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A quantitative method for assessing the sensitizing potency of low molecular weight chemicals using a local lymph node assay: employment of a regression method that includes determination of the uncertainty margins

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Abstract

Risk assessment of sensitizing chemicals requires, besides hazard identification, the assessment of potency. To examine the sensitizing capacity of low molecular weight chemicals, a murine local lymph node assay (LLNA) was used. The sensitizing capacity of known allergens was quantified by dose-response modeling. At a stimulatory index (SI) of 3, the corresponding estimated concentration was calculated (EC_3), together with a confidence interval to take account of the quality of the particular data set. We tested ten allergens (ethyl-p-aminobenzoate (benzocaine), diethylamine (DEA), 2,4-dinitrochlorobenzene (DNCB), 2-mercaptobenzothiazole (MBT), 4-ethoxymethylene 2-phenyl oxazol-5-one (oxazolone), phthalic anhydride (PA), toluene diisocyanate (TDI), trimellitic anhydride (TMA), tetramethylthiuramdisulfide (TMTD) and zincdimethyldithiocarbamate (ZDMC)). Oxazolone showed the strongest sensitizing potency followed in this order by DNCB, TDI, TMA, PA, TMTD, ZDMC, MBT, benzocaine and DEA. The approach performed in this study is a way to accurately assess the potency of sensitizing chemicals and thus a possibility for classification.

Keywords: EC_3 value; Local lymph node assay; Risk assessment; Sensitizers; Uncertainty distribution

1. Introduction

Methods for the identification of sensitization hazards have been available for many years. The tests most commonly used to identify skin sensitizing capacity are the guinea pig maximization test (GPMT) using adjuvant (Magnusson and Kligman, 1969) and the occluded patch test of Buehler without adjuvant (Buehler, 1965) in the guinea pig. More recently, the murine local lymph node assay (LLNA) (Kimber et al., 1986; Kimber and Weisenberger, 1989; Kimber and Basketter, 1992) was introduced and validated for various

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chemicals. The results obtained in these tests give the possibility for labeling and classification of sensitizing chemicals.

The GPMT has been used as the preferred method for predicting skin sensitization for over 25 years since it was first described. Although the GPMT is able not only to detect chemicals with moderate and strong sensitizing potential but also chemicals with relatively weak sensitizing potential (Robinson et al., 1990), it has its drawbacks compared to the LLNA. In contrast to the GPMT the LLNA is able to detect allergic potency based on a quantitative endpoint instead of visual (semi-quantitative) assessment of challenge induced erythema. Moreover, the GPMT has disadvantages including the use of an adjuvant and the length and complexity of the test.

The LLNA is a method for the predictive identification of chemicals that have the potential to cause sensitization. In the LLNA assessment of immune reactivity of an immune (allergy) response is determined in the induction phase. The uptake of [^{3}H]TdR by the local lymph node cells, as a response to the application of the test chemical to the mouse ear, is a measure for the immune response and thus can be used as a measure of sensitization.

Lymph node proliferative responses of treated animals are compared to those of non-treated or vehicle-treated animals. Chemicals that elicit a stimulation index (SI) of 3 or more in the LLNA are considered as being sensitizers. Currently, these EC_3 (estimated concentration in% required for $SI = 3$) values are used for the comparison of sensitizing potential derived from local lymph node responses (Kimber et al., 1995; Loveless et al., 1996; Kimber and Basketter, 1997).

In the GPMT the potency is based on the percentage of positive animals using a single dose. However, for determination of the potency based on lowest effective dose levels, dose-response studies are required. In the GPMT both for the induction and challenge phase dose-response studies would be needed. As the LLNA gives a more quantitative result, we feel that the LLNA is more suited for estimation of lowest effective doses in a dose-response study. To increase the sensitivity of the LLNA for weak allergens all animals were pretreated with sodium dodecyl sulphate (SDS).

In this study the known sensitizers ethyl-p-aminobenzoate (benzocaine), diethylamine (DEA), 2,4-dinitrochlorobenzene (DNCB), 2-mercaptobenzothiazole (MBT), 4-ethoxymethylene 2-phenyl oxazol-5-one (oxazolone), phthalic anhydride (PA), toluene diisocyanate (TDI), trimellitic anhydride (TMA), tetramethylthiuramdisulfide (TMTD) and zincdimethyldithiocarbamate (ZDMC) were evaluated.

2. Methods

2.1. Animals

Young adult (6–8 weeks old) male/female BALB/c strain mice were used for the experiments. They were obtained from our own breeding colony. The animals were bred specific pathogen free and kept under conventional conditions. The mice were fed Hope Farms chow pellets (Woerden, NL) and water ad libitum.

2.2. Chemicals

Benzocaine (ethyl-p-aminobenzoate; 99% purity; Sigma-Aldrich Chemie B.V., Zwijndrecht NL), DEA (diethylamine; 99.5%; free base solution; Sigma-Aldrich), DNCB (2,4-dinitrochlorobenzene; 98%; Sigma-Aldrich), MBT (2-mercaptobenzothiazole; 98%; Sigma-Aldrich), oxazolone (4-ethoxymethylene 2-phenyloxazol-5-one; 90%; Sigma-Aldrich), PA (phthalic anhydride; 99%; Sigma-Aldrich), TDI (toluene 2,4-diisocyanate; 99.8%; Sigma-Aldrich), TMA (trimellitic anhydride; 1, 2, 4- benzenetricarboxylic anhydride; 97%; Sigma-Aldrich), TMTD (tetramethylthiuramdisulfide; 98%; Sigma-Aldrich) and ZDMC (zincdimethyldithiocarbamate; 90%; Fluka, Zwijndrecht NL) were tested in 4:1 acetone/olive oil (AOO). SDS (sodium dodecyl sulphate; $>99\%$; Merck B.V., Amsterdam, NL) was dissolved in 4:1 acetone/olive oil (AOO).

2.3. Local lymph node assay (LLNA)

Groups of mice ($n = 3$, 4, or 6) were pretreated with 1% SDS (w/v) one hour before exposing the

animals to 25 μl of test solution in vehicle or vehicle alone on both ears daily for three consecutive days. A positive response is not seen at the SDS concentration that we used (1%). However, application of 1% SDS and the test chemical generally resulted in an increased response compared to the test chemical alone (data not shown).

The concentrations of the test chemicals used are presented in Table 1. Three days following the last topical application, the auricular lymph nodes were excised. The lymph nodes were weighed and pooled for each animal and suspended in 5 ml RPMI- 1640 (Gibco, Breda, NL) supplemented with 5% heat inactivated Fetal Calf Serum (PAA, Linz, Austria), 100 U/ml penicillin and 100 μg/ml streptomycin (standard medium). Single cell suspensions were prepared under aseptic conditions by pressing the lymph node through a sterile 70 μm nylon cell strainer (Falcon, Franklin Lakes, USA). The cells were washed twice in standard medium (10 min, 311 g, 4°C) and resuspended in 1 ml standard medium with 10% FCS. The cells were counted using a Coulter Counter (Coulter Electronics, Mijdrecht, NL) and cultured at a concentration of 1.10^7 cells/ml. When necessary, cell suspensions of several animals were pooled to obtain the concentration required. The cell suspensions (200 μl) were seeded in triplicate into round-bottomed 96-well microtitre plates (Greiner, Alphen a/d Rijn, NL). The cells were cultured with 10 μl of [^{3}H]TdR (Amersham, Buckinghamshire, UK; 37 kBq/ml) for 24 h at 37°C in a humidified atmosphere of 5% CO_2 in air. The [^{3}H]TdR incorporation was determined by liquid scintillation counting in a β plate counter (1205 Betaplate™ Wallac, Turku, Finland). The [^{3}H]TdR incorporation is expressed per animal, i.e. the [^{3}H]TdR incorporation is multiplied by the cell number of the two lymph nodes and devided by the cell number in culture.

Table 1
Concentrations used in the local lymph node assay (LLNA)

Chemical	% Concentration	Number (n) of animals (per concentration)
Benzocaine	7.5, 15, 22.5, 30	6
DEA	2.5, 5, 10, 20, 40	3
DNCB	0.05, 0.1, 0.25, 0.5, 1	3
MBT	0.1, 1, 5, 10, 17.5	4
Oxazolone	0.0004, 0.0012, 0.0037, 0.011, 0.033, 0.1	3
PA	0.25, 1, 2.5, 10, 25	3
TDI	0.25, 0.5, 1, 2.5, 5	3
TMA	2.5, 5, 10, 25, 50	3
TMTD	0.0312, 0.0625, 0.125, 0.25, 0.5, 1	3
ZDMC	0.375, 0.75, 1.5, 3, 6	3

To study the potency of sensitizers, we have used a range of test concentrations to determine dose-response relationships on which the quantitative estimation of the allergic potency was based. The estimated concentration in% required for SI = 3 (EC_3) was determined as the estimated dose inducing a stimulation index of three between treated versus control.

2.4. Statistical analysis

The dose-response data were analysed by non-linear regression analysis, using the following family of models:

model 1: $y = a$
model 2: $y = a\ \exp(bx)$
model 3: $y = a\ \exp(bx^d)$
model 4: $y = a(c\text{-}(c\text{-}1)\ \exp(bx)$
model 5: $y = a(c\text{-}(c\text{-}1)\ \exp(bx^d))$.

where y represents the response ([^{3}H]TdR incorporation) and x the applied concentration.

In these models the parameter a represents the background [^{3}H]TdR incorporation of the particular assay. The parameter b reflects the 'slope' or the 'strength' of the response with increasing dose. The selection of the model to be used for a particular data set follows from a procedure of successively fitting the above models, and applying likelihood ratio tests to establish if an increase in the number of parameters leads to a significantly better fit to the data. A model with more parameters is considered better only if this leads

to a significantly better fit (Slob, 1999). Then the selected model is used to derive the concentration (EC_3) associated with a stimulation index of 3. The uncertainty in the estimate of the EC_3 is assessed by a bootstrap method (Slob and Pieters, 1998), resulting in an uncertainty distribution from which any desired confidence interval can be derived. In this paper the 5% and 95% confidence limits are reported (i.e. 90%-confidence intervals).

3. Results

The results obtained with the various chemicals are shown in Fig. 1. The left panels show the [^{3}H]TdR incorporation as a function of the concentration with the fitted regression function, and the estimated concentration at a stimulation index of three (EC_3) in the [^{3}H]TdR incorporation per animal. The uncertainty distributions for the EC_3 values are shown in the right panels. The dose-response data were analysed by nonlinear regression analysis. Based on the criteria, namely a threefold increase over vehicle control, every compound tested proved to be positive and can therefore be regarded as a sensitizer. Differences in potency were observed between different chemicals. An exponentially shaped curve was seen for six of the chemicals, three of the chemicals showed a sigmoïdally shaped curve and one curve showed a logarithmic dose-response profile. These differences in the shapes of the dose-response curves result from the selection of the model to be used for the particular data set, as discussed in Section 2.4.

Lymph node weights and cell counts are presented in Table 2. Based on these data DNCB, oxazolone, PA, TDI and TMA are considered as being sensitizers according to the criteria for a positive response. ZDMC is regarded as a sensitizer based on the cell counts alone. In general, the data in Table 2 shows a ranking quite similar to the data shown in Fig. 1 and presented in Table 3. However, lymph node proliferation instead of lymph node weights and cell counts seems to be a better indicator of the sensitizing potential of a chemical, as was first suggested by Kimber and Dearman (1991).

The EC_3 values of [^{3}H]TdR incorporation per animal and the associated confidence intervals derived from the experiments are summarized in Table 3. The chemicals are ranked according to their EC_3 values. The classification formerly obtained with the GPMT, and the LLNA as performed by other groups, and information on whether the test compounds are known sensitizers in humans are also presented in Table 3. The ranking according to the estimated EC_3 values presented in Table 3 is quite similar to the classification derived from data formerly obtained in the GPMT and the LLNA.

4. Discussion

The LLNA is used as a test for predicting sensitization in humans. A chemical that induces an SI of three or more is regarded as a sensitizer. Recently, it has been suggested that simple linear interpolation between the observed responses on either side of the treefold stimulation index provides a robust assessment of the EC_3, without the need for recourse to more sophisticated statistical techniques (Basketter et al., 1999). Rather than using the SI of three as a cut-off point or limit, our method evaluates all data points (animals) contributing to the dose-response curve to determine the EC_3. In addition, information on the reliability of the data is obtained using the bootstrap approach, providing confidence limits. This renders the potency assessment more reliable. Also for very weak sensitizers inducing responses below an SI of three, the concentration producing an SI of three can be estimated based on the curve fitting method, together with its confidence limits. In such a case very high, and possibly unrealistic, sensitizing concentrations may be obtained.

It has been reported that the LLNA can detect contact allergens defined as moderate, strong or extreme, but not those defined as mild or weak (Basketter and Scholes, 1992). In the present study all chemicals reached an SI of three within the observed dose ranges and were thus identified as sensitizers. This may be due to the fact that in order to increase the sensitivity for weak allergens the animals were pretreated with SDS. This chem-

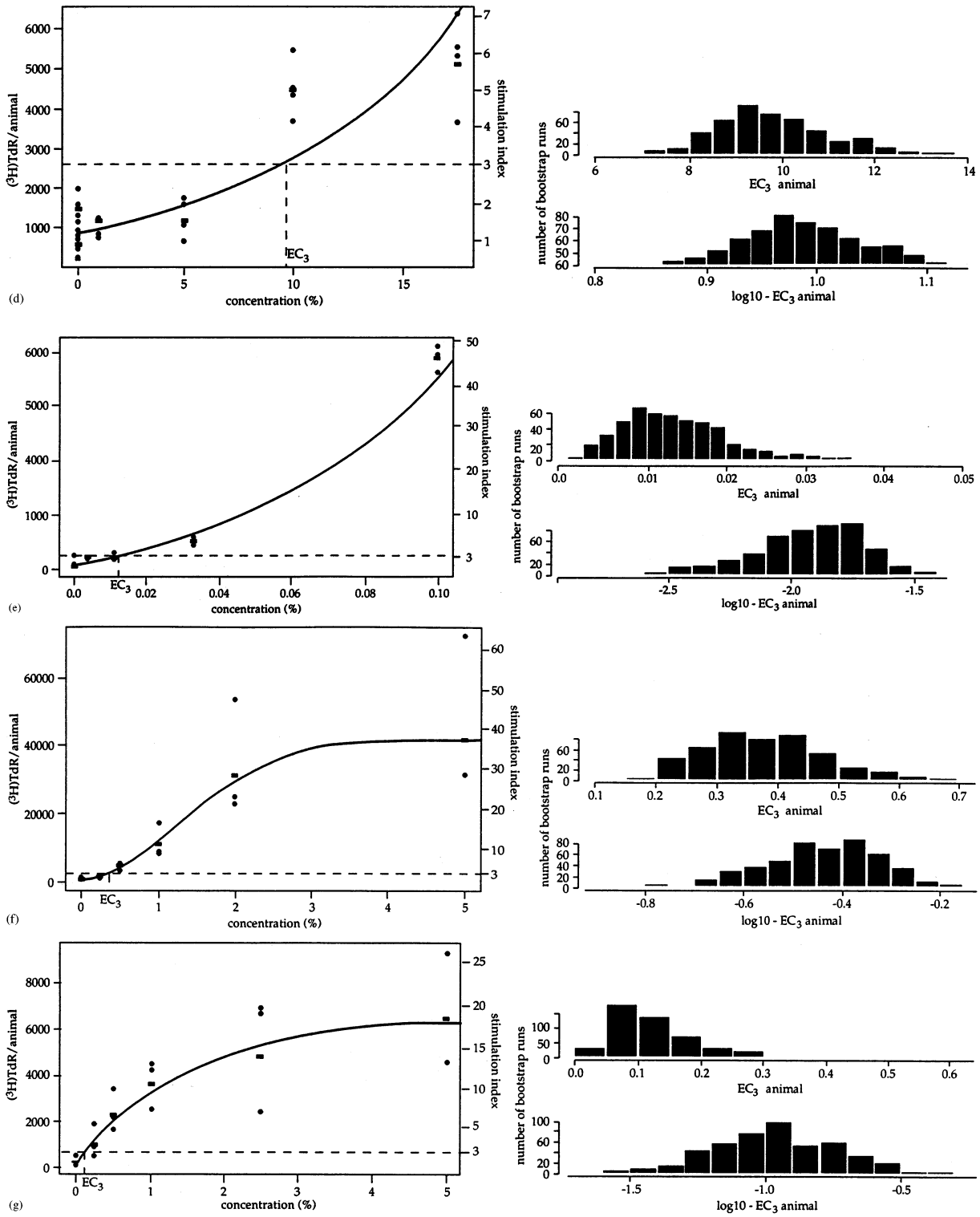

Fig. 1. (*Continued*)

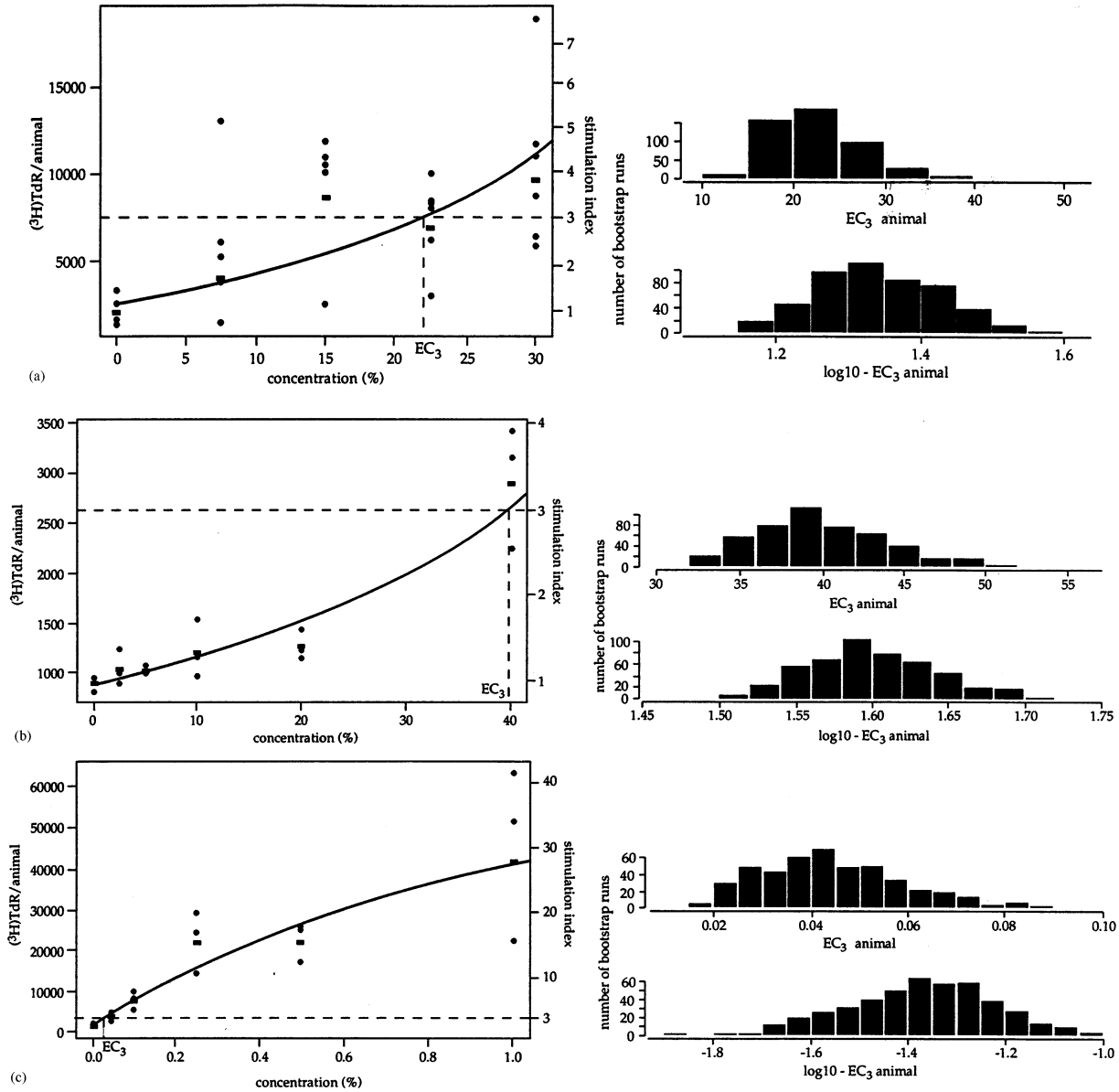

Fig. 1. Local lymph node dose response curves. Left panels: [^{3}H]TdR incorporation (cpm per animal) of the local lymph node cells as a function of concentration (dots refer to individual animals; lines refer to averages per concentration), with fitted regression function at a stimulation index of three and the estimated EC_3 values (in%). The models used for fitting were model 2 for benzocaine (a), DEA (b), MBT (d), TMTD (i) and ZDMC (j); model 3 for oxazolone (e); model 4 for DNCB (c), TDI (g) and TMA (h); model 5 for PA (f). Right panels: the associated uncertainty distribution (obtained with 500 bootstrap runs from the fitted regression function) for the EC_3 values, shown on a linear scale (upper graph) and a log-scale (lower graph).

ical is an example of an irritant that can give a positive response in the LLNA (Gerberick et al., 1992; Montelius et al., 1994). The pretreatment with SDS was done to increase the sensitivity of the LLNA, aiming at obtaining positive results for weak allergens. Pretreatment with SDS combined with application of the chemical showed an increased reponse compared to treatment with the

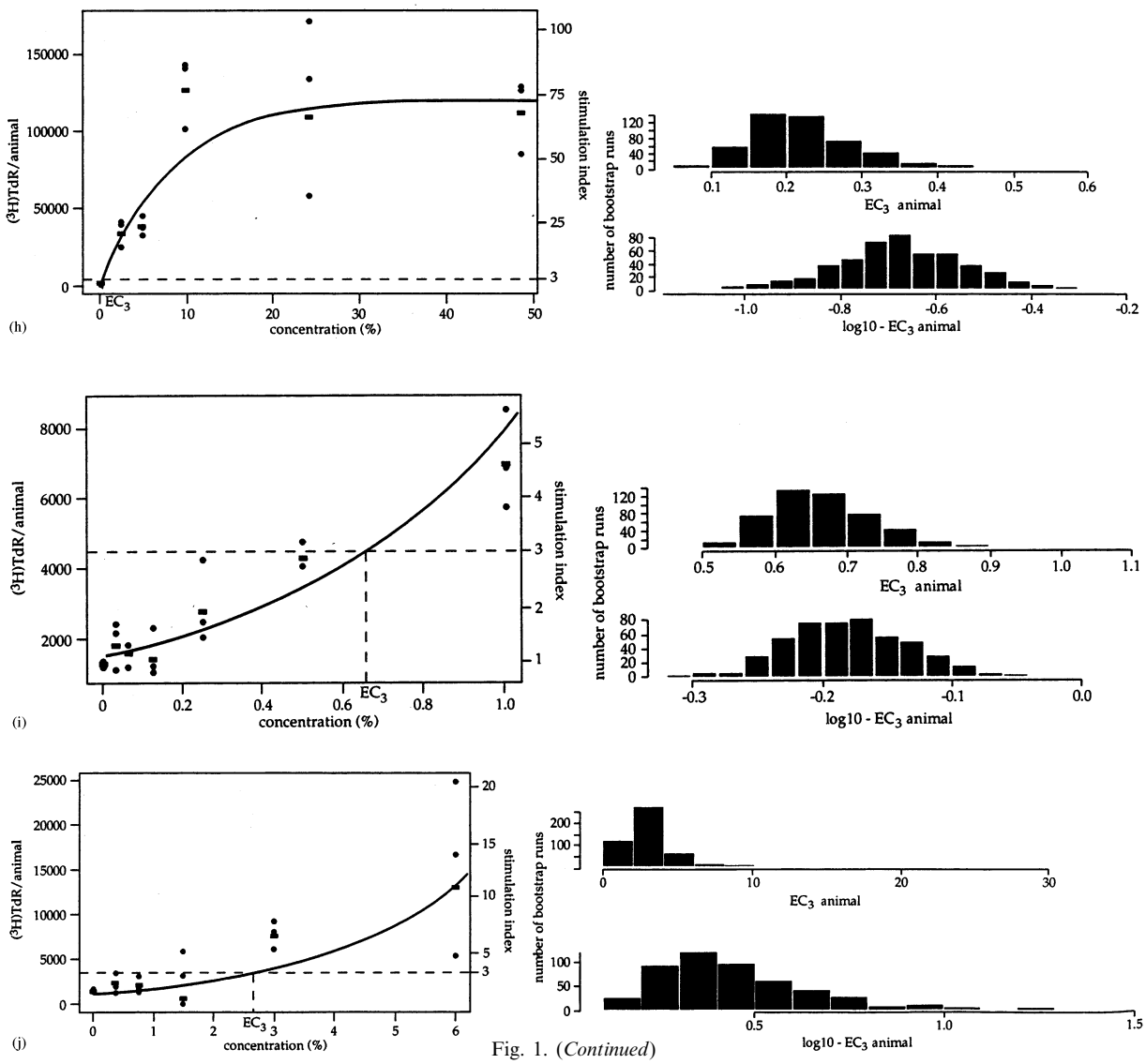

Fig. 1. (*Continued*)

chemical alone. In addition, the application of 1% SDS only gave no stimulation in the LLNA (data not shown).

Oxazolone was the most potent sensitizer in this study with an estimated EC_3 of 0.013% and an uncertainty range of 0.004–0.025%. This is approximately one order of magnitude higher than previous findings in the LLNA with predicted values ranging from 0.0007 to 0.0026% (Loveless et al., 1996). DNCB, known to cause allergic contact dermatitis in man (Kligman and Epstein, 1959), showed an estimated EC_3 of 0.044%, consistent with earlier findings obtained in several laboratories (Kimber et al., 1995; Loveless et al., 1996). Also TDI, TMA, PA and TMTD showed an estimated EC_3 below 1%. PA and TMA can penetrate the skin of humans and thus have the potential to elicit an allergic reaction of the skin (Bernstein et al., 1982). The contact sensitivity of TDI has also been demonstrated by some epidemiological investigations and animal experiments (Malten, 1979; Tominaga et al., 1985).

Table 2
Lymph node weights and cell counts after epicutaneous treatment of BALB/c mice

Chemical	% Concentration	Lymph node weight/animal (mean ± SD, in mg)	Cell counts/animal (mean ± SD × 10^6)
Benzocaine	0	5.20 ± 1.31	4.91 ± 1.12
	7.5	9.22 ± 1.75	6.83 ± 2.50
	15	6.87 ± 1.24	11.04 ± 2.71
	22.5	8.67 ± 0.60	9.77 ± 2.00
	30	8.69 ± 0.92	11.07 ± 1.59
DEA	0	2.42 ± 0.49	3.09 ± 0.26
	2.5	2.63 ± 0.20	3.20 ± 0.56
	5	2.65 ± 0.23	3.18 ± 0.05
	10	2.82 ± 0.19	3.87 ± 0.91
	20	2.87 ± 0.30	4.12 ± 0.48
	40	4.20 ± 0.57	7.54 ± 1.64
DNCB	0	2.23 ± 0.54	4.40 ± 1.11
	0.05	3.21 ± 0.50	7.52 ± 1.54
	0.1	4.95 ± 0.63	11.69 ± 0.34
	0.25	9.87 ± 1.39	23.00 ± 3.22
	0.5	10.15 ± 0.88	21.20 ± 5.10
	1	13.93 ± 1.31	29.60 ± 5.54
MBT	0	2.91 ± 0.41	4.22 ± 1.10
	0.1	2.93 ± 0.20	4.62 ± 1.13
	1	2.95 ± 0.49	5.07 ± 1.17
	5	3.70 ± 0.44	4.78 ± 1.00
	10	4.00 ± 0.42	5.61 ± 0.83
	17.5	4.24 ± 0.91	4.80 ± 0.71
Oxazolone	0	2.42 ± 0.49	3.09 ± 0.26
	0.0004	2.52 ± 0.56	3.28 ± 0.91
	0.0012	2.80 ± 0.21	3.69 ± 0.43
	0.0037	3.68 ± 0.79	5.33 ± 0.15
	0.011	4.60 ± 0.32	6.35 ± 1.51
	0.033	6.08 ± 0.83	10.30 ± 2.52
	0.1	11.40 ± 1.22	21.40 ± 2.51
PA	0	2.53 ± 0.46	5.42 ± 0.91
	0.25	4.10 ± 0.58	9.53 ± 2.00
	1	5.83 ± 0.90	13.19 ± 3.61
	2.5	6.12 ± 1.55	16.28 ± 4.12
	10	10.77 ± 1.43	25.01 ± 8.36
	25	11.10 ± 2.56	29.44 ± 2.10
TDI	0	2.75 ± 0.58	4.60 ± 0.50
	0.25	5.17 ± 1.31	12.78 ± 4.30
	0.5	9.78 ± 1.79	21.72 ± 6.79
	1	13.57 ± 2.36	33.20 ± 7.24
	2.5	13.13 ± 2.52	26.71 ± 6.58
	5	14.30 ± 3.06	25.57 ± 9.22
TMA	0	2.23 ± 0.54	4.40 ± 1.11
	2.5	7.78 ± 1.16	15.29 ± 1.60
	5	9.68 ± 1.14	22.00 ± 0.54
	10	12.37 ± 1.07	31.04 ± 5.22
	25	11.20 ± 1.11	23.70 ± 6.64
	50	10.73 ± 1.16	27.91 ± 2.57

Table 2 (*Continued*)

Chemical	% Concentration	Lymph node weight/animal (mean ± SD, in mg)	Cell counts/animal (mean ± SD × 10^6)
TMTD	0	2.87 ± 0.20	3.43 ± 0.25
	0.0312	3.38 ± 0.31	4.43 ± 1.61
	0.0625	2.70 ± 0.49	3.38 ± 0.74
	0.125	3.15 ± 0.42	4.25 ± 0.45
	0.25	3.27 ± 0.26	4.36 ± 1.41
	0.5	3.95 ± 0.58	6.23 ± 1.08
	1	4.75 ± 0.81	7.06 ± 2.30
ZDMC	0	2.87 ± 0.20	3.43 ± 0.25
	0.375	3.28 ± 0.35	4.94 ± 1.76
	0.75	3.47 ± 0.51	5.00 ± 2.03
	1.5	4.78 ± 0.76	7.61 ± 1.32
	3	5.35 ± 1.45	10.50 ± 3.48
	6	8.02 ± 2.23	16.00 ± 5.20

Table 3
The EC_3 values and uncertainty distribution of the chemicals tested, classification of chemicals in the local lymph node assay (LLNA) and the guinea pig maximization test (GPMT), and information on sensitizing capacity in humans[a]

			Classification of sensitizing potential[d]		
Chemical	EC_3[b](%)	L05-L95[c]	GPMT	LLNA	Human
Oxazolone	0.013	0.004–0.025	Extreme[e]	+[f]	NA
DNCB	0.044	0.025–0.078	Extreme	+	Yes[l]
TDI	0.109	0.048–0.263	NA	+[j]	Yes[m]
TMA	0.218	0.128–0.405	Moderate	+	Yes[n]
PA	0.357	0.226–0.560	Extreme	+	Yes[n]
TMTD	0.659	0.554–0.815	Moderate[g]	±[i]	Yes[p]
ZDMC	2.670	1.631–8.326	Moderate[h]	NA	Yes[o]
MBT	9.669	8.020–12.189	Moderate[g]	±[i]	Yes[o]
Benzocaine	22.026	16.576–33.953	Mild[g]/Moderate	−[d]/±[k]	Yes[p]
DEA	39.784	34.078–47.703	NA	NA	Yes[o]

[a] +, Strong; ±, moderate; −, weak; NA, data not available.
[b] EC_3, estimated concentration in% required for SI = 3.
[c] 5th and 95th percentile.
[d] Basketter and Scholes (1992), except where indicated.
[e] Gad et al. (1986).
[f] Loveless et al. (1996).
[g] Magnusson and Kligman (1969, 1970).
[h] Matsushita et al. (1977, 1978).
[i] Ikarashi et al. (1993).
[j] Dearman et al. (1996); (SI = 12 for TDI 0.75%).
[k] Basketter et al. (1995).
[l] Kligman and Epstein (1959).
[m] Malten (1979), Tominaga et al. (1985).
[n] Bernstein et al. (1982).
[o] Kaniwa et al. (1993).
[p] Cronin (1980).

TMTD is regarded as a moderate sensitizer based on the results obtained in the GPMT (Magnusson and Kligman, 1969, 1970). This was also found for ZDMC (Matsushita et al., 1977, 1978). Besides, it has been reported that ZDMC as well as DEA, MBT and TMTD are able to cause allergic contact dermatitis by rubber gloves in humans (Kaniwa et al., 1993). In summary, all the chemicals tested in this paper have proven their sensitizing potency based on experimental and/or clinical data.

The magnitude of the effect in the animal is estimated from the dose-response data obtained from the LLNA. The endpoint of the LLNA is proliferation per animal measured by [^{3}H]TdR incorporation and the criteria for a positive response is a threefold increase in proliferation over vehicle control. The precision of the estimated EC_3 is mainly governed by the total number of animals in the study. In most cases, three animals per group and five concentrations were used. Testing more concentrations benefits the benchmark approach (Slob and Pieters, 1997), making the results more reliable. More reliable results can be obtained by increasing the number of concentrations investigated and, compared to conventional toxicology, reducing the number of animals per group. However, a minimum of three animals per group seems warranted in order to exclude individual extreme (outlier) responses.

The chemicals tested can now be ranked based on the results presented in this paper. The ranking of the chemicals shown in Table 3 is quite similar to the ranking according to their sensitizing potential in the GPMT, and LLNA established by other groups. An exception is the relative potency of TMA and PA; the former is classified as more potent according to our approach, whereas the GPMT identifies PA as the more potent sensitizer (Basketter and Scholes, 1992). Benzocaine and TMTD are both known as moderate sensitizers in the classification of sensitizing potential performed in the GPMT and the LLNA (Table 3). However, according to the data presented in this paper EC_3 values showed a considerable (30-fold) difference between these two sensitizers. So, a more accurate distinction can be made between benzocaine and TMTD in terms of classification as sensitizers. In addition, the observed differences in EC_3 values also have an impact when doing risk assessment for these compounds. The quality of the data is translated to uncertainty margins which benefits the comparison between the sensitizers. For reasons of safety it would be better to make use of the calculated 5% confidence limit instead of the point estimate of the EC_3. The lower 5% confidence limit, predicts that below that particular dose there still is a 5% chance for a positive response in the test with an SI of three. It should be kept in mind that even when sensitizers are classified as strong inducers of allergy, the actual risk for developing allergy is also determined by exposure.

In conclusion, the sensitizing potential of ten chemicals was evaluated using a dose-response analysis for LLNA data. The sensitization ability was expressed as the concentration able to elicit a stimulation index of three compared to the vehicle control. The sensitizing potential of oxazolone was strongest, followed by DNCB, TDI, TMA, PA, TMTD, ZDMC, MBT, benzocaine and DEA. The approach performed in this study is a way to accurately assess the potency of sensitizing chemicals. This enables the estimation of the lowest concentration needed for sensitization, and to use these data for risk evaluation.

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