–	–	–
Sulfanilamide	–	–	+	–
Sulfanilic acid	–	+	–	–
Tartaric acid	–	–*	–	–
Tixocortol pivalate	–	+	+	+
Toluene sulfoamide formaldehyde resin	–	–		–
Zinc sulfate	–		–	–

[#]Positive results based on European classification threshold (EC, 1996).*Result obtained in a non-standard guinea pig test.

This represents the cellular basis of skin sensitization and it has been demonstrated previously that the vigour of T lymphocyte responses in skin draining lymph nodes correlates closely with the extent to which contact sensitization will develop (Kimber and Dearman, 1991).

In practical terms, an appreciation of the immunobiology of skin sensitization has been applied to the development of a relatively simple process for the predictive identification of contact allergens using the LLNA. Test chemical is applied topically daily, for 3 consecutive days, to groups of mice and the auricular lymph nodes draining the site of application are examined for evidence of cell division, measured by the incorporation of tritiated thymidine. The standard protocol is described in detail elsewhere (Kimber and Basketter, 1992). Activity in the LLNA is considered as a function of the vigour of lymph node cell (LNC) proliferative responses induced by the test chemical, compared with concurrent vehicle treated controls. Currently, the criterion for a positive LLNA response, and for classification of a chemical as a skin sensitizer, is when threefold or greater increase in LNC proliferative activity compared with concurrent vehicle treated controls is induced by one or more application concentrations of the test material. The selection of a stimulation index (SI) of 3 for the

definition of skin sensitization potential in the LLNA was empirical, being based on experimental observations and experience. Nevertheless, this criterion has proved in practice to provide a reliable arbiter of sensitizing activity.

It is appropriate now to consider whether this empirically derived measure of sensitizing activity in the LLNA provides the best possible means of discriminating between sensitizing and non-sensitizing chemicals. To this end, in the present work we have used an extensive dataset of LLNA results, produced according to the standard protocol (Kimber and Basketter, 1992) and where there exists either:

1. Guinea pig test results which would lead to a classification decision based on European criteria (EC, 1993),

or

2. Sufficient human data to permit an expert judgement of whether the substance should be regarded as a significant human skin sensitizer.

These data have been analysed using a statistical method that generates an objective assessment of the threshold stimulation index which would provide the optimum discrimination between those materials which represent a significant skin sensitization hazard from those chemicals which do not. It should be borne in mind that even chemicals generally not regarded as significant skin sensitizers, such as copper (Karlberg *et al.*, 1983) may under certain conditions give rise to episodes of allergic contact dermatitis. In practical terms, a distinction has to be made between those chemicals which present a significant hazard (and which should be identified in any test method for identification of contact allergens) and those which do not.

MATERIALS AND METHODS

Skin sensitization data

The data on which the statistical analyses have been based is collated in Table 1. Here data on 134 chemicals have been summarized from a number of sources. (It should be noted that this data was different to that used initially to derive the threefold criterion.) In every case, the LLNA was conducted using the standard protocol. Stimulation indices were derived by comparing the thymidine incorporation in the treated animals with that of the vehicle treated control group. The criterion for a positive LLNA response is that a SI of 3 or more is induced by one or more application concentrations of the test material compared with concurrent controls. Guinea pig data were derived from either the guinea pig maximization test (Magnusson and Kligman, 1970), the Buehler test (Buehler, 1965), or from a modified guinea pig maximization test (Roberts and Basketter, 1990). The results of guinea pig tests have been interpreted according to European criteria, but would also fit broadly with other regulatory criteria (reviewed in Basketter *et al.*, 1999), where the aim is to place the chemical into one of two categories "skin sensitizer" or "not classified". For the sake of simplicity, this latter category has been referred to as "non-sensitizer" in this paper. A total of 119 guinea pig tests were available for comparison.

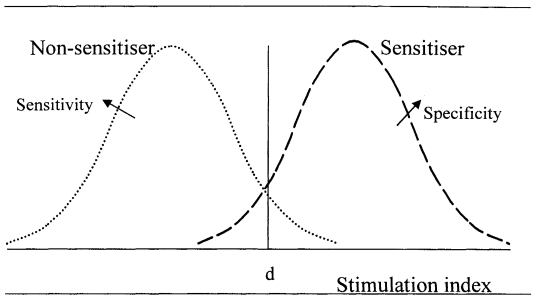

Fig. 1. This shows in theoretical form the distribution of SI values for groups of sensitizers and non-sensitizers tested in the LLNA. The line marked "d" indicates the SI value adopted as the cut-off to discriminate between these two groups. Movement of the line to the left enhances sensitivity, while movement to the right will enhance specificity.

Human data for comparison with the LLNA results, were taken from published work (Basketter *et al.*, 1994, 1996; Gerberick *et al.*, 1999; Kimber *et al.*, 1994; Kligman, 1966) aligned with out judgement, based on the available clinical studies, regarding evidence for significant skin sensitisation (De Groot *et al.*, 1994; Rietschel and Fowler, 1995).

In order to assign the chemicals to a regulatory classification as a skin sensitizer, the general principles outlined for such purposes by the European Union (EC, 1996) and the World Health Organization (WHO, 1997). Classification depends effectively on potency; substances which are weak skin sensitizers are not classified. The outcome of these considerations is reported in the final column of Table 1.

Statistical analyses

The basic methodology adopted was that described by Albert and Harris (1987), who describe statistical methods for measuring the efficiency of laboratory tests and interpreting individual test results which distinguish between two categories. For convenience, these categories have been designated as D and D^c and the assumption is that all chemicals fall into either one or other of these groups but not both. In the present case, D is the group of sensitizers and D^c the non-sensitizers and the test response being considered is the maximum stimulation index (SI) obtained for each chemical. Figure 1 illustrates hypothetical distributions of test values in each of the two groups of chemicals.

Although the stimulation index is a continuous variable, it is dichotomized by selecting a cut-off

Table 2. Theoretical considerations

	Stimulation index (SI)	
Population	$x \leqslant d$	$x > d$
Non-sensitizers	SP (d)	1-SP (d)
Sensitizers	1-SE(d)	SE

point (d) and defining a binary variable (Y), such that:

$$y = 0 \text{ when } x \leqslant d$$

$$y = 1 \text{ when } x > d$$

The specificity (SP(d)) of a test is defined to be the proportion of non-sensitizing chemicals for which $y = 0$ (i.e. $x \leqslant d$) and is the area under the non-sensitizer distribution of SI values to the left of the $x = d$ line. Conversely, the sensitivity (SE(d)) of a test is defined to be the proportion of sensitizing chemicals for which $y = 1$ (i.e. $x > d$) and is the area under the sensitizer distribution to the right of the $x = d$ line. These probabilities and their complements are displayed in Table 2.

Specificity and sensitivity depend on the choice of cut-off point d and vary inversely. Moving the cut-off point in Fig. 1 from right to left decreases specificity while increasing sensitivity. The classical graphical way of relating specificity and sensitivity to point d is called the receiver operating characteristic (ROC) curve (Swets, 1979). As illustrated in Fig. 2, the ROC curve is obtained by plotting the false positive rate (1-SP) versus the true positive rate (SE) for various values of d. A test is efficient it its ROC concentrates in the upper left corner where both specificity and sensitivity are high.

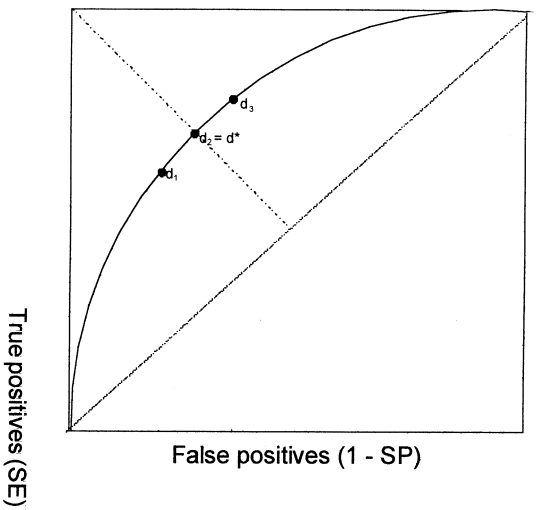

Fig. 2. The ROC plot curve for the LLNA is concentrated in the upper left corner, indicating a high degree of sensitivity and specificity for this assay.

Conversely, an inefficient test would have a ROC close to the line SE(d) = 1-SP(d).

Our aim is to find the cut-off point d on the SI scale that will provide on the basis of LLNA data an optimal discrimination between sensitizers and non-sensitizers, where these have been defined by either guinea pig or human data or by an EU classification. In practice, the optimal value depends on the relative importance of false positive and false negative rates.

Assuming equal weights on false positive and false negative rates there will exist a value of d* such that SP(d*) = SE (d*) =, designated S*, where $0.5 \leqslant S^* < 1$. The values 0.5 and 1 are unlikely to occur as 0.5 would imply the distributions of SI values for sensitizers are the same, whereas 1 implies total separation between sensitizers and non-sensitizers which is ideal in theory but unlikely in practice. The value of d* can be obtained by drawing a line perpendicular to the dotted line SE(d) = 1-SP(d). The point of intersection between the line and the ROC curve provides the required d*.

RESULTS

The mathematical analyses were performed using a large LLNA dataset of 134 chemicals where it was possible to identify whether the substance would be classified as a skin sensitizer on the basis of guinea pig and/or human data. It is important to note that this classification follows the general principles articulated in the legislation of the European Union and in the guidelines provided by WHO (reviewed in Basketter *et al.*, 1999). The chemicals tested their classification (" + " = skin sensitizer; "–" = non-sensitizer) based on either LLNA data, guinea pig tests or on human information are presented in alphabetical order, positives followed by negatives, in Table 1. Also included is our consideration of the ultimate classification as a skin sensitizer according to current regulatory criteria. Of the chemicals tested, only 14 gave discordant results when the LLNA classification was compared with the guinea pig, while nine chemicals gave discordant results in the human/LLNA comparison. Just six chemicals were represented in both discordant groups. The distribution of all 134 chemicals in terms of their maximum SI value in the LLNA and whether they were regarded as sensitizers in terms of EU classification standards is displayed in Fig. 3.

The results of the ROC analyses based on the guinea pig data, human data and EU classification are presented in Table 3(a). These demonstrate that for the guinea pig data, a threshold stimulation index for a classification, which is not biased either to false positives or false negatives and which minimises the number of misclassifications, is 3.6. A similar ROC analysis carried out using the classification decisions derived from the human data

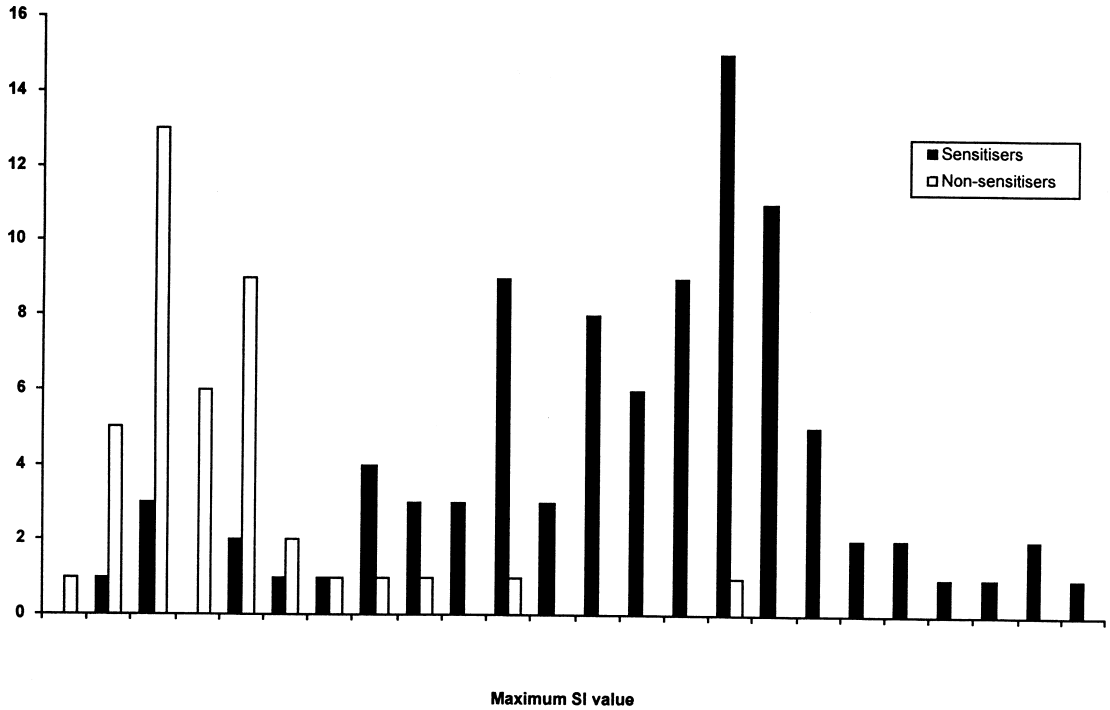

Fig. 3. Distribution of maximum SI values for the 134 chemicals tested. Chemicals regarded in EU classification terms as skin sensitizers are represented by the shaded columns, those regarded as non-sensitizers are represented by the open columns.

shows that as with the guinea pig, the optimum cut-off point on the stimulation index scale occurred at a value of 3.6.

As the LLNA is intended for use specifically for hazard identification in the context of regulatory toxicology, it is of interest to see how classification based on LLNA data compare to those made on the basis of judgement on the existing guinea pig/human data. This optimum threshold SI value from this ROC analysis is 3.4. In this case, the sensitivity and specificity of predictions are 90% or greater. In practical terms, with the dataset used, there are only two substances for which the LLNA classification would actually be changed by the adoption of a threshold value of 3.6, rather than the value of 3 used at present. These are xylene, which would no longer be a false positive, and α-trimethylammonium-4-tolyloxy-4-benzene-sulfonate, which would become a false negative. For comparative purposes, Table 3(b) shows how the sensitivity and specificity of the ROC analyses are affected by using a cut-off point of 3.0 rather than the values of 3.6 for guinea pig and human data or 3.4 for the EU classification. As is shown, the actual difference is very small, with for example the LLNA based prediction of skin sensitizers rising from 92% to 93% in comparison with EU classification when the threshold cut-off value is held at 3.0.

DISCUSSION

The first step in a toxicological evaluation is hazardous identification. For skin sensitization, this step has normally been achieved using one of two guinea pig assays (Buehler, 1965; Magnusson and Kligman, 1970). These protocols have been adopted by regulatory authorities such as those embodied in the European Union and criteria for test interpretation have been defined (EC, 1996). The approach adopted has been to discriminate substances which possess significant skin sensitization hazard from those which do not. Thus, a chemical which gives at least a 30% positive response in the Magnusson and Kligman test is classified as a skin sensitizer. A cut-off value of 15% was selected for the slightly less rigorous test protocol described by Buehler.

During the development of the LLNA, experience indicated that when the stimulation index of 3 was reached or exceeded, sensitizing chemicals could be effectively distinguished from non-sensitizers. It is true to say also that at this "threshold" the result emerged from the "background noise" in the assay. With increasing experience, but always based on observation, the use of a value of 3 to distinguish sensitizers was challenged, but was found in practice to be extremely satisfactory (see for example Kimber *et al.*, 1994). Ultimately, using this criterion

Table 3a. ROC analysis based on guinea pig data

	Guinea pig data		Human data		EU classification	
	SI value		SI value		SI value	
Population	$x \leqslant 3.6$	$x > 3.6$	$x \leqslant 3.6$	$x > 3.6$	$x \leqslant 3.6$	$x > 3.6$
Non-sensitizers	27 (84%)	5 (16%)	25 (86%)	4 (14%)	38 (90%)	4 (10%)
Sensitizers	11 (13%)	76 (87%)	7 (11%)	57 (89%)	7 (8%)	85 (92%)

Table 3b. Comparative ROC analysis of the data in Table 3(a) using a cut-off point of 3.0

	Guinea pig data		Human data		EU classification	
	SI value		SI value		SI value	
Population	$x < 3.0$	$x \geqslant 3.0$	$x < 3.0$	$x \geqslant 3.0$	$x < 3.0$	$x \geqslant 3.0$
Non-sensitizers	27 (84%)	5 (16%)	24 (83%)	5 (17%)	37 (88%)	5 (12%)
Sensitizers	9 (10%)	78 (90%)	6 (9%)	58 (91%)	6 (7%)	86 (93%)

alone, the assay was considered to be valid as a stand-alone method for the predictive identification of skin sensitizers (Basketter *et al*., 1996). Subsequently, the LLNA incorporating the empirically derived threefold criterion was submitted for formal assessment of validation status (Gerberick *et al*., 1999). In the latter, the predictive accuracy of the LLNA was shown to be approximately 90%, making it equivalent to the guinea pig assays in terms of correctly identifying which chemicals were significant human skin sensitizers.

In the present investigation, we have carried out retrospective statistical analyses on a body of data based on that used for the validation submission (Gerberick *et al*., 1999). Applying no bias to the analysis in terms of a preference for false positives or false negatives, when either guinea pig or human data are used for comparison, the threshold stimulation index value can be calculated to be 3.6. This outcome is very close to, and supports as a practical figure, the value of 3 which was adopted on the basis of experimental observations over many years. By using the value of 3, rather than 3.6, the LLNA will have a slight tendency to identify more chemicals as skin sensitizers, at the expense of a small number of false positives. In the case of the present dataset, the overall sensitivity and specificity are hardly changed at all. When the statistical analysis was carried out using the EU classification of the chemicals based on the combined guinea pig and human evidence, then the optimum threshold SI value was 3.4. It seems that to err slightly on the side of caution is appropriate in order to ensure proper health protection; to fail to detect a contact allergen is potentially an important omission since once an individual is sensitized it is a lifelong potential health problem. The small cost of a cautious approach is mitigated by effective strategies for the identification of potential false positives which have been published and include examination of chemical structure, use of information on skin irritation potential and consideration of the magnitude of the LLNA response in relation to test concentration (Basketter *et al*., 1998). These should ensure that there is, in practice, no substantial problem of classifying as skin sensitizers substances which in reality possess no significant sensitizing potential. While a threshold SI value slightly above 3 might provide an equal balance between false positives and false negatives the adoption of the empirically derived threshold value of 3 of the identification of skin sensitizers in the LLNA has been endorsed fully by a rigorous statistical evaluation of the available data.

In consequence, it is recommended that a threshold value of 3 is adopted formally as the appropriate threshold for the identification of skin sensitizers in the LLNA.

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