

In vitro testing of tensides employing monolayer cultures: a comparison with results of patch tests on human volunteers

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Evaluation of the irritant potential of new products or ingredients prior to human testing is generally performed in vivo on animals. However, according to the 6th amendment and following updates of the European Community directive on cosmetic products (93/35/EEC), animal testing will be banned when suitable substitutes will be available. To know whether in vitro tests for assessment of skin irritancy provide results approaching human conditions, comparisons have to be made between data deriving from in vitro tests and skin response in humans. The aim of our study was to assess the validity of the monolayer culture system of normal human keratinocytes as a model for the evaluation of the irritant effects of detergents, by comparing in vitro cell culture data to in vivo acute skin irritancy effects of cocamidopropyl betaine (CAPB), an amphoteric compound, Tween 20 (TW20) (polysorbate 20) and Tween 80 (TW80) (polysorbate 80), representing nonionic compounds, applied to the skin of 24 healthy volunteers at a concentration similar to that employed in commercial products. As parameters for cytotoxicity, cell proliferation, cell membrane integrity and cell metabolism were assessed by cell counts, thymidine incorporation, MTT conversion, and Neutral Red uptake. In order to increase the sensitivity of the in vivo evaluation, bioengineering methods for assessment of the effects of test products on the skin were employed. Whereas all 4 in vitro methods ranked the tensides according to their toxicity in the following order: CAPB>SLS>TW20>TW80, both in vivo methods agreed in identifying SLS as the most irritating substance. Moreover, as compared with the irritation potential on human skin, all 4 in vitro tests overestimated the toxicity of CAPB. This suggests that the keratinocyte monolayer cell culture technique cannot directly replace in vivo methods, and that data obtained by this method should be interpreted cautiously.

Key words: emulsifier; tenside; surfactant; monolayer culture; in vitro tests; human patch tests; cytotoxicity; transepidermal water loss; colorimetry; skin hydration. © Munksgaard, 1999.

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Evaluation of the irritant potential of new products or ingredients prior to human testing is generally performed in vivo on animals. This enables a cautious approach to human exposure and the avoidance of severe damage to the skin of human volunteers. However, according to the 6th amendment and following updates of the European Community directive on cosmetic products (93/35/EEC), animal testing will be banned when suitable substitutes can be identified, validated and accepted. Therefore, the search for alternatives to animal testing is urgent. The cell culture technique has been gradually introduced as an in vitro technique for the assessment of skin irritancy (1, 2). The monolayer culture system of normal human keratinocytes seems to be ideal for this purpose,

because keratinocytes represent the first target for irritants, when they come into contact with the skin (3, 4). To ascertain whether in vitro tests for assessment of skin irritancy provide results approaching human conditions, comparisons should be made between data derived from in vitro tests and from skin response in humans (5–9). The aim of our study was to compare in vivo acute skin irritancy data referring to 4 emulsifiers, applied to the skin at a concentration similar to that employed in commercial products, to in vitro cell culture data. As parameters for cytotoxicity, cell proliferation, cell membrane integrity and cell metabolism were assessed by cell counts, thymidine incorporation, MTT conversion, and Neutral Red uptake. In order to increase the sensitivity of the

in vivo evaluation, bioengineering methods for assessment of the effects of test products on the skin were employed.

Materials and Methods

Test substances

Cocamidopropyl betaine (Tegobetaine F50, Goldschmidt, Italy) (CAPB), an amphoteric compound, polyoxyethylene sorbitan monolaurate (polysorbate 20; Tween 20, Merck Schuchardt, Germany) (TW20) and polyoxyethylene sorbitan monooleate (polysorbate 80; Tween 80, Merck Schuchardt, Germany) (TW80), representing non-ionic compounds, and sodium lauryl sulfate (Sigma, St. Louis, MO, USA) (SLS), an anionic compound, were tested in vivo and in vitro. The same batch of each surfactant was used for both in vivo and in vitro assays.

Cell cultures

Human skin specimens were obtained from plastic surgery procedures. For all experiments a mixture of normal human keratinocytes from 4 different donors were employed. Keratinocyte cultures were prepared according to the method of Rheinwald and Green (10). The skin was minced and trypsinized (0.25% trypsin, 0.02% EDTA) at 37°C for 30 min and keratinocytes were grown in 75 cm² culture flasks (Costar, Cambridge, MA) with mitomycin-treated [10 mg/ml for 2 h at 37°C (Sigma)] 3T3 fibroblasts. Keratinocytes were cultured in Dulbecco's modified Eagle's medium/Ham's F12 medium (DMEM/F12, 3:1) (Seromed-Biochrom KG, Berlin, Germany) containing insulin (5 mg/ml, Sigma), transferrin (5 mg/ml, Sigma), triiodothyronine (2 nM, Sigma), hydrocortisone (0.4 mg/ml, Sigma), adenine (180 mM, Sigma), mouse epidermal growth factor (EGF, 10 ng/ml, Sigma) and 10% fetal calf serum (FCS) (Seromed-Biochrom). Subconfluent primary cultures were passaged in secondary cultures. Cells were plated at $1 \times 10^4/\text{cm}^2$ and grown in a serum-free medium containing bovine pituitary extract (BPE) (KBM, Clonetics, San Diego, CA, USA) until near confluence (3). For the experiments, keratinocytes were starved in medium depleted of growth factors and BPE (KBM, Clonetics) for 24 h before exposure to detergents.

Cytotoxicity assays

For each test 8 experiments with 6 replicates per concentration were performed. Stock solutions were prepared by dissolving tensides in KBM. Cells were exposed to serial dilutions of the irritants (Table 1). After 1 h, the media were changed.

Control wells were treated with KBM alone. After a 24h incubation at 37°C in 5% CO₂, the cytotoxicity tests were performed.

Cell counts. Cells were seeded onto Falcon 6-well plates (1×10^4 cells/cm²), harvested by using a trypsin/EDTA solution (0.05%/0.02%) for 15 min and counted by means of a hemocytometer chamber.

³H-thymidine incorporation. ³H-thymidine incorporation is a well-known technique for checking variations in DNA synthesis and cell proliferation (11, 12). After inoculation of keratinocytes in 96 multiwell plates (Falcon), ³H thymidine was added to each well at a final concentration of 5 µCi/ml (methyl ³H thymidine, Amersham Pharmacia Biotech, Rainham, UK) and cells were incubated for an additional 12 h. Cells were collected by using a trypsin/EDTA solution with the aid of cell harvester, and the radioactivity incorporation was determined using a liquid scintillation β counter (Packard 1600 TR). Results were expressed in counts/min (cpm).

Neutral Red uptake. The Neutral Red (NR) assay utilizes the capacity of NR (3-amino-7-dimethylamino-2-methyl-phenazine hydrochloride), a water-soluble vital dye, to pass through the intact plasma membrane and become concentrated in lysosomes of viable cells (13). Test agents that damage the cell surface and lysosomal membranes inhibit dye incorporation. The amount of NR taken up by a culture is therefore proportional to the number of viable cells. Keratinocytes were incubated with the NR solution for 3 h at 37°C. The NR solution was then removed and the culture was washed rapidly with 1% formaldehyde-1% calcium chloride. A solution of 1% acetic acid/50% ethanol was added to the cell monolayers to extract the NR from keratinocytes at room temperature for 30 min. Samples were read in an automatic spectrophotometer (BIO-RAD 550 microplate reader) at 540 nm.

Table 1. Concentration of tensides (%) employed for in vitro tests

SLS	CAPB	TW 20	TW 80
0.00001	0.00001	0.0001	0.0001
0.0001	0.00005	0.001	0.001
0.001	0.0001	0.003	0.01
0.00125	0.0005	0.006	0.025
0.00175	0.001	0.0075	0.05
0.0025	0.005	0.01	0.1
0.005	0.01	0.015	1
		0.03	
		0.06	
		0.1	
		1	

SLS: Sodium lauryl sulfate. CAPB: Cocamidopropyl betaine. TW 20: Tween 20. TW 80: Tween 80.

MTT test. In the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay, a yellow tetrazolium salt is reduced to a blue formazan product by mitochondrial succinic dehydrogenase present only in metabolically active cells (14). The crystals formed are solubilized and the resulting coloured solution is quantified. 20 μ l of MTT (5 mg/ml) were added to each well for 4 h. After removing the supernatant, the crystals formed were solubilized with 200 μ l of dimethyl sulfoxide (DMSO) and shaken for 10 min. The resulting colored solution was quantified at 540nm using a Bio-Rad 550 microplate reader.

In vivo study

Volunteers and test procedure. 24 healthy female volunteers, aged 18 to 45, were included in the study after informed consent. 40 μ l of 24% CAPB, 5% TW20, 5% TW80 and 5% SLS aqueous solutions were pipetted on filter paper discs in large (11 mm in diameter) Finn Chambers on Scanpor tape. Patches were applied on 5 different sites on both volar forearms, following a rotating pattern of application. After 30 min, patches were removed and the skin was rinsed with tap water. Test sites were covered again with an empty chamber until the 1st measurement and between measurements.

Instrumental evaluations. Instrumental evaluations were performed before the beginning of the experiment, and 24 h and 72 h after patch test removal. The Evaporimeter EP1 (Servomed, Stockholm, Sweden) was used to measure transepidermal water loss, and the Chroma Meter CR200 (Minolta, Osaka, Japan) to evaluate erythema employing the parameter a^* . Instruments and methods have already been described in detail elsewhere (15, 16). TEWL measurements were performed according to the guidelines of the Standardization Group of the European Society of Contact Dermatitis (17).

Statistics

Student *t*-test was used to compare values referring to control cultures to those referring to cells cultured with different concentrations of the test substances. Controls having received KBM alone were set at 100%. Test agent concentrations yielding a 50% decrease in cell viability in respect to control cultures were calculated for each assay (CC_{50} , NR_{50} , MTT_{50} and $^3[H]_{50}$) and the toxicity ranking carried out according to these results. Correlations between the 4 cytotoxicity tests were performed by comparing percentage reductions obtained by the different test methods for the same tenside concentration. For the *in vivo* study, the ANOVA test for

repeated values and the SNK test were used to compare 24-h and 72-h values to baseline values. The Student *t*-test was employed for the comparison of instrumental values at the same time point for different substances. A *p* value <0.05 was considered statistically significant.

Results

Cytotoxicity of surfactants in vitro

Data summarized in Fig. 1–4 show the results of *in vitro* evaluations. All values presented represent mean and standard deviation of 8 experiments with 6 replicates per concentration group in each experiment. A clear dose-response relationship was established for all test substances. SLS and CAPB gave a gradual change in cell viability on increas-

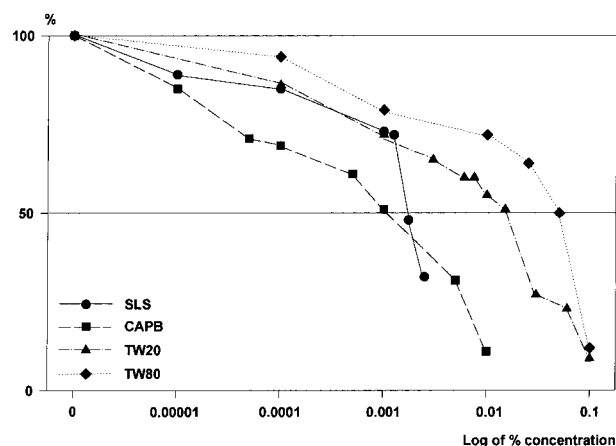


Fig. 1. Dose-response curve (log of % concentration versus % decrease in cell viability) referring to the cell count method for sodium lauryl sulfate (SLS), cocamidopropyl betaine (CAPB), Tween 20 (TW20) and Tween 80 (TW80).

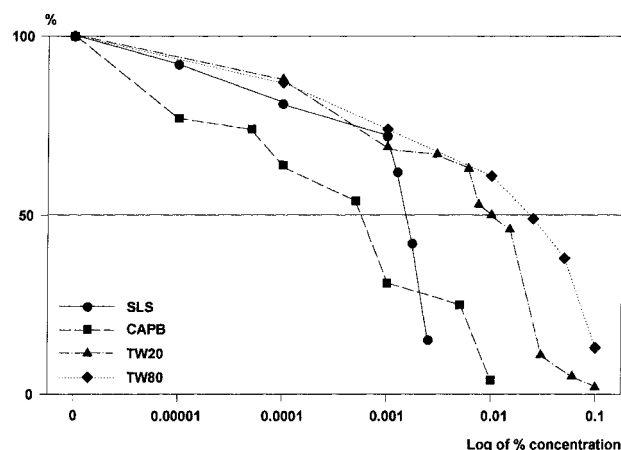


Fig. 2. Dose-response curve (log of % concentration versus % decrease in cell viability) referring to thymidine incorporation for sodium lauryl sulfate (SLS), cocamidopropyl betaine (CAPB), Tween 20 (TW20) and Tween 80 (TW80).

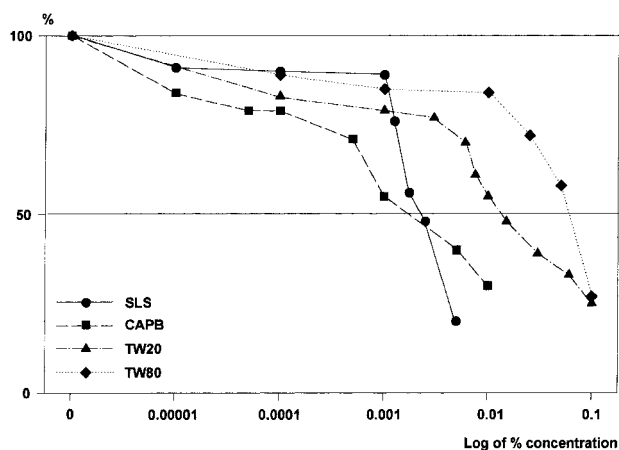


Fig. 3. Dose-response curve (log of % concentration versus % decrease in cell viability) referring to Neutral Red uptake for sodium lauryl sulfate (SLS), cocamidopropyl betaine (CAPB), Tween 20 (TW20) and Tween 80 (TW80).

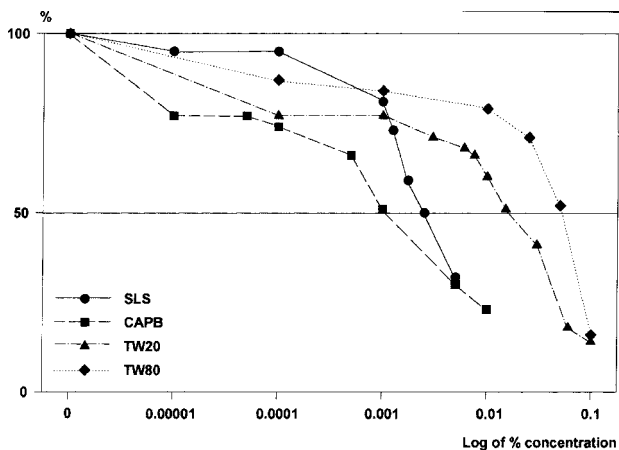


Fig. 4. Dose-response curve (log of % concentration versus % decrease in cell viability) referring to MTT conversion for sodium lauryl sulfate (SLS), cocamidopropyl betaine (CAPB), Tween 20 (TW20) and Tween 80 (TW80).

ing the concentration, whereas curves referring to TW20 and TW80, while showing a slightly decreasing slope with concentrations of up to 0.015% and 0.025%, respectively, presented an abrupt decreasing trend with higher concentrations. The $^3\text{H}\text{T}$ method was the most sensitive, evincing a 50% reduction in cell viability at the lowest concentrations (Table 2). Moreover, a 0.001% CAPB concentration, representing the CC_{50} , NR_{50} and MTT_{50} , induced a 70% reduction, as evaluated by the $^3\text{H}\text{T}$ method.

When comparing the surfactants' effects as evaluated by different cytotoxicity tests, we observed an analogous toxicity ranking in vitro. Correlation coefficients were extremely high, ranging from 0.969 to 0.996.

Table 2. % concentration of tensides inducing a 50% reduction in cell viability

	SLS	CAPB	TW 20	TW 80
CC_{50}	0.00175	0.001	0.015	0.05
$^3\text{H}\text{T}_{50}$	0.00125/ 0.00175	0.0005	0.01	0.025
NR_{50}	0.0025	0.001	0.015	0.05
MTT_{50}	0.0025	0.001	0.015	0.05

SLS: Sodium lauryl sulfate. CAPB: Cocamidopropyl betaine. TW 20: Tween 20. TW 80: Tween 80.

In vivo study

All surfactants induced a significant increase as compared to baseline, at 24 h and 72 h for TEWL and at 72 h for a^* values (data not shown). Δ values, referring to TEWL and erythema measurements, expressing differences between 24-h and 72-h values and baseline values, are represented in Table 3. For both methods, 72-h SLS values significantly differed from those of CAPB, TW20 and TW80. However, although a trend versus a ranking was observed ($\text{TW80} > \text{TW20} > \text{CAPB}$), no significant differences were detectable between values referring to these surfactants, except for colorimetric 24-h values of CAPB and TW80.

Comparison of surfactant toxicity in vitro and in vivo

Whereas for all 4 in vitro tests the rank order of toxicity was the following: $\text{CAPB} > \text{SLS} > \text{TW20} > \text{TW80}$, both in vivo methods identified SLS as the most irritant substance. Moreover, as compared with the irritation potential on human skin, all 4 in vitro tests overestimated the toxicity of CAPB.

Discussion

Emulsifiers are common constituents of most topical medicaments and cosmetics, which enables the solubilization of otherwise insoluble compounds. Tego compounds are amphoteric compounds, which combine both the detergent properties of an anionic part and the bactericidal characteristics of a cationic part. Several preparations, each different in chain length, are in use. They are employed as cleansing agents, shampoos, bath foams, liquid soaps, cosmetic cleansers, etc. Tweens (polysorbates) are nonionic compounds that are soluble in water. The addition of Tweens makes an emulsion more fluid (18).

The premarket testing of emulsifiers for dermal irritancy has been achieved mainly via animal testing, although many detergents, because of their

Table 3. Results of in vivo tests with different tensides; values represent mean $\pm$ SD of 24-h and 72-h differences with respect to baseline

	Δ TEWL		Δ a^*	
	24-h – baseline	72-h – baseline	24-h – baseline	72-h – baseline
CAPB	1.39 $\pm$ 1.64	1.82 $\pm$ 1.87	0.57 $\pm$ 0.74	1.03 $\pm$ 0.52
TW 20	2.16 $\pm$ 1.33	2.33 $\pm$ 1.63	0.96 $\pm$ 0.89	1.01 $\pm$ 0.44
TW 80	2.17 $\pm$ 1.12	2.70 $\pm$ 1.51	1.03 $\pm$ 0.49	1.02 $\pm$ 0.39
SLS	4.12 $\pm$ 2.01	4.08 $\pm$ 1.91	1.28 $\pm$ 0.86	1.39 $\pm$ 0.44

mildly irritant characteristics, can appropriately be tested for dermal safety using volunteer human subjects. However, screening for possible dermal hazard of totally new products or ingredients requires prehuman exposure, in order to avoid the induction of potential severe damage to the skin of human volunteers. In the near future, this exposure will not be obtained employing animal tests, since EC directives impose their total abolition. Moreover, besides ethical and financial objections to animal use, there are also scientific drawbacks, such as intra- and interlaboratory variability, reliable predictability only for strong irritants, and the fundamental difference between animal and human skin (2). In vitro tests have recently been proposed as methods to replace the use of animals (1). Although representing an oversimplified skin model not fully reproducing the in vivo situation, monolayered cultures are usually chosen to assess the biological effects of potential toxic compounds, since they provide rapid results while being relatively easy and inexpensive analytical techniques (19). Keratinocytes are the target cells for irritants and the cell type initiating the cytokine cascade in irritant contact dermatitis. Therefore, the monolayer keratinocyte culture technique should be ideal for assessment of the irritant potential of substances to be applied on the skin (1). Attempts have been made to improve skin substrates by introducing 3-dimensional models, enabling cell-to-cell interactions and orientation within the extracellular matrix, in order to approach the living skin structure (20). However, these skin equivalents are expensive and difficult to provide, and are generally employed as 2nd step assays for substances preselected by tests performed on monolayer cultures (21, 22).

Owing to their solubility, emulsifiers are an ideal product category for the submerged cell culture model. Surfactants exert their cytotoxic effects in vitro by reacting directly with cell membrane proteins and inhibiting enzymes (23). Their toxic potential has been evaluated on monolayer cultures (5, 24) and on 3-dimensional human fibroblast substrates (25, 26). Low concentrations of SLS were demonstrated to increase proliferative activity and mitochondrial metabolism of cultured human

keratinocytes (27, 28), whereas the opposite was observed for higher concentrations. Yet, in spite of technical improvements in the production of skin equivalents, before accepting an in vitro test as equivalent to a human assay, and extending the results obtained by in vitro experiments to the in vivo situation, parallel in vitro/in vivo investigations should be performed, to validate the in vitro procedure. So far, investigations dealing with the simultaneous performance of in vitro tests and in vivo experiments on humans are scarce (5–9). Gueniche & Poncet (2) investigated the toxic potential of SLS and other surfactants on human keratinocytes and fibroblasts and on SV40-transformed keratinocytes, evaluating changes in cell morphology and cell number, inhibition of cell proliferation, and modulation of IL-6 production. The authors did not perform simultaneous tests on humans, but compared their in vitro results to literature data. Of all the types of cells tested, only SVK14 cells showed the same ranking order of cytotoxicity response as that observed in vivo. Korting et al. evaluated the cytotoxic effects of surfactants on normal human keratinocytes, HaCaT cells and mouse fibroblasts (6). Cell membrane integrity, as assessed by the NR assay, and cell proliferation, as assessed by measurement of total protein content, were both affected in a dose-dependent manner in response to surfactant exposure. Results were compared with the responses of the soap chamber test performed on the forearms of healthy volunteers, which were evaluated by TEWL and skin redness. The highest correlation was observed for the NR release test on normal human keratinocytes. Eun et al. (5) assessed the irritant potential of shampoos, employing both the keratinocyte cell culture model and human patch tests as evaluated by laser Doppler flowmetry. Whereas good correlations were found for strong and weak irritant products, the rank order of shampoos of moderate toxicity was not the same for in vitro and in vivo assays.

In this study, we evaluated the irritant properties of some common substances, employing monolayer keratinocyte cultures and 30 min patch tests in human volunteers, performed with emulsifiers at a concentration contained in many topical prod-

ucts, simulating acute exposure as can result in daily practice. Since multiple mechanisms are involved in the process of skin irritation, and in order to predict human skin irritancy effectively, we employed different *in vitro* tests exploring various mechanisms of cell damage. Cell viability was evaluated by assessing the changes in mitochondrial activity using the MTT test and Neutral Red uptake. The proliferation was evaluated by ^3H -thymidine incorporation, a well-known technique for checking the reduction in DNA synthesis. Test substances were added at different concentrations and the test agent concentration yielding 50% endpoint decrease in respect of the control culture was calculated for each assay.

Employing human skin recombinants, Poncet (29) showed that SLS causes changes in epidermal morphology and in the expression and localization of differentiation specific protein-markers at concentrations similar to those inducing damage *in vivo*. On the contrary, with submerged cultures, results cannot immediately be transferred to the *in vivo* situation, where concentrations inducing irritation are mostly several orders of magnitude higher than those inducing changes *in vitro*. Therefore, the results have only a relative value, and, when investigating new substances, they should be compared with reference irritants (in our case SLS) and a toxicity rank order should be established.

We observed an analogous toxicity ranking *in vitro* for all 4 tests employed and the correlation between the 4 *in vitro* toxicity tests was extremely high. CAPB proved to be the most toxic substance, followed by SLS, whereas TW80 exhibited the mildest effect. Among the 4 methods investigated in this study, ^3H -thymidine incorporation was the most sensitive and early in evaluating tenside cytotoxicity.

In our study, skin responses to 30 min patch tests were evaluated instrumentally. Visual scoring, which is generally employed for the evaluation of irritancy, is quick and cheap, but can have the disadvantage of high intra- and interobserver variations and has low sensitivity. In this study, no visible reaction at most patch test sites was observable at different time points, while in some cases only slight erythema or dry skin were observed. In contrast to visual scores, instrumental measurements give quantitative results ranging on an equal statistical level to the data from most *in vitro* tests. Furthermore, TEWL measurements enable an earlier and more sensitive approach to barrier modifications after exposure to surfactants, and this is particularly useful when employing subeliciting doses of irritants. A 30 min exposure test to SLS has been employed by us for the evaluation of skin reactivity in subjects with atopic dermatitis

and allergic rhinitis (15). This type of contact, simulating an acute irritant exposure to surfactants without inducing visible damage in most volunteers, enabled us to discriminate between different populations. In this study, the same procedure was employed for discriminating between substances, that, by traditional tests, would have been classified as non-irritating to the skin. Significant differences with respect to baseline were observable for all substances at 72 h, as evaluated by TEWL and colorimetry. Moreover, this procedure allowed the identification of the most irritant product. In fact, SLS elicited the greatest increase in TEWL and in skin redness both at 24 h and 72 h, proving to be by far the most irritant substance *in vivo*. In our study, agreement between *in vitro* and *in vivo* tests was poor. Whereas both *in vivo* and *in vitro* assays agreed in identifying TW20 and TW80 as milder irritants with respect to SLS, the *in vitro* toxicity of CAPB was overestimated as compared with the irritation potential on human skin. This is in line with previous data. Korting et al. (6) observed that CAPB is highly cytotoxic *in vitro* while eliciting only a moderate response *in vivo*. Harvell et al. examined 10 substances with a broad range of structural diversity, performing *in vivo* human tests and 3 *in vitro* toxicity assays (7). Among 6 substances which were incorrectly classified in the *in vitro* systems, cocamidopropyl betaine gave a false positive result. These findings may be partly explained by the fact that the severity of the irritant reaction is also dependent on the amount of substance reaching viable cells and is therefore influenced by the resistance of the stratum corneum and its barrier capacity. One may assume that betaines are insufficiently absorbed and thus unable to damage target cells, while *in vitro* their toxic potential can be expressed by direct contact with viable keratinocytes. Moreover, it is also possible that the irritant effect of different surfactants is based upon a different interplay in the complex pattern of events involved in the inflammatory response, which is only partially reproduced by the *in vitro* model employed in our study. It is therefore unlikely that one single *in vitro* model will be able to mimic and predict *in vivo* exposure, and several aspects reflecting different steps of the irritant reaction should be investigated, employing different *in vitro* tests.

Our data suggest that, whereas for some categories of surfactants a close correlation exists between human patch test responses and damage to cultured monolayer cells, for others reproducibility of the *in vivo* irritant potential is not achieved by *in vitro* tests. Thus, replacement of *in vivo* tests by *in vitro* assays for dermatotoxicity should be cautious and the need for impartial validation

studies involving a wide spectrum of chemicals, to be simultaneously tested both in human assays and in vitro, is urgent.

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