

Assessment of the sensitizing potential of textile disperse dyes and some of their metabolites by the loose-fit coculture-based sensitization assay (LCSA)

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Abstract Certain textile disperse dyes are known to cause allergic reactions of the human skin. Here, we examined 8 disperse dyes and 7 products of azo-cleavage of these dyes in an in vitro assay. We used the loose-fit coculture-based sensitization assay (LCSA) of primary human keratinocytes and of allogenic dendritic cell-related cells for combined testing of the sensitizing and irritative properties of these substances. The obtained data were compared to data generated in a modified version of the local lymph node assay by our working group. Disperse Blue 1 (DB1), *p*-nitroaniline (*p*NA) and *p*-aminoacetanilide (AAA) showed no sensitizing potential under our experimental conditions. Disperse Blue 124 (DB124), Disperse Yellow 3 (DY3), Disperse Orange 37/76 (DO37), Disperse Blue 106 (DB106), Disperse Red 1 (DR1), 2-amino-*p*-cresol (*Ap*C), Disperse Orange 3 (DO3) and 2,6-dichloro-4-nitroaniline (DCh) were categorized as extreme sensitizers. Para-phenylenediamine (*p*PD) was categorized as

strong sensitizer, and 2-amino-5-nitrothiazole (ANT) and 2-(*N*-ethylanilino)-ethanol (EAE) as weak sensitizers. All dyes, except for DB1, and *Ap*C turned out to be strong irritants. DB1, ANT and DCh showed only weak irritative potential. PPD, *p*NA, EAE and AAA did not show any irritative effect at the concentration range tested. These results correlate with data derived from the modified version of LLNA and human data. Therefore, the LCSA represents a suitable test system to simultaneously analyse two crucial properties of substances relevant for allergy induction.

Keywords Disperse dyes · Sensitizing potential · In vitro sensitization assay · LCSA

Abbreviations

AAA	<i>p</i> -Aminoacetanilide (CAS No. 122-80-5)
7-AAD	7-Aminoactinomycine D (CAS No. 7240-37-1)
ANT	2-Amino-5-nitrothiazole (CAS No. 121-66-4)
<i>Ap</i> C	2-Amino- <i>p</i> -cresol (CAS No. 95-84-1)
DB1	Disperse Blue 1 (CAS No. 2475-45-8)
DB106	Disperse Blue 106 (CAS No. 12223-01-7)
DB124	Disperse Blue 124 (CAS No. 61951-51-7)
DB35	Disperse Blue 35 (CAS No. 12222-75-2)
DCs	Dendritic cells
DCh	2,6-Dichloro-4-nitroaniline (CAS No. 99-30-9)
DCrc	Dendritic cell-related cells
DMSO	Dimethylsulfoxide
DO3	Disperse Orange 3 (CAS No. 730-40-5)
DO37	Disperse Orange 37/76 (CAS No. 13301-61-6)
DR1	Disperse Red 1 (CAS No. 2872-52-8)
DY3	Disperse Yellow 3 (CAS No. 2832-40-8)
EAE	2-(<i>N</i> -ethylanilino)-ethanol (CAS No. 95-50-2)
EC50	Substance concentration causing a half-maximal increase of CD86 expression
FACS	Fluorescence-activated cell sorting

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FCS	Foetal calf serum
GMPT	Guinea pig maximization test
KCs	Keratinocytes
KGM-2	Keratinocyte growth medium 2
LCSA	Loose-fit coculture-based sensitization assay
LLNA	Murine local lymph node assay
MFI	Mean fluorescence intensity
PBMCs	Peripheral blood mononuclear cells
PE	Phycoerythrin
<i>p</i> NA	<i>p</i> -Nitroaniline (CAS No. 100-01-6)
<i>p</i> PD	1,4-Phenylenediamine (CAS No. 106-50-3)
TNBS	2,4,6-Trinitrobenzenesulfonic acid (CAS No. 2508-19-2)

Introduction

Textile dyes exhibit various chemical structures and physico-chemical properties. They are widely used in production of garments, and their close contact to the skin may pose a danger to human health. Of all textile dyes, disperse dyes—particularly disperse azo dyes—are the most important group (Federal Institute for Risk Assessment 2007). It has been known for a long time that disperse textile dyes can cause allergic reactions on the human skin, such as allergic contact dermatitis and contact urticaria (Hatch 1984; Hausen and Menezes 1986; Hausen 1993). Although azo dyes, which can be cleaved to carcinogenic amines by skin or intestinal bacteria, are banned by EU legislation (Directive 2002/61/EG), no such regulation exists for sensitizing disperse dyes. Producers are not obliged to provide information about auxiliaries or dyes used. As a consequence, consumers are not fully protected from contact with these dyes, especially when wearing imported clothes. In Germany, the Federal Institute for Risk Assessment recommends not to use certain dyes with sensitizing potential in textiles (BfR 2007). With regard to tattoos, these substances are banned in Germany by the Ordinance on Tattoos, 2008. The international textile and clothing industry uses the so-called Restricted substances list (RSL) that provides information about substances that are banned or restricted in different countries. The RSL was first published in 2007 by the American Apparel and Footwear Association (AAfA). It lists the eight disperse dyes we have tested in the experiments described here (AAfA 2011).

While many clinical data about contact allergies to textile dyes have been published (Hausen 1993; Hatch et al. 2003), only few quantitative data exist, which were gathered by animal testing (Ahuja et al. 2010; Hausen and Sawall 1989). However, ethical concerns have been raised regarding animal experimentation. Furthermore, the European REACH regulation demands replacement of animal

testing for chemicals by in vitro methods, if possible. Currently, no OECD-validated in vitro method for the assessment of the sensitizing potential of chemicals is available. It is hence of great interest to develop and validate new in vitro methods for this purpose.

We have recently developed an in vitro assay based on a coculture of primary human keratinocytes and allogenic dendritic cell-related cells (DCrc), which is capable of identifying the sensitizing and irritative potential of substances—the loose-fit coculture-based sensitization assay (LCSA) (Schreiner et al. 2007; Wanner et al. 2010). Using the LCSA, we have tested eight textile dyes, which are listed in the latest RSL (AAfA 2011) and which the Federal Institute for Risk Assessment recommends not to use in textiles (BfR 2007). In addition, we have tested products of azo-cleavage of these dyes as far as commercially available. The eight disperse dyes have been tested before in a modified local lymph node assay in mice (Ahuja et al. 2010). Thus, we are able to compare the results obtained by in vitro experiments with those animal data.

Materials and methods

Media and reagents

All tested substances as listed in Table 1 were purchased from Sigma-Aldrich (Steinheim, Germany) and were dissolved in DMSO (DMSO Hybrimax, Sigma-Aldrich). Final concentration of DMSO in cell culture medium did not exceed 0.1%. Cells were cultured in serum-free KGM-2 (PromoCell, Heidelberg, Germany) supplemented with complete SupplementPack (PromoCell), 100 U/ml penicillin and 100 µg/ml streptomycin (Biochrom, Berlin, Germany). TNBS was also obtained from Sigma-Aldrich. The cytokines IL-4 and GM-CSF were purchased from ImmunoTools (Friesoythe, Germany) and TGF-β1 from R&D Systems (Minneapolis, USA). For FACS analysis, cells were stained with PE-conjugated anti-CD86 and PE-conjugated isotype control (clone: MOPC-21, mouse IgG1, BD Biosciences, Heidelberg, Germany) and 7-AAD (Cell Viability Solution, BD Biosciences).

Cryopreservation of primary human keratinocytes

Human skin from healthy donors was received as anonymous residual material from plastic surgery. After 18 h of incubation in PBS containing 2 U/ml of dispase I (Roche, Mannheim, Germany) at 4°C, epidermal sheets were stripped off the dermal layer and dissociated in PBS containing 0.25% trypsin (Biochrom) and 0.01% DNase (Roche) for 15 min at 37°C. Single-cell suspension was obtained by passing through a 40-µm cell strainer (BD,

Table 1 Tested substances (LCSA)

Dye	Azo-cleavage products tested
Disperse Blue 106	2-Amino-5-nitrothiazole
Disperse Blue 124	2-Amino-5-nitrothiazole
Disperse Orange 3	<i>p</i> -Phenylenediamine <i>p</i> -Nitroaniline
Disperse Orange 37/76	2,6-Dichloro-4-nitroaniline
Disperse Red 1	<i>p</i> -Nitroaniline 2-(<i>N</i> -ethylanilino)-ethanol
Disperse Yellow 3	<i>p</i> -Aminoactanilide 2-Amino- <i>p</i> -cresol
Disperse Blue 1	(Anthraquinone dyes do not form azo-cleavage products)
Disperse Blue 35	

Heidelberg, Germany). Cells were washed with PBS, resuspended in KGM-2 and seeded on Costar Cell Culture Flasks (Corning, Shiphoh-Rijk, the Netherlands) at a density of 5×10^5 cells/cm². Cells were cultured until confluence with medium changes on alternate days. KCs were harvested by trypsinization and cryopreserved in FCS with 10% DMSO (Hybrimax, Sigma) at a density of 2×10^6 cells/ml.

Cryopreservation of human PBMCs

PBMCs were enriched from fresh buffy coat preparations (German Red Cross, Berlin, Germany) by density centrifugation on Ficoll-Paque (Biochrom). PBMCs were cryopreserved as described above for KCs at a density of 4×10^7 cells/ml.

Loose-fit coculture-based sensitization assay

KCs were defrosted at 37°C, washed with PBS and resuspended in KGM-2. KCs were seeded in 12-well plates (Costar Cell Culture Cluster, Corning) at a density of 6×10^4 cells/cm². When cells were adherent, wells were rinsed with PBS to remove dead cells. PBMCs were first defrosted and resuspended in KGM-2, then seeded onto KCs at a density of 3×10^6 cells/cm². IL-4, GM-CSF and TGF- β were added to the coculture at final concentrations of 100, 100 and 10 ng/ml, respectively. After 48 h of incubation, test substances were added. Increasing concentrations of test substances were tested until significant cytotoxicity occurred as measured by 7-AAD staining. Every substance was tested with three different DC-rc/KC-donor pairs to account for interindividual variability.

TNBS at a concentration of 100 μ mol/l served as positive control. After another 48 h, floating cells were pipetted off the coculture and prepared for FACS analysis.

FACS analysis

FACS analysis was performed on a FACS Calibur flow cytometer (BD Biosciences). Cells were gated to a distinct population of DCrc according to their scatter properties (Fig. 1). Of the three cell populations to be seen in a scatter dot plot, one is CD14⁺, one CD4⁺CD8⁺ and one CD86⁺ (data not shown). The latter is considered to be the DC-rc population and is therefore gated (for details, see earlier publications: Schreiner et al. 2007, 2008). The experiments were assumed to be reliable, when the addition of TNBS led to a 1.5- to 3-fold increase in CD86 expression compared with zero controls (Fig. 1). Relative upregulation of CD86 expression was calculated by the following equation: MFI (CD86 of treated cells)/ MFI (CD86 of vehicle control). 7-AAD staining was used to determine and exclude dead cells from the calculation. Dose-effect relationships for relative CD86 expression and viability were determined using GraphPad Prism[®] software (GraphPad Software, San Diego, USA).

Categorization

For categorization of sensitizing potential, the substance concentration that led to half-maximal increase of CD86 expression (EC₅₀) was assessed. Substances were categorized as follows: half-maximal increase in CD86 expression <12.5 μ M, extreme; <50 μ M, strong; <100 μ M, moderate; and >100 μ M, weak. Substances failing to elicit significant rise in CD86 expression up to the maximum test concentration were deemed non-sensitizers.

Likewise, categorization of irritative potential is based on the concentration that resulted in cell death of 50% (EC₅₀%) of DC-rc as compared to vehicle controls. Substances eliciting 50% cell death at concentrations below 50 μ M were considered to be irritative. Those with an EC₅₀% value from 50 to 1,000 μ M were considered to be weakly irritative. Substances that did not reach the 50% threshold or with EC₅₀% values above 1,000 μ M were rated as non-irritative (Wanner et al. 2010).

Results

Sensitizing potential of substances

Of the eight disperse dyes tested, only Disperse Blue 1 showed no measurable sensitizing potential up to a concentration of 100 μ M. Disperse Orange 3 was categorized as a strong sensitizer with an EC₅₀ value of 18 μ mol/l. All other dyes were categorized as extreme sensitizers with EC₅₀ values <12.5 μ mol/l. Based on the assessed EC₅₀ values, disperse dyes could be arranged in the following descending

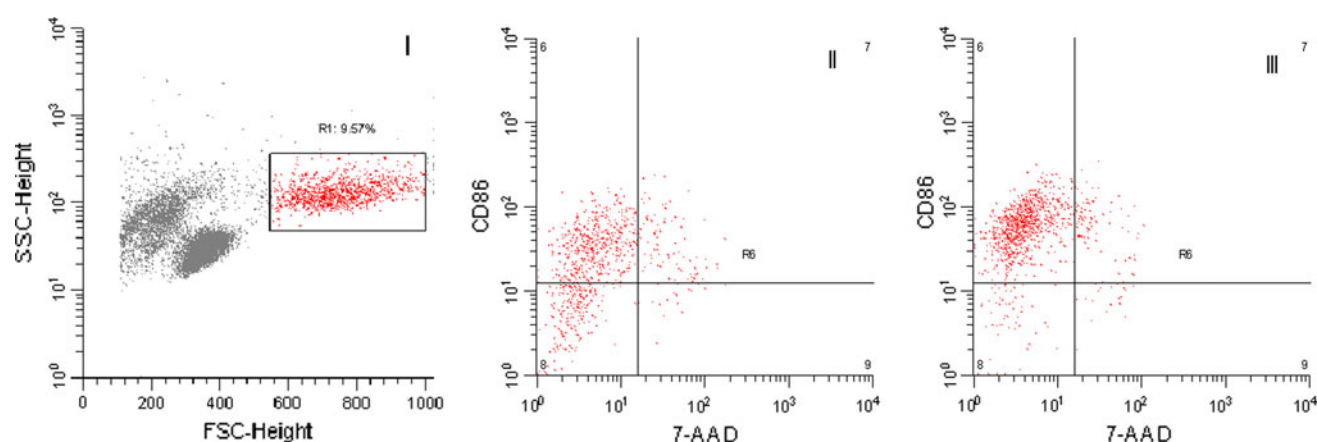


Fig. 1 Scatter dot plot (I) and CD86/7-AAD plots (II and III) of a representative experiment showing three distinct cell populations, one of which is CD86⁺ and gated (I) and the change in CD86-MFI in the

gated population between a zero control (II) and a positive control with 100 µmol/l TNBS (III)

Table 2 EC50 and EC50% values and categorization of tested substances (LCSA)

Substance	Sensitization EC50 (µmol/l)	Irritation EC50% (µmol/l)	Category	
			Sensitization	Irritation
Disperse Blue 1 (DB1)	/	85.0	None	Weak
<i>p</i> -Aminoacetanilide (AAA)	/	/	None	None
<i>p</i> -Nitroaniline (<i>p</i> NA)	/	/	None	None
Disperse Orange 37/76 (DO37)	1.0	18.2	Extreme	Irritative
Disperse Red 1 (DR1)	3.0	31.0	Extreme	Irritative
Disperse Yellow 3 (DY3)	0.5	36.5	Extreme	Irritative
Disperse Blue 35 (DB35)	6.0	40.0	Extreme	Irritative
Disperse Blue 106 (DB106)	2.5	12.0	Extreme	Irritative
2-Amino- <i>p</i> -cresol (<i>Ap</i> C)	6.7	17.3	Extreme	Irritative
Disperse Orange 3 (DO3)	18.0	22.0	Strong	Irritative
Disperse Blue 124 (DB124)	0.25	23.0	Extreme	Irritative
2,6-Dichloro-4-nitroaniline (DCh)	30.0	95.0	Strong	Weak
<i>p</i> -Phenylenediamine (<i>p</i> PD)	42.0	100.0	Strong	Weak
2-Amino-5-nitrothiazol (ANT)	120.0	126.0	Weak	Weak
2-(<i>N</i> -Ethylanilino)-ethanol (EAE)	128.0	/	Weak	None

order according to their sensitizing potential: Disperse Blue 124 > Disperse Yellow 3 > Disperse Orange 37 > Disperse Blue 106 > Disperse Red 1 > Disperse Blue 35 > Disperse Orange 3 > Disperse Blue 1. The corresponding EC50 values are listed in Table 2.

Para-nitroaniline (*p*NA) and *p*-aminoacetanilide (AAA)—azo-cleavage products of Disperse Orange 3 and Disperse Yellow 3, respectively—showed no sensitizing properties up to concentrations of 200 and 300 µM, respectively. An azo-cleavage product of both Disperse Orange 3 and Disperse Red 1—2-amino-*p*-cresol (*Ap*C)—was categorized as an extreme sensitizer with an EC50 value of 6.7 µmol/l. For *p*-phenylenediamine (*p*PD), the second cleavage product of Disperse Orange 3, and 2,6-dichloro-4-nitroaniline (DCh), a cleavage product of Disperse Orange

37, we assessed EC50 values of 42 and 30 µmol/l, respectively. The substances were therefore categorized as strong sensitizers. As weak sensitizers, we categorized 2-amino-5-nitrothiazol (ANT, cleavage product of Disperse Blue 106 and Disperse Blue 124) and 2-(*N*-ethylanilino)-ethanol (EAE, second cleavage product of Disperse Red 1) with EC50 values of 120 and 128 µmol/l, respectively.

Irritative potential of substances

Baseline of cell viability in zero controls was $63.89 \pm 9.56\%$ viable cells. All disperse dyes except for Disperse Blue 1 were classified as irritative with EC50% values ranging from 12 to 40 µM (see Table 2). Disperse Blue 1 was categorized as a weak irritant with an EC50% value of

85 μM . The irritative potential of cleavage products was weak (DCh, *p*PD and ANT) or not assessable (*p*NA, AAA, and EAE). Only *Ap*C was classified as irritative with an EC50% value of 18 μM .

Strong or extreme sensitizers tended to be irritants as well, except for *p*PD and DCh, both having little irritative potential (Table 2).

Discussion

Contact dermatitis is a common skin disease with major socio-economic impact. Many reports have described textile dye-related contact sensitization, especially in work-related contexts (Marks 1986; Sadhra et al. 1989; Bordel-Gomez et al. 2010). The gold standard for predictive testing of contact allergens are still animal tests, such as the guinea pig maximization test (GPMT) and the local lymph node assay (LLNA) in mice. The LLNA has advantages over conventional guinea pig assays in measuring an objective endpoint, that is, lymph node cell proliferation measured by ^3H -thymidine incorporation (Sailstad 2002). Several modifications of the LLNA have been suggested over the past years to improve its reliability and practicability. Non-radioactive endpoints, such as lymph node weight, cell proliferation and ear swelling, have been described by several authors (Ehling et al. 2005a, b; Suda et al. 2002; for review: Anderson et al. 2011). Two versions of the LLNA with non-radioactive endpoints, the LLNA: DA and the BrdU-ELISA-version, are currently validated and available as draft OECD guidelines (Omori et al. 2008; Kojima et al. 2011). Ulrich et al. (2001) described a two-tiered murine local lymph node assay and proposed that it is useful to discriminate between contact allergenic potential and skin irritation potential. In earlier studies, we established a biphasic sensitization-challenge protocol with an induction and an elicitation phase using non-radioactive endpoints, such as ear thickness, ear punch weight, lymph node weight, lymph node cell count and flow cytometric measurement of various lymphocyte subpopulations (Stahlmann et al. 2006; Ahuja et al. 2009a, b, 2010).

The present experiments were undertaken with the aim to study the sensitization potential of various disperse dyes and their azo-cleavage products using an in vitro assay, the LCSA. We compared our results to data derived from two earlier studies by our work group, which used a modified biphasic LLNA protocol on the same set of disperse dyes (Ahuja et al. 2010) and two azo-cleavage products (Stahlmann et al. 2006). Based on our LLNA data, substances were grouped according to the lowest concentration eliciting a significant increase in lymph node cell count (Table 3).

Table 3 Comparison of LLNA and LCSA sensitization data for the tested textile dyes and cleavage products

Substance	LLNA (%*)	LCSA	LCSA EC50 (μM)
Disperse Blue 124	0.003	Extreme	0.25
ANT		Weak	120
Disperse Blue 106	0.003	Extreme	2.5
ANT		Weak	120
Disperse Blue 1	3	None	–
Disperse Red 1	3	Extreme	3
<i>p</i> NA		None	–
EAE		Weak	128
Disperse Orange 37	10	Extreme	1
DCh		Strong	30
Disperse Blue 35	10	Extreme	6
Disperse Orange 3	30	Strong	18
<i>p</i> NA		None	–
<i>p</i> PD		Strong	42
Disperse Yellow 3	30	Extreme	0.5
AAA	30	None	–
<i>Ap</i> C	10**	Extreme	6.7

* Ahuja et al. (2010), ** Stahlmann et al. (2006), not tested in lower concentrations

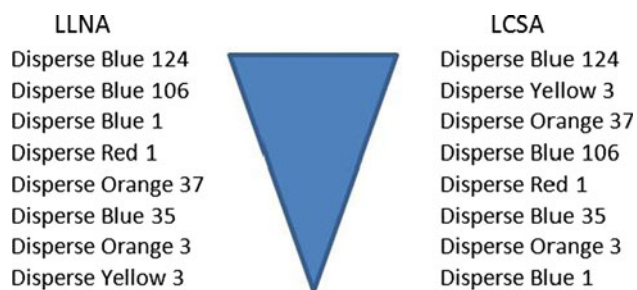


Fig. 2 Ranking of disperse dyes according to their sensitization potential as derived from modified LLNA data (Ahuja et al. 2010) and LCSA data. The most potent sensitizers are listed at top

Comparing the ranking of dyes according to their sensitization potential, modified LLNA and LCSA gave similar results (Fig. 2).

An obvious mismatch is found in Disperse Blue 1, which is categorized as a strong sensitizer in the modified LLNA but a non-sensitizer in the LCSA.

A sensitizing potential for this dye was demonstrated in the GPMT (Hausen and Sawall 1989). However, primary human data about contact allergy against Disperse Blue 1 have not been published. In the publication by Hausen and Sawall, it was only reported as having the ‘least frequency’ of reported contact allergies against dyes in humans. Considering this, it seems unlikely that DB1 is a strong sensitizer in humans. The use of cells of the relevant species (human) in the LCSA could be of advantage.

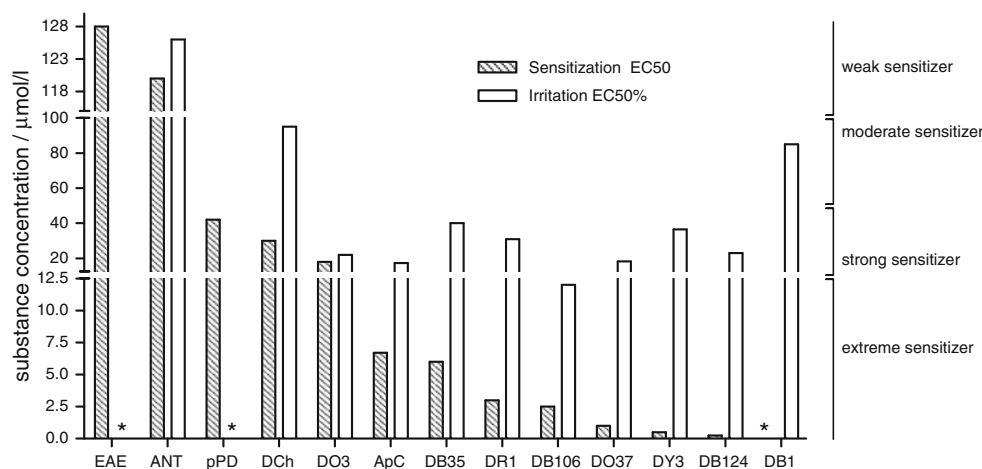


Fig. 3 EC50 and EC50% values obtained by graphical analysis of LCSA data. Substances with EC50% values of $>50 \mu\text{M}$ are considered as irritative, with EC50% values of $<50 \mu\text{M}$ as weakly irritative. *Irritative or sensitizing potential not measurable

Other strong mismatches are found in the results for Disperse Yellow 3 and Disperse Orange 37/76. LCSA classified DY3 as extreme sensitizer, whereas in the modified LLNA, it exhibited only weak activity. The azo-cleavage products of DY3, AAA and ApC, showed no and extreme sensitizing potential in LCSA, respectively. LLNA data revealed weak and moderate sensitizing potential for AAA and ApC, respectively, but ApC was not tested in lower concentrations than 10%. Disperse Orange 37/76 was categorized as an extreme sensitizer by LCSA but as a moderate sensitizer by LLNA. We have also tested an azo-cleavage product of DO37, 2,6-dichloro-4-nitroaniline, which was identified as a strong sensitizer.

Possibly, disperse dyes are cleaved by human keratinocytes in the LCSA to a higher degree than in animal skin. We assume that in this way ApC contributes to the effects of DY3 found in LCSA and that the stronger reaction of DO37 in the LCSA is due to synergistic effects of the cleavage product and the mother substance. However, this hypothesis needs to be proved in further experiments.

The missing skin barrier in the in vitro assay could also explain that apparently most substances were classified as stronger sensitizers by LCSA compared with the modified LLNA.

Considering the remarks above, the sensitizing potential of substances as measured in the in vitro assay LCSA showed a good correlation with animal data using a modified LLNA protocol. Where available, human data were also congruent with our findings in vitro. In a study from Italy, Seidenari et al. (2002) observed Disperse Blue 124 and 106 and Disperse Orange 3 as the most common sensitizers among the various textile dyes tested.

We observed that the sensitizing potential of mother substances was consistently stronger than that of their

corresponding cleavage products. A possible explanation could be the cleavage of dyes by metabolically active keratinocytes in the in vitro system. The presence of both the mother substance and its cleavage products in the cell culture medium could have an additive effect on the activation of dendritic cells. This additive effect could furthermore explain the tendency of the LCSA to classify substances as stronger sensitizers as compared to the modified LLNA.

The LCSA is designed to provide some indication on the irritative potential of a test substance besides its sensitizing potential. Obtaining data of irritative and sensitizing potential concomitantly is an intriguing concept because a correlation between these two entities has often been proposed (for review: McFadden and Basketter 2000). However, with the results from this panel of textile dyes and some of their cleavage products, we were not able to prove a strict correlation (Fig. 3). Linear regression of log/log-plotted sensitization and irritation data and calculated Pearson correlation coefficient ($r = 0.009867$, $p = 0.9745$) showed no significant correlation between sensitizing and irritative potentials (Fig. 4). Statistical analysis of a larger set of 55 substances using data from LLNA and GPMT was also not conclusive (Basketter et al. 2007).

Several other in vitro assays for sensitization have been proposed, mostly based on continuous cell lines. The human cell lines U937 and THP-1 have been extensively studied (Python et al. 2007; Sakaguchi et al. 2006). To our knowledge, disperse dyes have never been tested with one of these cell lines. So a direct comparison was not possible. It is often stated that in vitro assays based on continuous cell lines do not have the problem of donor-to-donor variability (Aeby et al. 2010). However, in our experience donor-to-donor variability has little impact on the LCSA. A post hoc analysis of CD86 expression after application of

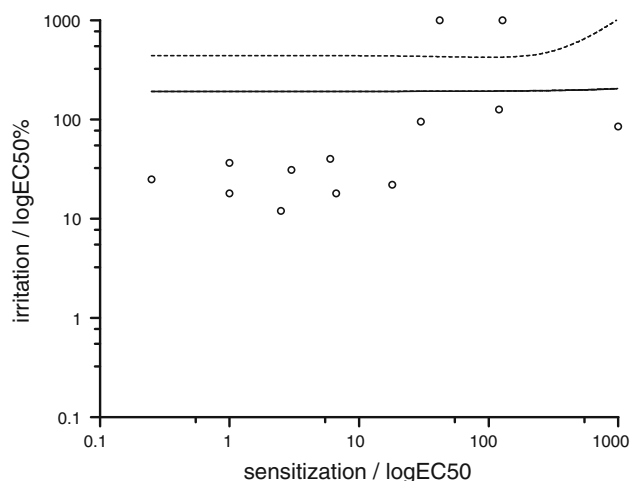


Fig. 4 Log/log plot of data for sensitization and irritative potentials. Linear regression (with 95% confidence band) reveals no significant correlation between the two properties

100 μ M TNBS (which served as positive control in all test series) revealed a relatively low variability: 2.3 ± 0.48 -fold increase in CD86 ($\pm$ SEM) with 12 different donor pairs of keratinocytes and dendritic cell-related cells. While unspecific exhibitory effects due to the allogenicity of KC and monocyte donors cannot be excluded, the low donor-to-donor variability shows that this is not of practical relevance.

In conclusion, our study showed that the results obtained with the LCSA gave comparable results to data derived from LLNA experiments, as well as data from humans. Some discrepancies might be explainable by species differences in the metabolic capacities of the keratinocytes. The LCSA is designed to measure both irritative and sensitizing potential of substances in one step. Hence, the LCSA provides a robust test system to simultaneously analyse two crucial properties of chemicals relevant for induction of contact allergy.

Acknowledgments In memoriam. The in vitro assay LCSA (loose-fit coculture-based sensitization assay) is a result of the research activities of Priv.-Doz. Dr. rer. nat. Reinhard Wanner, who passed away unexpectedly at the age of 51 on 2nd April 2010. Since 2008, Dr. Wanner had been the coordinator of the Master Degree Program in Toxicology at the Charité—Universitätsmedizin Berlin. His scientific activities focussed on the immunologic aspects of the skin. Over the last few years, he and his co-workers published a series of papers on LCSA experiments and described the usefulness of this in vitro test, which has great potential to be used as an alternative to the LLNA or other animal experiments. Data presented in this manuscript further underline the robustness and usefulness of the test; they derive from experiments that he had initiated in early 2010. We feel deeply obliged to our colleague, friend and mentor, and we will carry on his ideas into the future. Studies were supported by a grant from the Federal Institute for Risk Assessment (Bundesinstitut für Riskobewertung, BfR), Berlin, Germany. We are also grateful for a grant of the Sonnenfeld Stiftung, Berlin, which financed the FACS Calibur.

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