



Comparison of the skin sensitizing potential of unsaturated compounds as assessed by the murine local lymph node assay (LLNA) and the guinea pig maximization test (GPMT)

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Abstract

The skin sensitization potential of eight unsaturated and one saturated lipid (bio)chemicals was tested in both the LLNA and the GPMT to address the hypothesis that chemicals with unsaturated carbon–carbon double bonds may result in a higher number of unspecific (false positive) results in the LLNA compared to the GPMT. Seven substances (oleic acid, linoleic acid, linolenic acid, undecylenic acid, maleic acid, squalene and octinol) gave clear positive results in the LLNA (stimulation index (SI) ≥ 3) and thus would require labelling as skin sensitizer. Fumaric acid and succinic acid gave clearly negative results. In the GPMT, besides some sporadic skin reactions, reproducible skin reactions indicating an allergic response were found in a few animals for four test substances. Based on the GPMT results, only undecylenic acid would have to be classified and labelled as a skin sensitizer according to the European Dangerous Substance Directive (67/548/EEC) (results for linoleic acid were inconclusive), while the other seven test substances would not require labelling. Possible mechanisms for unspecific skin cell stimulation and lymph node responses are discussed. In conclusion, the suitability of the LLNA for unsaturated compounds bearing structural similarity to the tested substances should be carefully considered and the GPMT should remain available as an accepted test method for skin sensitization hazard identification.

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1. Introduction

The local lymph node assay (LLNA) was first described by Kimber and Weisenberger (1989) as an alternative to

guinea pig tests for the detection of the skin sensitizing potential of chemicals. It was recognized in 1999 by ICC-VAM, the Interagency Coordinating Committee on the Validation of Alternative Methods, as a valid alternative to the GPMT (ICCVAM, 1999; Dean et al., 2001; Haneke et al., 2001; Sailstad et al., 2001). In 2002, the LLNA was published by the OECD in Test Guideline No. 429 (OECD, 2002) as a stand alone method to assess the skin sensitizing properties of chemicals and is listed as such in Annex V of Directive 67/548/EEC. In context of the 3R principles

Abbreviations: GPMT, guinea pig maximization test; LLNA, local lymph node assay; SI, stimulation index; LNWI, lymph node weight index.

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(Refinement, Reduction, Replacement), the LLNA is the preferred method for the evaluation of skin sensitization in the European Union. Furthermore, the LLNA enables a more objective readout of results and facilitates the determination of threshold doses. Results generated in the LLNA are used for hazard identification on which classification and labelling of a chemical and subsequent risk assessment are based. The occurrence of apparent false negative and false positive results from the LLNA, and, of course, from other sensitization tests, too, needs to be avoided as much as possible, because false negatives or false positive results could have substantial impact on worker and consumer protection issues as well as important economic implications.

The OECD Guideline 429 states, that the LLNA has its limitations by referring to examples of false negative findings with certain metals and false positive findings with certain skin irritants (Ikarashi et al., 1993; Montelius et al., 1994, 1996, 1998). Based on comparison of the percentages of positive and negative findings in LLNA vs. GPMT tests submitted to authorities in data packages generated as part of new chemical notifications in the EU, it was discussed at two workshops (HSE, 2004; CTL, 2005), whether the LLNA may be more sensitive than the GPMT. However, a study suggested that the proportion of new substances classified as skin sensitizers based on the LLNA was not higher than that based on the GPMT (Cockshott et al., 2006).

Vohr and Ahr (2005) have drawn attention to the problem of false positive results of the LLNA caused by non-specific cell activation as a result of inflammatory processes in the skin (irritation). A non-specific activation of dendritic cells can lead to cell migration and non-specific proliferation of lymph node cells. The authors compared test results using as readout parameters either cell proliferation alone or cell proliferation in combination with irritation (local inflammatory reactions in the skin measured as increases in ear thickness or ear weight) and found that a considerable number of test compounds gave 'false positive' results.

Unpublished data indicated that several substances used in consumer products, which have been on the market for decades without any indication of sensitizing property, unexpectedly showed positive results in the LLNA. Regardless of whether these results were confirmed by other skin sensitization tests in either guinea pigs (GPMT, Buehler Test) or humans (HRIPT, human repeated insult patch test), under current regulatory practice such results would necessitate a classification and labelling as "skin sensitizer" (R43). In view of this apparent dilemma, a reappraisal of the potential limitations of the LLNA for some classes of compounds seemed necessary. In order to extend the scientific basis on possible limitations of the LLNA for substance classes which have not been covered in previous validation trials, this paper presents a comparison of results obtained with nine (bio)chemical compounds tested in both, the LLNA and the GPMT and

addresses the hypothesis, that chemicals with unsaturated carbon-carbon double bonds may result in a higher number of unspecific, i.e., false positive, results in the LLNA compared to the GPMT.

2. Materials and methods

2.1. Chemicals

Oleic acid (Aldrich No. O1008), linoleic acid (Aldrich No. L1376), undecylenic acid (Aldrich No. 124672), fumaric acid (Aldrich No. 240745), maleic acid (Aldrich No. M153), succinic acid (Aldrich No. 398055) and octinol were obtained from Sigma-Aldrich (Munich, Germany). Linolenic acid (Acros No. 227240050) was purchased by Acros Organics (Geel, Belgium) and Squalene (Merck No. 821068.0100) was obtained from Merck Schuchardt (Hohenbrunn, Germany). The chemical description, CAS number, structure and purity of the test chemicals are shown in Table 1. Other materials used were acetone (Biesterfeld Spezialchemie), olive oil highly refined (Sigma Chemicals Co.), DMSO (dimethylsulphoxide, AppliChem, Darmstadt, Germany), ³H-methyl thymidine (TRK 300, 25 Ci/mmol; Amersham Pharmacia Biotech) diluted to a working concentration of 80 µCi/mL, Trichloroacetic acid (TCA, Sigma GmbH), phosphate-buffered saline (PBS, BSL Bioservice), *p*-phenylenediamine (purity >98%, Sigma GmbH), Freund's complete adjuvant (FCA, Sigma Chemicals Co.), NaCl 0.9% (B. Braun Melsungen), sodium lauryl sulphate (Sigma Chemicals Co.) and vaseline (Fluka Chemika).

2.2. Animals

Female SPF-CBA/Ca01aHsd mice (6–12 weeks) and non-pregnant, nulliparous female SPF-Hsd Poc: DH guinea pigs (300–500 g at the commencement of the study) were purchased from Harlan Winkelmann (Borchen, Germany). The mice were housed in groups in Macrolon cages on Altromin saw fiber bedding. The guinea pigs were housed in groups in Terluran cages on Altromin saw fiber bedding or Lignocel bedding. The animals were barrier-maintained (semi-barrier) in an air conditioned room (temperature 22 ± 3 °C, relative humidity 55 ± 10%) with a 12-h light/dark cycle and air change rate of at least 10 × h⁻¹. The acclimatization period was at least five days. Food (Altromin 1324 maintenance diet for rats and mice, totally-pathogen-free; Altromin 3122 maintenance diet for guinea pigs or ssniff Ms-H, 4mm V2233-000 complete diet for guinea pigs, rich in crude fiber, totally-pathogen-free) and tap water were provided ad libitum.

2.3. Skin sensitization tests

All experiments were performed by Bioservice Scientific Laboratories GmbH (Planegg, Germany) under the guidance of Dr. Achim Albrecht in compliance with the OECD Principles of Good Laboratory Practice (as revised in 1997). According to the legal regulations in Germany, a project proposal for tests carried out on experimental animals had been approved by the Government of Upper Bavaria, Germany.

2.3.1. Local lymph node assay (LLNA)

The test was performed according to OECD Test Guideline No. 429 (OECD, 2002), EC guideline B.42 (EC, 2004) and US EPA Guideline OPPTS 870.2600 (US EPA, 2003). The test compounds were assayed at three concentrations. If technically possible, the vehicle was AOO (3 + 1 (v/v) acetone/olive oil). Due to their solubility properties, fumaric acid, maleic acid and succinic acid were tested using DMSO as vehicle. A preliminary experiment was performed with three animals to detect the highest tolerable exposure concentration. Three different concentrations of the test compound were applied daily to the ears of the animals, which were observed for systemic effects and local skin irritation. Local irritant effects expressed as ear swelling were assessed by measuring the ear

Table 1
Chemical description of test substances

Substance	Chemical structure	Purity (%)	Occurrence and use
Oleic acid CAS 112-80-1		99	Natural food ingredient, endogenous substance
Linoleic acid CAS 60-33-3		99	Essential fatty acid, natural food ingredient, endogenous substance
Linolenic acid CAS 463-40-1		90 ^a	Essential fatty acid, natural food ingredient, endogenous substance
Undecylenic acid CAS 112-38-9		98	Cosmetic ingredient
Fumaric acid CAS 110-17-8		99	Endogenous substance
Maleic acid CAS 110-16-7		99	Endogenous substance
Succinic acid CAS 110-15-6		99	Endogenous substance
Squalene CAS 111-02-4		98	Endogenous substance, natural food ingredient, cosmetic ingredient
1-Octin-3-ol CAS 818-72-4		96	Flavor and fragrance ingredient

^a The purity is given as 90% linolenic acid, the remainder being linoleic acid.

thickness on day 1 and day 4. Test concentrations used in the main test are given in the results table (Table 3).

In the main test, groups of 5 mice were treated by topical applications of 25 µl test or control solution to the entire dorsal surface of each ear. Topical applications were performed once daily over three consecutive days. A negative control group was treated with the vehicle alone. The positive control group received *p*-phenylenediamine at 1% in the respective vehicle. On day 6, all mice were dosed with 20 µCi ³H-methyl thymidine by intravenous injection into the tail vein. Approximately 5 h after ³H-methyl thymidine injection, all mice were sacrificed. The draining auricular lymph nodes were excised, weighed (the left and right lymph nodes were weighed and the mean calculated for each mouse), individually pooled for each animal (two lymph nodes per animal) and collected in PBS. A single cell suspension of pooled lymph node cells was prepared by gentle mechanical disaggregation through polyamide gauze (200 mesh size). After washing the gauze with PBS the cell suspension was pelleted in a centrifuge. The supernatant was discarded and the pellets were re-suspended with PBS. This washing procedure was repeated. After the final wash, each pellet was re-suspended in approx. 1 ml 5% TCA and incubated at 4 °C overnight for precipitation of macromolecules. Each precipitate was once washed again, re-suspended in 10 ml scintillation fluid, transferred into scintillation vials and stored at room temperature overnight.

The ³H-methyl thymidine incorporation was measured in a scintillation counter and expressed as the number of disintegrations per minute (DPM). Similarly, background ³H-methyl thymidine levels were also measured (5% TCA). The proliferative response of lymph node cells was expressed as the number of radioactive disintegrations per minute per lymph node (DPM/node). Before DPM/node values were determined, the background value was subtracted. For each dose group, the stimulation index (SI) was calculated as the ratio of the arithmetic mean DPM/node values of the test group and the negative control group.

In addition to the requirements of the OECD guideline, a lymph node weight index (LNWI) was calculated as the ratio of the arithmetic mean lymph node weight values of the test group and the negative control group. A one-sided Student's *t*-test was used to identify a statistically significant (at *p* < 0.01) lymph node weight increase in a treated group vs. the vehicle control group. Also in addition to the guideline, ear thickness was measured in all groups of mice using a mechanical caliper on day 1 (prior to first treatment) and on day 5 (prior to ³H-methyl thymidine injection) in order to determine local irritant effects as ear swelling response.

2.3.2. Guinea pig maximization test (GPMT)

The guinea pig maximization test was performed according to OECD Test Guideline No. 406 (OECD, 1992), EC guideline B.6 (EC, 1996) and the US EPA Guideline OPPTS 870.2600 (US EPA, 2003). A range finding for the test concentrations was performed in a preliminary experiment assessing the irritant effects of the test compounds after intradermal and topical application. Test concentrations of the different test substances in the main test are shown in Table 2. Each test was performed with 10 test and five control animals because this number of animals has been reported as sufficient for the GPMT and is, in general, accepted by authorities (Hofmann et al., 1987; Shillaker et al., 1989; Steiling et al., 2001). The animals received two induction treatments, an intradermal injection (day 0) and a topical application (day 7), and a topical challenge treatment on day 20. In addition to the requirements of the OECD guideline, a second topical challenge was performed on day 28 in all experiments. The intradermal induction comprised three pairs of injections of 0.1 ml each of the test compound in vehicle, the test compound in a 1:1 (v/v) mixture of FCA/physiological saline and a 1:1 (v/v) mixture of FCA/physiological saline without test compound. The control animals received applications

Table 2
Test concentrations used in the guinea pig maximization test

Test substance	Test concentrations		
	Intradermal induction ^a (%)	Topical induction ^b (%)	Challenge ^b (%)
Oleic acid	5	50	25
Linoleic acid	5	100	50
Linolenic acid	5	100	50
Undecylenic acid	1	25	25
Fumaric acid	5	25	10
Maleic acid	0.5	25	25
Succinic acid	0.5	25	10
Squalene	5	100	100
Octinol	0.25	10	5

^a Isotonic saline (NaCl 0.9%) was used as vehicle, except for undecylenic acid, fumaric acid and squalene for which cottonseed oil was used.

^b Vaseline was used as vehicle, unless pure substance (100%) was applied.

without test substance. Approximately 24 h before the topical induction treatment (day 6) the test animals received a topical application of 0.5 ml of 10% sodium lauryl sulphate in Vaseline in order to create a mild local skin irritation. For test compounds which themselves caused a clear skin irritation at the topical induction concentration used (oleic acid, linoleic acid, linolenic acid and octinol) no pretreatment with sodium lauryl sulphate was done. For the topical induction, a gauze patch with 0.5 g of the test compound was applied to the clipped skin and held under occlusion for 48 h. Control animals received the vehicle without test compound. After a resting period, topical challenge treatments were performed on day 20 and on day 28 by applying 0.5 g test compound for 24 h under occlusion onto the clipped skin of both test and control animals.

Approximately 21 h after removal of the challenge patch, the skin area was cleaned and cleared of hair. About 24 and 48 h after removal of the patch, the skin reaction was observed and recorded according to the

Magnusson and Kligman grading scale: grade 0, no reaction, grade 1, slight edema and/or erythema; grade 2, well defined edema and/or erythema; grade 3, moderate edema and/or erythema; grade 4, severe edema and/or erythema. In addition to the requirements of the OECD guideline, an additional reading of skin reactions was performed at 72 h after patch removal.

3. Results

3.1. Local lymph node assay

In the LLNAs, no signs of systemic toxic effects were observed for any of the test substances, the only exception being the death of a single animal treated with octinol. However, this death was not considered to be caused by the test substance treatment. No clinical signs of local irritation were observed by visual inspection of the ears. Similarly, no significant ear swelling response was measured for any of the test substances at any of the test concentrations (data not shown). The stimulation indices (SI) and lymph node weight indices (LNWI) obtained at the tested concentrations are shown in Table 3 below. While only the SI will be discussed in the following, it is evident from the table that there was a good agreement between SI values above three and statistically significant lymph node weight increases.

Seven of the nine test substances, oleic acid, linoleic acid, linolenic acid, undecylenic acid, maleic acid, squalene and octinol, gave clear positive results in the LLNA with the highest SI value of each substance lying in the range between 4.4 and 16.1. All substances showed a conventional dose–response relationship, i.e., the SI values increased with increasing test concentration, except for

Table 3
Stimulation indices (SI) $\pm$ SD and lymph node weight indices (LNWI) $\pm$ SD obtained in the LLNA

Test substance	Vehicle	Parameter	Index $\pm$ SD at test concentration			
			50%	25%	10%	5%
Oleic acid	AOO ^a	SI	6.9 $\pm$ 3.6	14.9 $\pm$ 6.8	2.6 $\pm$ 1.6	n.t. ^c
		LNWI	2.2 $\pm$ 0.2*	2.3 $\pm$ 0.2*	1.1 $\pm$ 0.3	n.t.
Linoleic acid	AOO	SI	9.1 $\pm$ 2.4	7.0 $\pm$ 1.2	1.5 $\pm$ 0.7	n.t.
		LNWI	1.9 $\pm$ 0.2*	1.56 $\pm$ 0.09*	1.1 $\pm$ 0.2	n.t.
Linolenic acid	AOO	SI	10.3 $\pm$ 4.7	9.3 $\pm$ 1.4	3.1 $\pm$ 1.2	n.t.
		LNWI	1.7 $\pm$ 0.3*	1.7 $\pm$ 0.1*	1.0 $\pm$ 0.2	n.t.
Undecylenic acid	AOO	SI	4.4 $\pm$ 0.6	3.3 $\pm$ 1.0	2.5 $\pm$ 0.6	n.t.
		LNWI	1.4 $\pm$ 0.1*	1.3 $\pm$ 0.1*	1.1 $\pm$ 0.1	n.t.
Fumaric acid	DMSO ^b	SI	n.t.	1.4 $\pm$ 0.6	2.3 $\pm$ 0.8	1.3 $\pm$ 0.3
		LNWI	n.t.	1.3 $\pm$ 0.1	1.5 $\pm$ 0.1*	1.3 $\pm$ 0.2
Maleic acid	DMSO	SI	16.1 $\pm$ 6.3	16.1 $\pm$ 1.9	6.7 $\pm$ 2.6	n.t.
		LNWI	2.9 $\pm$ 0.7*	2.9 $\pm$ 0.3*	2.1 $\pm$ 0.3*	n.t.
Succinic acid	DMSO	SI	n.t.	1.3 $\pm$ 0.7	1.2 $\pm$ 0.3	1.2 $\pm$ 0.4
		LNWI	n.t.	1.1 $\pm$ 0.1	1.1 $\pm$ 0.1	1.12 $\pm$ 0.03
Squalene	AOO	SI	8.2 $\pm$ 1.3	6.9 $\pm$ 0.8	3.8 $\pm$ 1.0	n.t.
		LNWI	1.6 $\pm$ 0.3*	1.4 $\pm$ 0.1*	1.16 $\pm$ 0.06	n.t.
Octinol	AOO	SI	11.2 $\pm$ 1.0	8.8 $\pm$ 2.0	5.6 $\pm$ 1.2	n.t.
		LNWI	2.7 $\pm$ 0.1*	2.48 $\pm$ 0.08*	1.7 $\pm$ 0.1*	n.t.

^a AOO, acetone: olive oil (3 + 1, v:v).

^b DMSO, dimethylsulphoxide.

^c n.t., not tested.

* Statistically significant increase ($p < 0.01$) compared to vehicle control group.

oleic acid, which showed a bell-shaped dose–response curve with the SI at 50% being lower than the SI at 25% (but still clearly above 3). This type of dose–response curve has repeatedly been observed by others and documented in the literature (see e.g., Basketter et al., 1991; Kimber et al., 1998; Ryan et al., 2000). The seven substances resulting in SI values above 3 have to be considered as skin sensitizers according to the OECD test guideline 429.

Fumaric acid and succinic acid gave clearly negative results, i.e. SI values were below 3 for all tested concentrations. Fumaric acid produced a weak, but statistically significant LNWI increase at 10%, but not at 5% or 25%. As the lymph node weight reaction was weak and lacked a dose–response relationship, the SI result was given precedence. Therefore, these two substances were considered to show no skin sensitizing potential in the LLNA.

3.2. Guinea pig maximization test

No signs of systemic toxicity were observed for any of the test substances in any of the animals. The test concentrations used as well as the skin reactions observed at the 24, 48 and 72 h readings after the first and second challenges are shown in Table 4.

All test substances caused isolated grade 1 skin reactions in one or a few animals. Fumaric acid and maleic acid each caused a grade 1 skin reaction in one animal of the treatment group. The skin reaction was observed 24 and 48 h after patch removal. As from the visible inspection of the treated skin site, a sensitizing reaction cannot be differentiated from a skin irritation reaction, a re-challenge was performed in order to clarify this question. A sensitized animal is expected to react again upon re-challenge, i.e., when it is exposed to the test substance another time. After re-challenge with fumaric acid and maleic acid, the animals that had reacted after the first challenge failed to react. Since the skin reaction observed after the first challenge was not reproducible upon re-challenge the animals were not con-

sidered sensitized against the test substances. Similarly, octinol induced isolated skin reactions after the first challenge that could not be reproduced at re-challenge. The first challenge induced a skin reaction persisting from 24 to 72 h in one animal and a reaction only at 72 h in another animal. As both animals failed to react upon re-challenge, both animals were not considered to have been sensitized to octinol.

Also succinic acid caused skin reactions in two animals of the treatment group, one showing a reaction only at 24 h after patch removal and another only at 72 h. Moreover, one animal of the control group showed a skin reaction of the same grade and time course, i.e., a grade 1 reaction at 24 h, but not at 48 and 72 h. Neither of the two animals of the treatment group displayed a skin reaction upon re-challenge, supporting the conclusion that the reactions in these animals (as that of the control group) were non-specific, i.e., not caused by a specific immune reaction.

For linolenic acid, two test animals showed a skin reaction at the 24-h reading, but not after 48 and 72 h, suggesting a non-specific rather than an allergic reaction in these animals. This view was supported by the fact that also one control animal showed a skin reaction at the 48-h reading. Another test animal showed a skin reaction at 48 and 72 h. Unfortunately, for technical reasons, a re-challenge experiment could not be performed with linolenic acid, so that the nature of the skin reactions observed in the treatment group could not be clarified.

For squalene, one animal showed a grade 1 skin reaction at all reading time points after the first challenge as well as at all readings after the re-challenge. Thus, this animal clearly was sensitized to the test substance. Another two animals showed a skin reaction only at the 72 h reading after the first challenge. Upon re-challenge, the reaction was reproducible in only one of these two animals. Conclusively, two animals were considered sensitized to squalene.

Similar to squalene, for oleic acid one animal showed a skin reaction at 48 and 72 hours and an additional three

Table 4
Results of the guinea pig maximization tests

Test substance	Number of test animals showing skin reaction grade $\geq 1^a$ (reactions in control group)						Number of animals showing reproducible skin reactions in both challenges
	First challenge			Re-challenge			
	Reading time (h)			Reading time (h)			
	24	48	72	24	48	72	
Oleic acid	0/10 (0/5)	1/10 (0/5)	4/10 (0/5)	2/10 (0/5)	3/10 (0/5)	3/10 (0/5)	2
Linoleic acid	2/10 (4/5)	1/10 (3/5)	0/10 (3/5)	4/10 (2/5)	3/10 (2/5)	2/10 (2/5)	2
Linolenic acid	2/10 (0/5)	1/10 (1/5)	1/10 (0/5)	n.t. ^b	n.t.	n.t.	n.t.
Undecylenic acid	2/10 (0/5)	2/10 (0/5)	4/10 (0/5)	3/10 (0/5)	2/10 (0/5)	2/10 (0/5)	4
Fumaric acid	1/10 (0/5)	1/10 (0/5)	0/10 (0/5)	0/10 (0/5)	0/10 (0/5)	0/10 (0/5)	0
Maleic acid	1/10 (0/5)	1/10 (0/5)	0/10 (0/5)	1/10 (0/5)	0/10 (0/5)	0/10 (0/5)	0
Succinic acid	1/10 (1/5)	0/10 (0/5)	1/10 (0/5)	0/10 (0/5)	0/10 (0/5)	0/10 (0/5)	0
Squalene	1/10 (0/5)	1/10 (0/5)	3/10 (0/5)	2/10 (0/5)	2/10 (0/5)	2/10 (0/5)	2
Octinol	1/10 (0/5)	1/10 (0/5)	1/10 (0/5)	0/10 (0/5)	0/10 (0/5)	0/10 (0/5)	0

^a All skin reactions were of grade 1, except for grade 2 reactions in one animal of the linoleic acid control group at 24 h and in one animal of the octinol treatment group at 24 and 48 h.

^b n.t., not tested.

animals showed reactions only at the 72-h reading. Upon re-challenge a skin reaction was reproduced in only two of the four animals. Thus skin sensitization was confirmed in two animals. The skin reaction in another animal showing a reaction at all reading time points after re-challenge, but that had not reacted to the first challenge, remained equivocal.

Undecylenic acid caused skin reactions in six animals after the first challenge. The onset and persistence of the skin reaction was very variable. Upon re-challenge, skin reactions developed again in four out of the six animals. Additional reactions in other animals that had not reacted after the first challenge were not observed, suggesting that the animals that showed a reproducible, although sporadic, skin reaction had been sensitized.

After challenge and re-challenge with linoleic acid, skin reactions were observed in the control group as frequently or even at a higher incidence than in the test group. From this result it had to be concluded that the test concentration used for challenge and re-challenge was too high and induced non-specific, i.e., irritative, skin reactions. Therefore, no conclusion with regard to skin sensitization could be drawn and the result of this test had to be considered as inconclusive.

3.3. Consequences for classification and labelling

An important aspect of performing testing on substances is whether, on the basis of the experimental results, a relevant hazard is identified that requires classification and labeling of the substance as dangerous according to, e.g., the EU Dangerous Substances Directive 67/548/EEC.

For the LLNA, directive 67/548/EEC defines as the criterion for classification and labelling as skin sensitizer that a stimulation index of ≥ 3 is obtained, together with consideration of dose–response and, where appropriate, statistical significance. Under adherence to these criteria, in our study seven test compounds showed stimulation indices (SI) above 3 in the LLNA and thus would have to be classified and labelled as skin sensitizers. Two substances (fumaric acid and succinic acid) yielded negative results in the LLNA.

For the GPMT, the OECD test guideline 406 prescribes that skin reactions are to be observed and recorded at about 24 and 48 h after patch removal and describes how skin reactions are to be graded. It does not directly provide the criteria defining when a test substance has to be regarded as a skin sensitizer, however, the section ‘Reliability check’ defines that a response of at least 30% of the animals in an adjuvant test should be expected for mild/moderate sensitizers. Similarly, this criterion is found in Directive 67/548/EEC: “Positive results from appropriate animal tests are:” ... “in the case of the adjuvant type test method for skin sensitization detailed in Annex V or in the case of other adjuvant-type test methods, a response of at least 30% of the animals is considered positive, ...”

In the GPMT, sporadic skin reactions were observed for all test substances in both test animals and some control

Table 5

Theoretical classification of test substances on either the LLNA or GPMT

Test substance	Classification required on the basis of LLNA results	Classification required on the basis of GPMT results
Oleic acid	R43 – May cause skin sensitization	None
Linoleic acid	R43 – May cause skin sensitization	Data inconclusive
Linolenic acid	R43 – May cause skin sensitization	None
Undecylenic acid	R43 – May cause skin sensitization	R43 – May cause skin sensitization
Fumaric acid	None	None
Maleic acid	R43 – May cause skin sensitization	None
Succinic acid	None	None
Squalene	R43 – May cause skin sensitization	None
Octinol	R43 – May cause skin sensitization	None

animals. However, reproducible skin reactions that would point towards an allergic response were found in a few animals, indicating that a weak sensitizing potential may be attributed to four tested substances. Seven test substances (oleic acid, linolenic acid, fumaric acid, maleic acid, succinic acid, squalene and octinol) would not require classification and labelling. Undecylenic acid would have to be classified and labelled as a skin sensitizer and for linoleic acid no conclusion could be derived due to inconclusive test results.

Table 5 summarises the consequences for classification and labelling as skin sensitizer according to the EU Dangerous Substances Directive if each substance were classified on the basis of either the LLNA alone or the GPMT alone. It should be noted that this classification attempt is hypothetical because, normally, the classification and labelling decision takes into account all available data on human experience and animal testing results in a weight of evidence approach.

4. Discussion

This study compared the activity of several unsaturated aliphatic and fatty acids, including also some other related substances, in the LLNA and the GPMT. Since all test substances were either endogenous physiological constituents of the human body or common and widely used natural constituents of food and/or cosmetics, the high number of positive findings in the LLNA and also some positive reactions in the GPMT were unexpected. For example, oleic acid, a fatty acid occurring at high amounts in fatty constituents of foods and as a natural building block in membrane phospholipids and triglycerides, was clearly positive in the LLNA and also caused skin sensitization reactions in two out of ten animals in the GPMT.

In the LLNA, except for fumaric acid, all seven unsaturated compounds tested gave clear positive results, i.e.,

caused stimulation indices of ≥ 3 compared to the vehicle control group: four substances (linolenic acid, maleic acid, squalene and octinol) were already positive at the lowest tested concentration of 10% and three substances (oleic acid, linoleic acid and undecylenic acid) resulted in stimulation indices above 3 at the mid test concentration of 25%. These reactions occurred without any significant ear swelling response or clinical signs of local irritation on the ears.

Since a widespread exposure of humans to these substances occurs either endogenously or from food and cosmetics, the public available literature databases (PubMed, TOXLINE, STN) were queried for human experience regarding contact dermatitis from exposure to these substances. No case reports on allergic contact dermatitis or other studies regarding skin sensitization in humans were found for oleic acid, linoleic acid, linolenic acid, maleic acid, squalene and octinol. Only limited information about predictive skin sensitization tests with these substances were located: for oleic acid no skin sensitization potential was found when tested in a total of about 600 subjects in several human repeat insult patch tests using cosmetic formulations containing between 2% and 6% oleic acid (CIR, 1987). Only for undecylenic acid two case studies were located, which reported contact dermatitis to undecylenic acid from use in topically applied medicaments (Gelfarb and Leider, 1960; Anguita et al., 2002). Interestingly, undecylenic acid was also the only test substance that was not only positive in the LLNA, but also caused reactions in more than 30% of the guinea pigs and, thus, also would have to be classified as skin sensitizer on the basis of the GPMT results.

Another interesting observation was the occurrence of scattered, isolated slight skin reactions (grade 1) in the guinea pig tests, which were variable over time and, in general, not reproducible upon re-challenge with the test substance, and sometimes also occurred in control group animals. Although such observations are not uncommon in guinea pig tests, we wondered whether these reactions reflected mere unspecific membrane/cell damage or more specific mechanisms. In the published literature several reports suggest that unsaturated fatty acids may cause cell activation by a number of several specific mechanisms.

Several cellular second messenger cascades seem to get activated by unsaturated acids: oleic acid and palmitoleic acid induced intracellular calcium signals in cultured human keratinocytes and caused epidermal hyperplasia and abnormal keratinization in vivo, whereas the saturated stearic acid, palmitic acid and triglycerides did not affect intracellular calcium levels or skin morphology (Katsuta et al., 2005); oleic acid, but not the saturated stearic acid, directly activated purified protein kinase C in vitro (Murakami and Routtenberg, 1985); linoleic acid and oleic acid directly inhibited GTPase activity of isolated G-protein similar to arachidonic acid, while the saturated fatty acids myristic acid, palmitic acid and stearic acid had no effect (Mukhopadhyay et al., 1997).

These second messenger mechanisms may be involved in triggering release of pro-inflammatory cytokines. The

unsaturated fatty acids linoleic acid and oleic acid, but not the saturated palmitic acid, induced release of interleukin-1 (IL-1) from the human monocytic cell line U937 (Bal-die et al., 1993). Similarly, oleic acid induced IL-1 α mRNA expression in submerged human keratinocyte cultures and in reconstituted human epidermis (Boelsma et al., 1996) and caused IL-1 α release in the rat epidermal keratinocyte organotypic culture (Pappinen et al., 2005).

Interestingly, IL-1 α is able to stimulate the activation and migration of Langerhans cells from the epidermis and their subsequent accumulation in skin-draining lymph nodes, where the arriving cells showed phenotypic characteristics of dendritic cells, similar to those activated in the skin by topical application of a skin sensitizing chemical (Cumberbatch et al., 2002). In this way, activation of T lymphocytes in the lymph node can be induced by both skin sensitizers and skin cell stimulants (Gerberick et al., 1999). Taken together, it may be speculated that the unsaturated substances by one or more of the mechanisms described above, led to the activation of epidermal cells without visible signs of skin irritation. This stimulation could have activated Langerhans cells to migrate out of the skin to the auricular lymph node where they induced a hapten-unrelated cell proliferation of lymph node cells. The gathered information on possible activation of cells in the skin by mechanisms different from a hapten-specific activation suggests that, perhaps, at least part of the lymph node reactions observed after application of the unsaturated substances has been caused in an unspecific way and question whether the positive findings in the LLNA really indicate a skin sensitization potential of the test substances. A definitive clarification of this question would require additional experimental work such as the incorporation of additional parameters in the LLNA (see e.g., Vohr et al., 2000; Vohr and Ahr, 2005) and/or testing for specific secondary immune responses in mice with induction plus challenge-type designs, e.g., in the mouse ear swelling test or the challenge LLNA (Ulrich and Vohr, 2007). These studies might help to clarify whether the positive LLNA results obtained in this study reflect truly “false positive” findings. Regarding the situation in humans, it cannot be decided without further data whether the absence of reports on human cases of allergic contact dermatitis, despite of widespread exposure, indicate the absence of hazard or the absence of risk (Basketter et al., 2006; Basketter and Kimber, 2007).

The more relevant point for the European manufacturing, importing and downstream industries is the question whether a substance has to be classified as skin sensitizer and, thus, has to be regarded as a dangerous substance according to the EU Dangerous Substances Directive 67/548/EEC. If the substances investigated in this study were new chemical substances in the sense of EU legislation, either the LLNA or the GPMT would have to be performed and the classification and labeling would have to be derived on the performed animal test. It should be noted that for animal welfare reasons it is unlawful in the EU that

a company performs both tests with the same substance in order to do a hazard assessment. Of the seven unsaturated substances tested here (disregarding linoleic acid for which the GPMT test was inconclusive), oleic acid, linoleic acid, undecylenic acid, maleic acid, squalene and octinol would have to be labeled with R43 according to the LLNA tests, while only fumaric acid would not have to be labeled (see Table 5). In contrast, according to the GPMT tests, only undecylenic acid would require R43 labeling. From the discussion provided above we propose that the substances that caused a positive result in the LLNA, but a negative result in the GPMT, possess skin sensitization properties – if at all – to such a limited degree that formal classification and labeling would be inappropriate. In this case, the classification and labeling would lead to the scientifically unjustified ban of safe uses. Thus, for unsaturated compounds that bear structural similarity to the substances investigated here, the suitability of the LLNA should be carefully considered, e.g., by evaluating test results for structurally related substances, before deciding to use the LLNA for skin sensitization hazard testing. For this group of chemicals, the GPMT may give results that more adequately reflect the relevance in humans. It is acknowledged that the LLNA has advantages over the GPMT in terms of animal welfare, time need, objectivity of readout and quantitation of results, however, in light of our findings we do propose that as long as unspecific findings cannot be excluded in the LLNA, the GPMT should remain available as a recognized and accepted test method for identification of a skin sensitization hazard.

Conflict of interest statement

R. Kreiling, H. M. Hollnagel, L. Hareng, D. Eigler, M. S. Lee, P. Griem, B. Dreeßen C. Garcia and M. Kleber are employees of various companies of the chemical and cosmetic ingredient industry and all of them stated that they have no conflict of interest. The experimental part of the study was outsourced on a commercial basis to A. Albrecht by A. Wendel who had received a research grant from the European Federation of Cosmetic Industry, Frankfurt, Germany. All decisions about the experimental part as well as about the interpretation of the results were independent of the EFCI grant, hence both persons state that they have no conflict of interest.

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