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Quantitative relationship between the local lymph node assay and human skin sensitization assays

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Abstract

The local lymph node assay (LLNA) is a new test method which allows for the quantitative assessment of sensitizing potency in the mouse. Here, we investigate the quantitative correlation between results from the LLNA and two human sensitization tests—specifically, human repeat insult patch tests (HRIPTs) and human maximization tests (HMTs). Data for 57 substances were evaluated, of which 46 showed skin sensitizing properties in human tests, whereas 11 yielded negative results in humans. For better comparability data from mouse and human tests were transformed to applied doses per skin area, which ranged over four orders of magnitude for the substances considered. Regression analysis for the 46 human sensitizing substances revealed a significant positive correlation between the LLNA and human tests. The correlation was better between LLNA and HRIPT data ($n = 23$; $r = 0.77$) than between LLNA and HMT data ($n = 38$; $r = 0.65$). The observed scattering of data points is related to various uncertainties, in part associated with insufficiencies of data from older HMT studies. Predominantly negative results in the LLNA for another 11 substances which showed no skin sensitizing activity in human maximization tests further corroborate the correspondence between LLNA and human tests. Based on this analysis, the LLNA can be considered a reliable basis for relative potency assessments for skin sensitizers. Proposals are made for the regulatory exploitation of the LLNA: four potency groups can be established, and assignment of substances to these groups according to the outcome of the LLNA can be used to characterize skin sensitizing potency in substance-specific assessments. Moreover, based on these potency groups, a more adequate consideration of sensitizing substances in preparations becomes possible. It is proposed to replace the current single concentration limit for skin sensitizers in preparations, which leads to an all or nothing classification of a preparation as sensitizing to skin (“R43”) in the European Union, by differentiated concentration limits derived from the limits for the four potency groups.

Keywords: Allergic contact dermatitis; Skin sensitizers; Potency; Local lymph node assay; Human repeat insult patch test; Human maximization test; Classification and labeling; Preparations

1. Introduction

Regulatory aspects of skin sensitizing agents currently comprise mostly qualitative (i.e., “all or none”) considerations. Thus, Directive 67/548/EEC foresees in the European Union a classification as a skin sensitizer (R43), irrespective of the potency of a substance, and a concentration limit of 1% for a substance which is classified R43 is set for the classification of preparations. Several reasons can be identified for ignoring potency considerations for sensitizers in current risk assessment frameworks. Despite clear scientific evidence for dose–response relationships for both induction and elicitation of allergic skin reactions, effective doses vary substantially, due to considerable inter-individual differences in susceptibility (Jerschow et al., 2001; Robinson, 1999; Uter et al., 1995), and/or differences in exposure conditions, vehicle influences, and other factors modulating the severity of allergic symptoms (Felter et al., 2002). Furthermore, the guinea-pig models used for identifying skin sensitizers, i.e., the guinea-pig maximization test (GPMT) (Magnusson and Kligman, 1969) and the Buehler test (Buehler, 1965), are sensitive qualitative tests, well-established for decades, but do not allow for an objective measurement of potency. As in both the

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GPMT and the Buehler test only one concentration for induction and elicitation, respectively, is used, the dose–response information obtainable is limited. But van Och et al. (2001) showed that by varying the intradermal and topical concentrations in the GPMT dose–response relationships can also be observed in guinea-pig studies.

With the local lymph node assay (LLNA) a new mouse test for assessing skin sensitization has been established (Kimber et al., 1986). With this assay the induction reaction after repeated percutaneous exposure of the mouse ear is measured and the proliferation rate of nearby located lymph node cells is used to quantify the effect. The concentration leading to a threefold increase of the baseline proliferation (EC_3) is considered a "threshold" for sensitization. The qualitative concordance of the LLNA with guinea-pig and human data has been shown (Dean et al., 2001; NIEHS, 1999). To quantify the outcome of the LLNA, van Och et al. (2000) applied dose–response models to data on 10 sensitizers. In contrast, most others calculate EC_3 values to express potencies observed in the LLNA and Basketter et al. (1999a,b) emphasize that this straightforward linear procedure leads to similar results compared to dose–response modeling.

Gerberick et al. (2001) compared LLNA and human data for 15 substances, using NOAEL values from human repeat insult patch tests, stating a substantial concordance between mouse and human data. Based on such comparisons, there are suggestions for using the LLNA results to differentiate skin sensitizers according to their potency (European Commission Working Group on Sensitization, EC SEG, 2003; ECETOC, 2003). Still, the quantitative correlation between the LLNA and the skin sensitizing activity of chemicals in humans has not been investigated thoroughly, rendering the use of the LLNA for quantitative purposes provisional.

In this investigation, an analysis of the quantitative relationship of LLNA and human data is presented. For comparability with the LLNA, human data must include the induction phase of the sensitization process. Thus the human data base is limited to results from human predictive testing, namely human repeat insult patch tests (HRIPTs) or human maximization tests (HMTs). It has been shown both in animal models and in humans that allergic skin reactions relate to the amount of substance applied per skin area, not to the absolute amount of applied substance (Boukhman and Maibach, 2001; Magnusson and Kligman, 1969; Upadhye and Maibach, 1992). To reach comparability between mouse and human data the dose per skin area was calculated for all test results.

2. Methods

Literature search for chemicals was limited to compounds for which response on experimental sensitization has been tested in both predictive human tests and the local lymph node assay. Relevant publications were identified in the databases PubMed (http://www.ncbi.nlm.nih.gov.html) and ToxNet (http://toxnet.nlm. nih.-gov.html). Special sources for HMT data were the publications of Kligman (1966a,b), and for LLNA data the report of ICCVAM ("Interagency Coordinating Committee on the Validation of Alternative Methods") on evaluation of the LLNA (NIEHS, 1999). Furthermore, unpublished LLNA data were kindly provided by Dr. David Basketter (Unilever Colworth Laboratory).

The effective concentrations of a chemical in human and LLNA studies were converted to a unique comparable value following different approaches.

2.1. Human dose–response data

The lowest effective concentration applied during the induction phase of the study for each chemical was converted to a dose per unit area ($\mu g/cm^2$) using the information on substance concentration, application volume, and area of application given in the publication. Using the information on sensitization incidence given in the publications from this value a dose per skin area leading to a sensitization incidence of 5% (DSA_{05}) was derived by linear interpolation. This low but existent effect level was assumed to be comparable to the EC_3 effect level in the LLNA.

2.2. LLNA dose–response data

Published EC_3 values (i.e., the concentration of the test substance in the test solution leading to a threefold increase of lymph node cell proliferation) were used. If not reported, EC_3 values were derived by different approaches.

EC_3 values were calculated according to Basketter et al. (2001a) by linear interpolation between the reported values of the stimulation index (SI) (as a measure of the proliferative response induced in draining lymph node cells by a sensitizing chemical compared to vehicle-treated controls) with the coordinates (a, b) and (c, d) lying immediately above and below the SI value of 3, using the equation

$$EC_3 = c + \frac{3-d}{b-d}(a-c)$$

with a the concentration with $SI > 3$; b the SI at concentration a; c the concentration with $SI < 3$; and d the SI at concentration c.

If only SI values above the value of 3 were given for a chemical, the EC_3 was estimated by linear extrapolation using the lowest data point in the dose–response curve and the vehicle control with the coordinates "concentration 0" and "SI 1," using the equation

$$EC_3 = \frac{(SI3-1)}{(b-1)}a$$

with a the lowest concentration with SI > 3 and b the SI at concentration a.

If only values for the lymphocyte proliferative responses—measured as incorporation of [^{3}H]thymidine by draining lymph node cells and registered as disintegration per minute (dpm)—yielding an SI > 3 were available, the EC_3 was estimated by the ratio of dpm counts for the lowest dose group tested and the threefold dpm for the vehicle control group (identical to the SI 3), using the equation

$$EC_3 = \frac{3e}{f}a$$

with a the lowest concentration with SI > 3; e the dpm in control; and f the dpm at concentration a.

Priority was given to published EC_3 values or those calculated according to the original method of Basketter et al. (2001a) over estimations using only one data point (the two last methods mentioned above). EC_3 values (concentrations, in %) were converted to dose per skin area values (DSA_{EC3}, in μg/cm^2) using the dose volume of 25 μl according to the standard LLNA protocol and an estimated application area of 1 cm^2 for the mouse ear.

Where multiple results in both human and LLNA data (including multiple results for one ranking level in the LLNA) were present for a particular chemical, an arithmetic mean was calculated. Negative results were not considered in the calculation of the mean values. The mean values include comparable results with different vehicles except strikingly discordant results from tests with varying vehicles. Results from tests with modifications of the standard protocol were included (except results from HRIPTs using sodium lauryl sulfate).

Analysis of correlation and variance between LLNA and human test results was performed using Analyse-it, version 1.63, supplementary software to Microsoft Excel (Analyse-it Software, Ltd.).

3. Results

For 46 sensitizing substances positive test results from both the LLNA and human studies could be retrieved from various sources and publications (Table 1 and Fig. 1). For 16 substances we found both HMT and HRIPT data. Published test results were transformed into amount of substance applied per skin area as described in Section 2. Effective concentrations vary over four orders of magnitude, emphasizing the vast differences in potency between substances (Fig. 1).

Correlations between LLNA results and observed activity in humans were investigated separately for HRIPT and HMT data. Results from LLNA and HRIPT studies, expressed as applied dose per skin area, are significantly positively correlated ($r = 0.77$), and slope of the regression line of logarithmic values is 0.87 (Table 2, Fig. 2). Similarly there is also a significant correlation between LLNA and HMT data, but the correlation is poorer, with a regression line of slope 0.71 and intercept 1 (Table 2, Fig. 3). The correlation between HRIPT and HMT results is also poorer than that between LLNA and HRIPT (Table 2) indicating that uncertainties associated with available human data are more important than species differences.

In addition to the substances in Table 1, a further 11 substances could be identified which have been tested both in the LLNA and in a human maximization test (Table 3). These substances generally gave negative results when tested for skin sensitizing properties and therefore were not included in the above evaluation. Only with isopropyl myristate and linalool was a borderline positive result in the LLNA observed at very high concentrations. Application of isopropyl myristate and linalool in a concentration of 50% (corresponding to a dose per skin area of 12,500 μg/cm^2) led to a stimulation index of 3.3 and 4.8, respectively, whereas no activity was noted in the HMT. For the remaining nine substances the evaluated studies revealed no activity in both the mouse model and the human maximization test. The consistent negative results for these substances corroborate the positive correlation observed between LLNA and human data.

4. Discussion

The regression analysis reveals a clear positive correlation of the murine LLNA outcome with the results of human sensitization studies and hence is in agreement with more qualitative comparisons already performed by others (Dean et al., 2001).

In a recent publication Griem et al. (2003) compared human and LLNA data for 30 substances. For human data from HRIPT and HMT they used no observed adverse effect levels (NOAELs) and lowest observed adverse effect levels (LOAELs), the latter being divided by an arbitrary factor in cases of high observed incidences, for comparison with the dose per skin area equivalent to the LLNA EC_3. Of these substances, 23 are also included in our analysis, with those remaining being substances for which they used unpublished data. Gerberick et al. (2001) also compared NOAELs from HRIPT or HMT with EC_3 from LLNA, recalculated as dose per skin area, for 15 skin sensitizing substances.

Both groups concluded that there was a high concordance between murine and human data. Although we reached similar conclusions, we must note that the use of NOAELs for comparison can lead to misinterpretations. This can be exemplified by the data for glutaraldehyde. Gerberick et al. (2001) stated high concordance for this

Table 1
Data from LLNA (DSA_{EC3}) and human tests (DSA_{05}) (HRIPT or HMT) for 46 skin sensitizing substances used for regression analysis

Chemical name (CAS No.)	LLNA		Human data		
	DSA_{EC3}	References	$DSA_{05\text{-HRIPT}}$	$DSA_{05\text{-HMT}}$	References
Acetyl isovaleryl (13706-86-0)	6450	Ryan et al. (2000)		3541	Epstein (1979); Kligman (1978) (both unpublished data cited in Opdyke, 1982)
Aniline (62-53-3)	6658	Basketter et al. (1991)		2463	Kligman (1966a)
Benzocaine (94-09-7)	3338	Basketter et al. (1993, 1995); Kimber et al. (1989); Montelius et al. (1994); van Och et al. (2000)	29,167	3902	Marzulli and Maibach (1974); Kligman (1966a,b)
Benzoyl peroxide (94-36-0)	41	Basketter (2003); Kimber et al. (1998);	895	987	Leyden and Kligman (1977); Poole et al. (1970)
Benzilidene acetone (122-57-6)	883	Ryan et al. (2000)	619	144	Kligman (1972) (unpublished data cited in Opdyke, 1973); Marzulli and Maibach (1980)
Beryllium(II) sulfate (7787-56-6)	8.6[a]	Basketter et al. (1994); Mandervelt et al. (1997)		11[a]	Kligman (1966a)
n-Butyl glycidyl ether (2426-08-6)	7725	Basketter et al. (1994)		437	Kligman (1966a)
Chlorpromazine (69-09-0)	463	Basketter et al. (1994)		1150	Kligman (1966a)
Cinnamic alcohol (104-54-1)	5150	Basketter (2003)	3474	625	Jordan and King (1977); Steltenkamp et al. (1980c)
Cinnamic aldehyde (104-55-2)	359	Basketter et al. (2001b); Basketter and Scholes (1992); Kimber et al. (1989); Montelius et al. (1994); Wright et al. (2001)	639	216	Danneman et al. (1983); Kligman (1977) (unpublished data cited in Opdyke, 1979a); Marzulli and Maibach (1980)
Citral (5392-40-5)	2415	Ashby et al. (1995); Basketter et al. (1991); Basketter and Kimber (2001)	1266	862	Opdyke (1979b); Steltenkamp et al. (1980a)
Cobalt(II) salts (7440-48-4)	50[a]	Basketter and Scholes (1992); Ikarashi et al. (1992b); Mandervelt et al. (1997)		313[a]	Kligman (1966a,b)
Diethylenetriamine (111-40-0)	463	Basketter et al. (1994)		411	Kligman (1966a)
Diethyl maleate (141-05-9)	1175	Basketter (2003); Ryan et al. (2000)	1067	150	Marzulli and Maibach (1980)
3,4-Dihydrocoumarin (119-84-6)	1402	Ashby et al. (1995)	769	750	Marzulli and Maibach (1980)
2,4-Dinitrochlorobenzene (97-00-7)	14	Basketter et al. (1997); Kimber et al. (1995); Loveless et al. (1996); van Och et al. (2000)	5.5[b]		Friedmann et al. (1983b)
Ethyl acrylate (140-88-5)	7175	Warbrick et al. (2001)	1222	375	Marzulli and Maibach (1980)
Ethylenediamine (107-15-3)	550	Basketter (2003); Kimber et al. (1998)	732		Maibach (1975) (unpublished data cited in Marzulli and Maibach (1976))
Eugenol (97-53-0)	4780	Basketter (2003); Basketter and Kimber (2001); Basketter and Scholes (1992); Bertrand et al. (1997); Gerberick et al. (1992); Kimber and Weisenberger (1991)	5926		Marzulli and Maibach (1980)
Formaldehyde (50-00-0)	102	Basketter et al. (2001b); Basketter and Scholes (1992); Hilton et al. (1998); Sailstad et al. (1995)	411	89	Kligman (1966a); Marzulli and Maibach (1974)
Geraniol (106-24-1)	5100	Basketter and Kimber (1997); Kimber and Weisenberger (1989, 1991)	7407	216	Malten et al. (1984); Marzulli and Maibach (1980)
Glutaraldehyde (111-30-8)	26	Basketter (2003); Gerberick et al. (1992); Hilton et al. (1998); Sailstad et al. (1995)	1073		Marzulli and Maibach (1974)
Glyoxal (107-22-2)	150	Basketter et al. (1994); Basketter (2003)		345	Kligman (1966a)

Gold(III) chloride (7440-57-5)	78[a]	Basketter et al. (1999b)		65[a]	Kligman (1966a)
Hydroxycitronellal (107-75-5)	6054	Basketter (2003); Basketter et al. (1994, 2001b); Basketter and Kimber (2001); Basketter and Scholes (1992); Montelius et al. (1994)	3937	4311	Jordan and King (1977); Ford et al. (1988); Marzulli and Maibach (1980); Steltenkamp et al. (1980b)
Imidazolidinyl urea (39236-46-9)	6952	Basketter (2003); Basketter and Scholes (1992)	3846		Jordan and King (1977)
Isoeugenol (97-54-1)	524	Basketter (2003); Basketter and Kimber (2001); Basketter and Scholes (1992); Kimber and Weisenberger (1991); Loveless et al. (1996); Wright et al. (2001)	657		Marzulli and Maibach (1980); Thompson et al. (1983)
Kanamycin (59-01-8)	2075	Basketter (2003)		1874	Kligman (1966a)
Kathon CG (55965-84-9)	1.8	Botham et al. (1991); Warbrick et al. (1999a)	5.8		Cardin et al. (1986)
2-Mercaptobenzothiazole (149-30-4)	1214	Basketter et al. (1993); De Jong et al. (2002); Montelius et al. (1994); Scholes et al. (1992)		2269	Kligman (1966a)
Mercuric(II) chloride (7487-94-7)	98[a]	Basketter et al. (1994)	924[a]	55[a]	Kligman (1966a); Marzulli and Maibach (1973)
Methylanisyliden acetone (1-(*p*-methoxyphenyl)-1-penten-3-on) (104-27-8)	2123	Ryan et al. (2000)		412	Epstein (1977) (unpublished data cited in Opdyke, 1979c)
Neomycin sulfate (1404-04-2)	1500	Boussiquet-Leroux et al. (1995); Gerberick et al. (2000)	15,625	2028	Kligman (1966b); Marzulli and Maibach (1973, 1974)
Nickel(II) salts (7786-81-4)	350[a]	Basketter et al. (1994); Basketter and Scholes (1992); Gerberick et al. (1992); Ikarashi et al. (1992a,b); Mandervelt et al. (1997); Scholes et al. (1992)		28[a]	Kligman (1966b)
Penicillin G (61-33-6)	5606	Basketter and Scholes (1992); Kimber et al. (1998); Kimber and Weisenberger (1989); Scholes et al. (1992)		76	Kligman (1966b)
Pentachlorophenol (87-86-5)	5000	Basketter (2003)		2155	Kligman (1966a)
Phenylacetaldehyde (122-78-1)	963	Basketter et al. (2001b); Basketter and Kimber (2001); Ryan et al. (2000)		415	Epstein (1973); Kligman (1971); Maibach (1971) (all unpublished data cited in Opdyke, 1979d)
p-Phenylenediamine (122-78-1)	23	Basketter and Kimber (2001); Basketter and Scholes (1992); Montelius et al. (1994); Warbrick et al. (1999b)	6.9	16.4	Kligman (1966b); Marzulli and Maibach (1974)
2-Phenylpropionaldehyde (93-53-8)	1575	Basketter (2003)	692		Anon. (1991)
Potassium dichromate (7778-50-9)	116	Basketter et al. (1999a); Basketter and Scholes (1992); Ikarashi et al. (1992b, 1993); Kimber et al. (1991); Mandervelt et al. (1997)		111	Kligman (1966a,b)
Propylidene phthalide (17369-59-4)	775	Ryan et al. (2000)		1150	Kligman (1975) (unpublished data cited in Opdyke, 1978b)
Pyridine (110-86-1)	17,975	Basketter (2003)		41,051	Kligman (1966a)
Streptomycin (57-92-1)	6750	Kimber et al. (1998)		82	Kligman (1966b)
Tetrachlorosalicylanilide (11554-59-2)	7.75	Basketter et al. (1994); Scholes et al. (1991)		14.4	Kligman (1966b)
Tetramethyl thiuram disulfide (97-77-8)	785	Basketter (2003); Basketter and Kimber (2001); De Jong et al. (2002); van Och et al. (2000)		4610	Kligman (1966a,b)
1-Thioglycerol (96-27-5)	878	Voss (1958)	661	1724	Kligman (1966a)

All data are expressed as dose per skin area ($\mu g/cm^2$).

[a] Doses refer to the amount of metal cation only.

[b] Result from an induction-elicitation patch test, not included in the regression analysis.

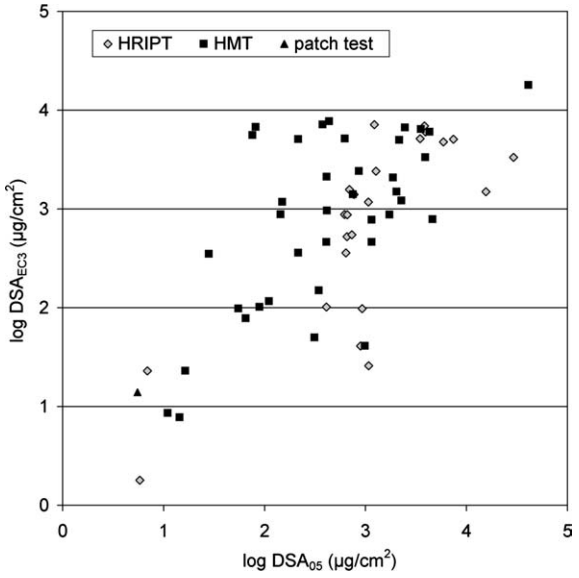

Fig. 1. Comparison of data from LLNA (DSA_{EC3}) and human tests (DSA_{05}) for 46 skin sensitizing substances (data from Table 1).

substance based on a NOAEL of 100 µg/cm^2 observed in a HRIPT, compared with an EC_3 area dose of 23 µg/cm^2 from the LLNA. These HRIPT data were retrieved from a study by Marzulli and Maibach (1974), in which sensitization by glutaraldehyde was observed only at the higher concentration of 5000 µg/cm^2, leading to an incidence of 23%. Due to the large spacing between applied concentrations, the NOAEL reported for glutaraldehyde in this latter study may be misleading and a "real" NOAEL might be an order of magnitude higher.

Consequently, we applied a concept for evaluating the experimental data which is independent of the chosen test concentrations. Since in most human studies tests were performed with only one or two different concentrations, dose–response modeling could not be investigated. Instead, we interpolated linearly from the lowest observed effect level to a dose corresponding to an estimated sensitization incidence of 5% (DSA_{05}). Since in several cases only high incidences were reported and the shape of the dose–response curve in the lower range is unknown, this procedure holds significant uncertainties. Nevertheless, for comparison with EC_3 values from LLNA studies we argue that it is the best expression of potency observed in studies with limited dose–response information.

Other relevant uncertainties are related to limitations in the human data, which mostly come from older studies. First, the reporting of size of the skin area to which the test substance has been applied and of the volume of test solution used is often insufficient. In some cases, skin area and test solution volume could be deduced from information given on types of patches and application systems used. Moreover, in human HRIPT and HMT studies observed incidences for sensitization reactions depend on the concentrations applied during both the induction and elicitation phase (Friedmann et al., 1983a,b; Marzulli and Maibach, 1974). Often, but not in all cases, the same concentration was applied for both phases. Otherwise, the overall outcome of the test may have been influenced by different elicitation concentrations, a factor not considered in the regression analysis. Furthermore, insufficient dose–response data with only high SI values reported had to be used in some LLNA evaluations. Vehicle influences can be presumed for both LLNA and human tests. In addition, there are concerns that the LLNA might lead to false positive results with irritating substances (Basketter et al., 1998; Ikarashi et al., 1993; Montelius et al., 1994) and thus that the observed EC_3 might overestimate the risk, e.g., for benzoyl peroxide and glutaraldehyde (Table 1).

In summary, with the exception of irritating substances, quantitative uncertainties of the data used are judged to be more substantial for human data than for the LLNA, which has been performed according to a standardized and strict test protocol. Taking into account these various sources of uncertainties, the observed correlation between murine and human data seems to be reasonably good. The uncertainties for the human data must be accepted in at least some cases to attain a statistically meaningful number of substances. A substantial improvement of the human database cannot be expected in the future as HRIPT tests are performed for ethical reasons at doses expected not to lead to sensitization reactions, thereby confirming the absence of this kind of effects at a dose range proposed for human uses, e.g., with cosmetic products.

Table 2
Regression analysis of logarithmic DSA_{EC3}, DSA_{05} from HRIPT, and DSA_{05} from HMT

	log DSA_{EC3} versus log $DSA_{05\text{-HRIPT}}$	log DSA_{EC3} versus log $DSA_{05\text{-HMT}}$	log $DSA_{05\text{-HRIPT}}$ versus log $DSA_{05\text{-HMT}}$
n	23	38	16
Slope	0.87	0.71	0.62
95%-CI of slope	0.55–1.19	0.43–1.0	0.29–0.95
Intercept with *y*-axis	0.19	1.01	0.71
Pearson's coefficient of correlation *r*	0.77	0.65	0.73
p Value (2-sided, 95% CI)	<0.0001	<0.0001	0.0012

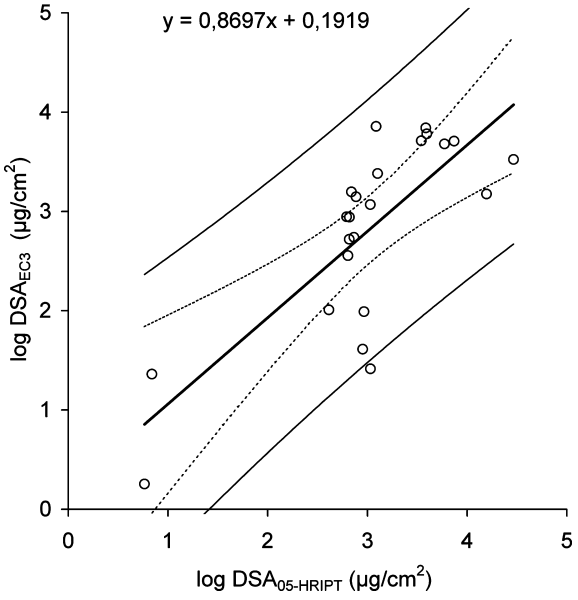

Fig. 2. Correlation of the logarithmic dose per skin area values from LLNA and HRIPT data (regression equation, regression line, confidence interval of the slope (inner lines), lower and upper bounds of the confidence interval (outer lines), calculated for a 95% confidence interval).

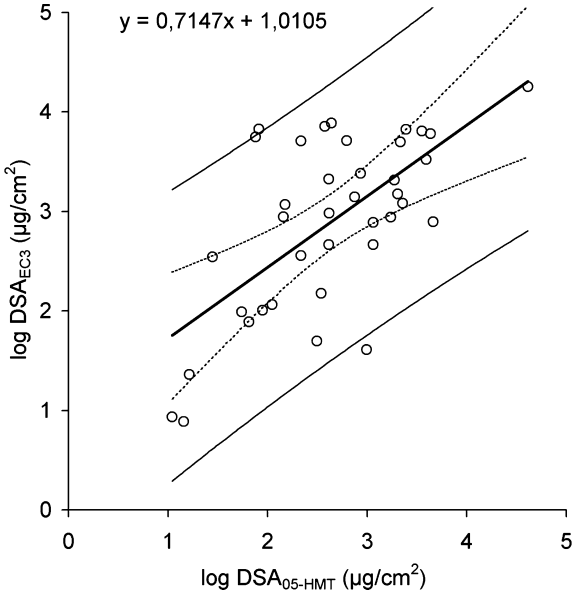

Fig. 3. Correlation of the logarithmic dose per skin area values from LLNA and HMT data (regression equation, regression line, confidence interval of the slope (inner lines), lower and upper bounds of the confidence interval (outer lines), calculated for a 95% confidence interval).

Based on the observed correlation, LLNA results can be used to differentiate skin sensitizers according to their potency. Table 4 presents a proposed assignment of sensitizers into four potency groups, according to their EC_3 values. Criteria for the setting of group limits are a division of the total range of observed potencies into parts of similar width and a comparable filling of the groups. According to these criteria, 11, 24, 39, and 26% of the 46 substances would be assigned to groups 1, 2, 3, and 4, respectively. Adequacy of the chosen group limits and representative group filling have also been checked by using 20 substances with LLNA data that have not been included in the evaluation above on account of lacking human data (data not shown).

It must be emphasized that in assigning substances to potency groups use should be made of all available information, e.g., from human elicitation patch test studies, structure considerations, and other supporting data (e.g., information on irritating properties influencing the LLNA result). Thus, in the case of specific additional scientific evidence, it should be permissible to deviate from the default assignment of substances to the potency groups.

Potency considerations can be helpful in the assessment of new chemical substances, of biocides within EC Directive 98/8/EEC, and in the classification of preparations. Currently, according to Directive 1999/45/EEC, in the European Union a preparation must be classified as sensitizing by itself if it contains more than 1% of a substance classified as a skin sensitizer. Using the established potency groups, the group limits can be translated to maximum concentrations of sensitizing substances in preparations (Table 5). Exceeding these limit concentrations would result in the classification of the preparation as sensitizing.

The proposed concentration limits are similar to recent suggestions by a European Commission expert group (EC SEG, 2003). The main difference of the EC SEG (2003) system is that it relies on only three groups, resulting in the major part of the substances investigated belonging to the "moderate" group (this group would comprise >70% of the 46 substances evaluated here). Consequently, also only three different concentration limits for preparations have been proposed. The rationale for the grouping by the EC SEG was not revealed in detail.

The concentration limits proposed in the present paper for preparations will lead to significant changes; e.g., *p*-phenylenediamine, a potent skin sensitizer with an EC_3 of 0.1% or 23 µg/cm^2, respectively, would be assigned to group 1 ("extremely sensitizing") according to the criteria laid down in Table 3. It is contained in hair dye formulations in concentrations of up to 1%, in rare cases even higher (BUA, 1993). According to current legislation in effect in the European Union, hair dye formulations have not to be classified as sensitizing if the content of *p*-phenylenediamine does not exceed 1%. By the criteria proposed here (see Table 4), all preparations with a concentration of the substance exceeding 0.01% must be classified as sensitizing. Another hair dye ingredient, resorcin, is classified as a sensitizing substance (R43) but is of very low potency. It was inactive in a

Table 3
HMT and LLNA data for 11 substances that showed no sensitizing activity in HMT

Chemical (CAS-No.)	LLNA			HMT		
	DSA applied ($\mu g/cm^2$)	SI	Reference	DSA applied ($\mu g/cm^2$)	Incidence	Reference
4-Aminobenzoic acid (150-13-0)	625	1.1	Basketter (2003)	17,242	0/25	Kligman (1966a)
	1250	1.6				
	2500	1.4				
Diethyl phthalate (84-66-2)	6250	1.0	Ryan et al. (2000)	6896	0/25	Greif (1967)
	12,500	1.3				
	25,000	1.5				
n-Hexane (110-54-3)	6250	0.8	Basketter (2003)	68,966	0/25	Kligman (1966a)
	12,500	0.8				
	25,000	2.2				
Hydrocortisone (50-23-7)	625	0.3	Basketter (2003)	17,242	0/25	Kligman (1966a)
	1250	0.1				
	2500	0.06				
Isopropyl myristate (110-27-0)	6250	2.1	Ryan et al. (2000)	13,793	0/25	Opdyke (1976a)
	12,500	3.3				
Linalool (78-70-6)	6250	2.5	Ryan et al. (2000)	55,176	0/25	Greif (1967)
	12,500	4.8				
4-Methoxyacetophenone (100-06-1)	2500	1.3	Ryan et al. (2000)	41,38	0/25	Opdyke (1974)
	6250	1.0				
	12,500	1.0				
6-Methylcoumarin (92-48-8)	1250	1.2	Scholes et al. (1992)	2759	0/25	Opdyke (1976b)
	2500	0.9				
	6250	0.8				
Methyl salicylate (119-36-8)	6250	0.9	Basketter (2003)	5517	0/27	Opdyke (1978a)
	12,500	1.0				
	25,000	2.6				
Resorcinol (108-46-3)	1250	2.2	Basketter (2003)	10,345	0/22	Kligman (1966a)
	2500	2.2				
	6250	2.7				
Salicylic acid (69-72-7)	1250	0.8	Basketter (2003)	13,793	0/25	Kligman (1966a)
	2500	1.5				
	6250	2.5				

Table 4
Potency groups for skin sensitizing agents according to the EC_3 observed in the local lymph node assay (LLNA)

Group	EC_3 (%)	Corresponding DSA_{EC3} ($\mu g/cm^2$)
Group 1: extreme	$\leqslant 0.2$	$\leqslant 50$
Group 2: strong	>0.2 to $\leqslant 2$	>50 to $\leqslant 500$
Group 3: moderate	>2 to $\leqslant 20$	>500 to $\leqslant 5000$
Group 4: weak	>20	>5000

HMT (Kligman, 1966a) and revealed no clear-cut response in the LLNA when tested at concentrations of up to 25% (Basketter et al., 1994). Assignment to group 4 would result in no need for classification of a preparation with a content of up to 20%, in contrast to currently prevailing legislation, which requires labeling if 1% is exceeded.

Table 5
Proposed concentration limits for the classification of preparations as "may cause sensitizing by skin contact" (R43), depending on assignment to potency groups

RL 1999/45/EG (%)	EC SEG (2003) (%)	This paper (%)
>1	moderate: >1	weak: >10
		moderate: >1
	strong: >0.1	strong: >0.1
	extreme: >0.001	extreme: >0.01[a]

[a] Concentration limits <0.01% should be derived substance-by-substance for very potent sensitizers with EC_3 values below 0.02%.

The proposed concentration limits for preparations are not meant to provide protection against the elicitation of allergic symptoms in sensitized individuals or even against primary sensitization. But by taking into account highly differing potencies of substances, protection against sensitization would be significantly im-

proved compared to current practice, very possibly leading in the long run to reduced numbers of sensitized individuals. Elicitation thresholds are not well-established in humans for most substances and no reliable method is currently available to estimate elicitation thresholds in animal assays. To protect people who are already sensitized the EC SEG (2003) proposed to list skin sensitizing substances on the label when they are present at a concentration of 10 ppm or above. In the case of extreme sensitizers (group 1), it was suggested to list substances in concentrations of 1 ppm and above.

Whereas potency considerations prove helpful for comparing potency between substances and drawing regulatory conclusions from this, the setting of exposure limit values for sensitizers still remains difficult. In addition to the mentioned uncertainties of experimental results, exposure conditions (air humidity, skin moisture, and skin occlusion), time and duration of exposure, and inter-individual differences due to age, gender, and genetic preposition significantly influence overall susceptibility and, therefore, sensitization and elicitation thresholds. Moreover, limit values meant to protect against elicitation in sensitized individuals must take into account the interdependency of induction and elicitation, which may cause considerable variability in allergic reaction outcome in individuals. Several authors recently proposed the use of LLNA as a starting point to estimate allowable doses aiming to protect humans from elicitation and/or sensitization (Felter et al., 2003; Griem et al., 2003). They covered the mentioned uncertainties by safety factors. To assess the reliability of these proposed risk assessment schemes more information has to be gathered (e.g., on inter-individual differences in susceptibility and on human thresholds for elicitation) and then applied to exemplary substances.

5. Conclusions

This analysis of the quantitative relationship between the outcome of the LLNA and human sensitization tests demonstrates that the correlation is sufficiently strong to permit relying on the LLNA for relative potency assessments for sensitizing substances. Proposed differentiation according to potency for assessing individual substances and classifying preparations would lead to more extensive use of available data and promise to significantly improve regulation of skin sensitizers. Its usefulness and practicability must to be demonstrated after implementation in regulatory frameworks.

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