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Characterization and applications of human epidermis reconstructed in vitro on de-epidermized derma

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Abstract

In recent years in vitro models have been developed to avoid the use of animals in cutaneous toxicological studies. Submerged human keratinocyte cultures in vitro could be so far employed as an alternative to animal testing and a good correlation between skin irritation and cytotoxicity has been demonstrated. Nevertheless, these submerged cultures are lacking in the stratum corneum which acts as a barrier to chemical toxicity, so that this type of culture is far from the in vivo situation. A better alternative method seems to be the use of in vitro reconstructed skin at the air–liquid interface that closely resembles the in vivo situation. In this work, in a first step we have characterized human epidermis reconstructed in vitro on de-epidermized derma (DED) after a two-week air exposure. Human skin reconstituted in vitro on DED was histologically similar to the in vivo skin. A stratified epidermis including the stratum corneum was obtained. The presence of basal lamina as well as of various important markers for epidermal differentiation (involucrin, K10 keratin, and filaggrin) were revealed. In a second step we have tested the cytotoxic and morphological effects of four surfactants on our model. A good rank correlation has been shown to exist between the irritation potency of surfactants on our model and reported ocular irritancy in vivo. From our results, in vitro reconstituted human skin could represent an attractive model for irritancy testing and could be an in vitro replacement for animal testing.
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1. Introduction

In recent years in vitro models have been developed to avoid the use of animals in cutaneous toxicological studies. Actually, apart from ethical and economic problems, in vivo tests, especially the Draize test, are above all scientifically criticable [1]. First of all, there is little correlation with the response observed in human patch tests as both the stratum corneum barrier properties and metabolic conversions of tested chemicals are different among species, most of all between animals and humans. Moreover, test results are based on subjective evaluations, thus bringing a lack of reproducibility of test results in different laboratories.

Submerged human keratinocyte cultures, on the other hand, could be employed as an alternative to animal testing and a good correlation between skin irritation and cytotoxicity has been demonstrated [2]. Nevertheless, these submerged cultures do not present any markers of terminal differentiation such as keratin K10 and are lacking in a stratum corneum [3]. This is the reason why this type of culture seems to be far from the in vivo situation where the stratum corneum acts as a barrier to chemical toxicity [4]: in fact, most of the irritating substances alter proteins and lipids playing a crucial role in the skin barrier function. Thus the lack of percutaneous absorption, which modulates toxic effects, makes this model unable to mimic the in vivo situation. Moreover, a significant limitation of this method is that the testing compounds must be soluble in a culture medium. A better alternative method seems to be the use of in vitro reconstructed skin at the air–liquid interface that resembles more closely the in vivo situation. In fact, besides cytotoxicity, even permeation through the stratum corneum [5] and the release of pro-inflammatory mediators [6,7] could be studied. Actually, skin equivalent obtained culturing keratinocytes on various substrates such as collagen matrix [8], dermal equivalent [9], de-epidermized dermis [10] and acetate cellulose–polycarbonate filter membrane [11] are avail-

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able. Nevertheless, validation of these in vitro models for the evaluation of skin irritation is still in progress.

In this work we have characterized in a first step human epidermis reconstructed in vitro on de-epidermized derma (DED) after a two-week air exposure, and in a second step we have tested the cytotoxic and morphological effects of four surfactants (benzalkonium chloride, sodium dodecylsulfate, Triton X-100, Tween 20) on our model, comparing the results with the in vivo ones.

2. Experimental

2.1. Keratinocyte cell culture

2.1.1. Submerged cultures

Human keratinocytes were isolated from foreskins of children aged 0–5 years by trypsinization and plated at high cell density (3×10^4cells/cm^2) onto a feeder monolayer of 3T3-J_2 cells previously treated with mitomicyn C (4 μg/ml) for 2 h. Keratinocytes were grown in Dulbecco's modified Eagle's medium (DMEM) and Nutrient mixture F-12 Ham's (3:1) (Sigma Chemical Co., Poole, Dorset, UK), containing hydrocortisone 0.5 ng/ml, insulin 5 μg/ml, adenine 24 μg/ml, cholera toxin 6 ng/ml, transferrin 10 μg/ml, triiodotyronin 10 μg/ml and epidermal growth factor (Sigma) 10 ng/ml supplemented with 10% HyClone calf serum, according to Rheinwald and Green [12].

Swiss 3T3-J_2 cells [13] were grown in DMEM supplemented with 10% HyClone calf serum. Antibiotics and amphotericin B were added to all media. Cells were cultured at 37°C in a humidified atmosphere with 5% CO_2.

2.1.2. Air exposed keratinocyte culture

(a) Human de-epidermized dermis (DED) was prepared according to Régnier et al. [10] from skin obtained from abdomen-plastics. Dermal cells were killed by 10 successive cycles of freezing and thawing, then skin was de-epidermized after a seven-day immersion in phosphate-buffer saline (PBS), containing an antibiotic–antimycotic solution (Sigma).

(b) Third passage cultures of human keratinocytes were seeded on de-epidermized dermis (6.2×10^5 cells/cm^2) [10] using stainless steel rings and cultured under submerged conditions with the medium previously described, supplemented with 5% of HyClone serum. After 3 days cultures were lifted up to the air–liquid interface on stainless steel grids and cultured for 1 or 2 weeks with medium supplemented with 10% of de-lipidized calf serum (Poiesys).

2.2. Immunohistochemical characterization

6 μm vertical sections of reconstructed epidermis were obtained with a cryostat. Immunofluorescence was performed using the following primary mouse monoclonal antibodies: anti-human cytokeratin K10 (Sigma Immuno Chemical), anti-human filaggrin (Biomedical Technologies Inc.). Then a secondary goat anti-mouse IgG fluorescein conjugated antibody (Sigma Immuno Chemical) was employed.

Immunoperoxidase staining was carried out using a primary rabbit monoclonal anti-human involucrin (Biomedical Technologies Inc.) and a secondary goat anti-rabbit IgG peroxidase conjugate (Biomedical Technologies Inc.).

2.3. Transmission electron microscopy

Samples of normal human skin and reconstructed human skin were fixed with 25% glutaraldehyde in cacodylate buffer (pH 7.4, 0.2 M), post-fixed with 1% osmium tetroxide/ruthenium tetroxide in cacodylate buffer and embedded in Epon 812. Thin sections were cut on a Reichart-Yung ultramicrotome and stained with uranyl acetate and lead citrate before being examined in a Hitachi H7000 transmission electron microscope.

2.4. Lipid analysis

2.4.1. Lipid extraction

Epidermal and dermal fractions were separated after normal human skin and reconstructed human epidermis had been heated at 60°C for 60 s [14].

Unbounded lipids were extracted for four times with 2 ml chloroform methanol (2:1), in all 1 h each time. These extracts were combined, dried under a stream of nitrogen at 40°C and redissolved in chloroform [15]. Covalently bounded lipids were extracted [16] from the saponification of the residue unbounded lipid-free, with 1 ml of 1 M NaOH in 90% methanol for 1 h at 60°C. The mixture was then acidified to pH 4 with 2 M HCl and shaken with chloroform. After filtration through a coarse sintered glass filter, the aqueous phase was re-extracted four times with chloroform. This extract was dried under a stream of nitrogen and redissolved in chloroform.

2.4.2. Thin layer chromatography (TLC)

Standard lipids (phosphatidylcholine, phosphatidilserine, galactocerebroside type I, galactocerebroside type II, cholesterol 3 sulfate, ceramide type IV, cholesterol, lanosterol, squalene) (Sigma Aldrich) were dissolved in chloroform (5 mg/ml). 100 μg of each standard solution and 100 μg of each extract were seeded on silica-gel plates (Merck) and TLC was performed using chloroform–acetone–methanol 90:5:5 (up to 4 cm) as the first, chloroform–ethylacetate–methanol–2-propanol–triethylammine–water 64:15:17:0.5:3:2 (up to 6.5 cm) as the second, hexane–diethyl ether 90:10 (up to 9 cm) as the third developing system. After lipid separation, the plate was first heated for 5 min at 130°C, subsequently sprayed with a mixture of acetic acid–sulfuric acid–water 4.9:0.1:95 containing 0.5% $CuSO_4$, heated at 150°C for 15 min and at 200°C for 15 min, respectively.

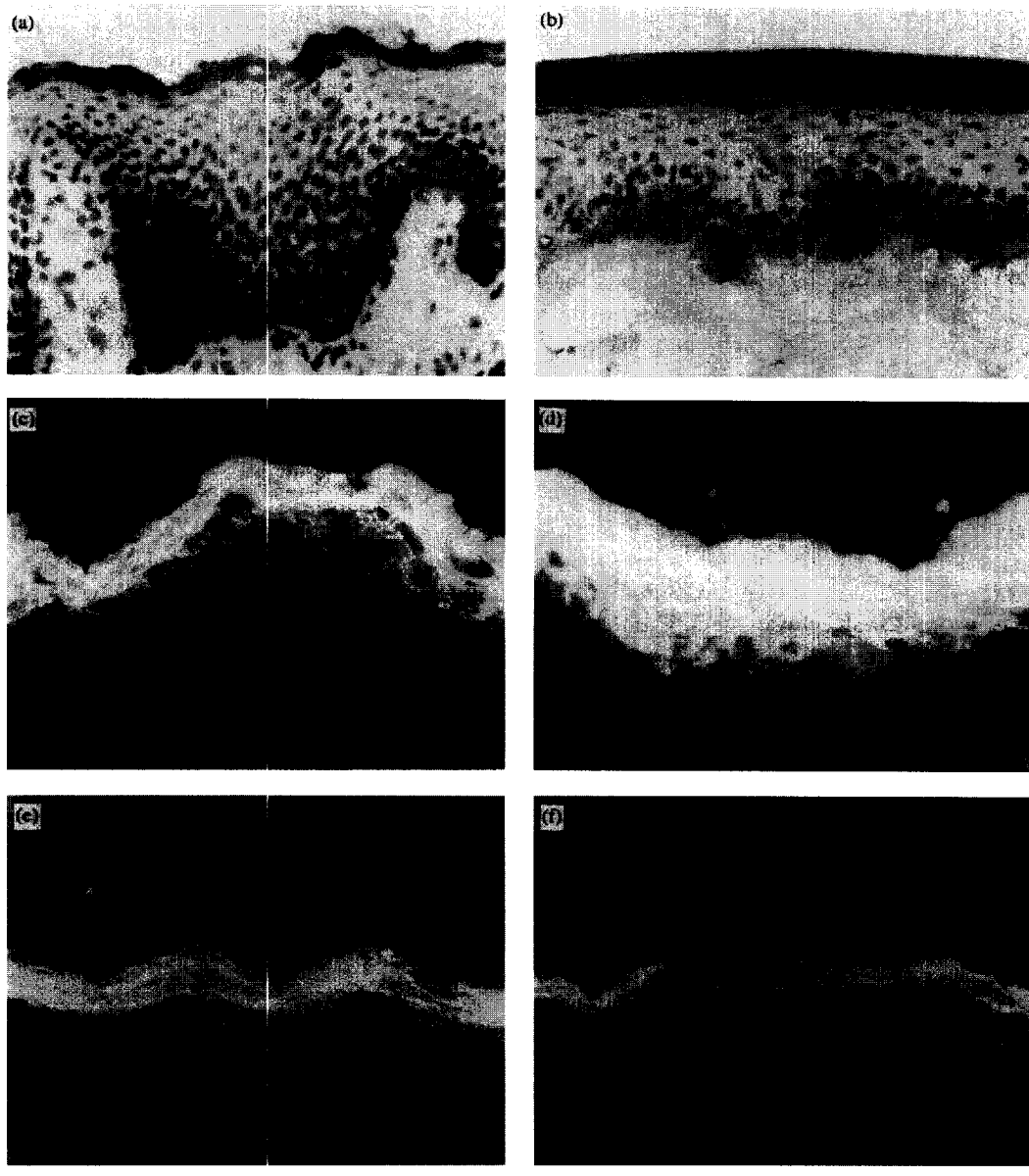

Fig. 1. Immunohistochemical characterization of reconstructed epidermis after 2 weeks of culture (b,d,f) compared to that observed in sections derived from human foreskin (a,c,e). Note the distribution of involucrin (a,b) (×250), immunofluorescence localization of fillagrin (c,d) (×400) and of cytokeratin K10 (e,f) (×400).

over, benzalkonium chloride caused necrotic damage in the suprabasal layers, while sodium dodecylsulfate induced parakeratosis and necrolysis of the basal layers.

4. Discussion

Human skin reconstituted in vitro on DED, which represents an optimum substrate for keratinocyte growth [19,20], was histologically quite similar to the in vivo skin as already reported by Régnier et al. [10]. Actually, in our model both a basal layer presenting cubic-shape cells and several suprabasal cell layers with cells joined by desmosomes, including the stratum corneum, are visible. Nevertheless the stratum corneum appeared thicker and basal layers were less numerous than observed in vivo. Transmission electron microscopy and immunohistochemistry have clearly shown the presence of the basal lamina and its two components (lamina lucida and lamina densa) between DED and the reconstructed epidermis. The importance of the basal lamina as an important element of both morphological and functional equivalence of reconstructed tissue compared to in vivo skin was already stressed by Ponec et al. [21].

Moreover appropriate expressions of various important markers for epidermal differentiation were observed in cultures exposed to the air. The cornified envelope protein involucrin [20] was only revealed in the stratum corneum layer, while basal cells were involucrin-negative. K10 keratin [20]

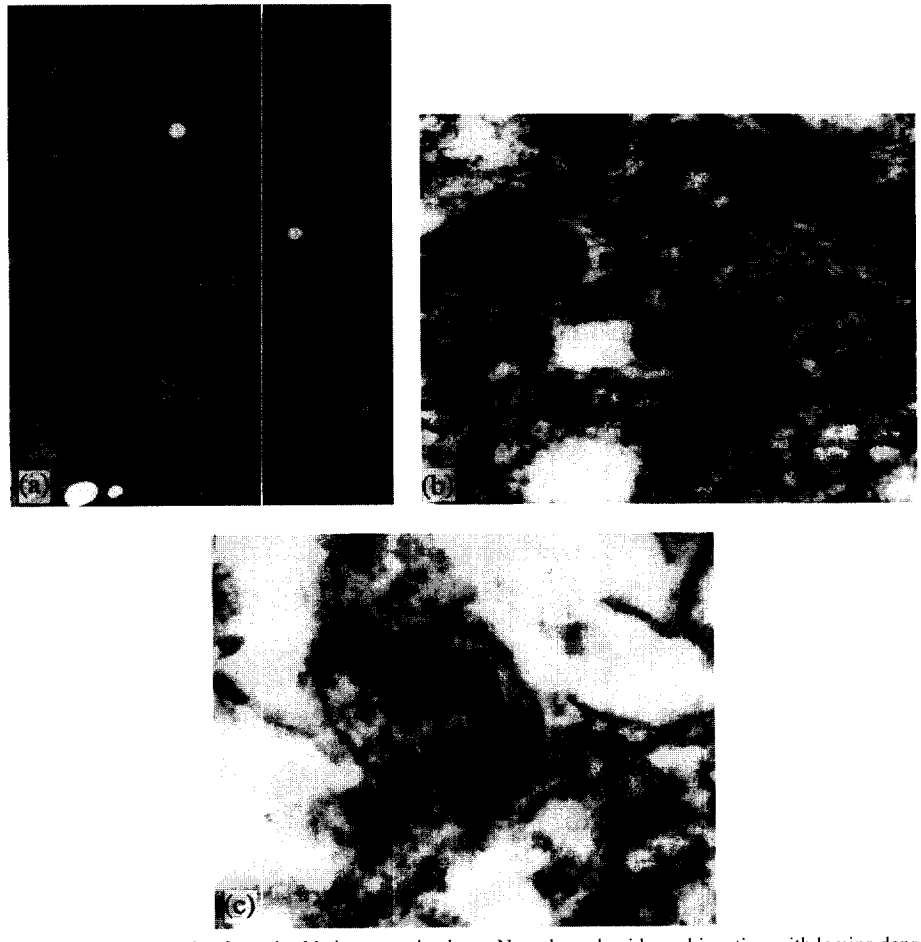

Fig. 2. Electron microscopy of sections through a 2-week-old air exposed culture. Note dermal epidermal junction with lamina densa (LD) and lamina lucida (LL) (a) (×11100), complete desmosomes (D) (b) (×72000) and Odland bodies (OB) (c) (×80000).

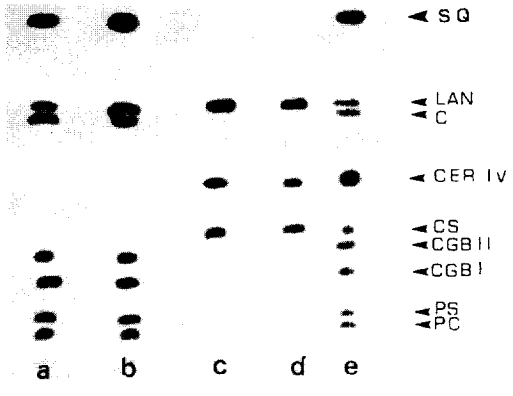

Fig. 3. One-dimensional TLC separation of lipid extracts. Lane a: unbounded lipids extracted from reconstructed skin in vitro; lane b: unbounded lipids extracted from normal human epidermis; lane c: covalently bounded lipids extracted from reconstructed skin in vitro; lane d: covalently bounded lipids extracted from normal human epidermis; lane e: standard lipids: PC phosphatidylcholine, PS phosphatidylserine, GCB I galactocerebroside type I, GCB II galactocerebroside type II, CS cholesterol 3 sulfate, CER IV ceramide type IV, C cholesterol, LAN lanosterol, SQ squalene.

(56.5 kDa) was detected as in vivo in the suprabasal layers. Filaggrin, which is synthesized in vivo in the granular layer [20], was revealed in the suprabasal layers and in keratinocytes just beneath the cornified layers. These observations confirm that cultures exposed to the air closely resemble the in vivo epidermis and are in accordance with several authors who have reconstructed human epidermis on DED [10].

As the epidermal lipids are known to be important for the cutaneous permeability barrier, we studied their intercellular arrangement and their composition. Odland's bodies were observed in the stratum granulosum of reconstructed skin and the results of lipid composition analysis revealed that keratinocytes synthesize all the lipids that are to be found in normal human epidermis according to Ponec et al. [21].

We also proved that epidermis reconstructed in vitro on DED shows as time passes an excellent morphological equivalence compared to the in vivo skin. In fact, the cultures after 1 (data not shown) and 2 weeks air exposure revealed the same tissue architecture, thus enabling long-term experiments.

From our results, in vitro reconstituted human skin could represent an attractive model for irritancy testing and could be an in vitro replacement for animal testing. Actually, a good rank correlation has been proved to exist between irritation potency in MTT data and reported ocular irritancy in vivo (Draize test [18]), confirming the results previously

Table 1
Comparison among MTT toxicity assays performed on submerged and air exposed human keratinocyte cultures, and in vivo ocular irritancy data

Surfactants	MTT_{50} [a]			Ocular irritancy [b]
	Submerged non-confluent cultures	Submerged confluent cultures	Air-exposed cultures	
Benzalkonium chloride	5.10 ± 0.03	10.25 ± 0.35	$0.12 \times 10^3 \pm 0.02 \times 10^3$	extreme
Triton X100	82.50 ± 2.50	115.50 ± 6.36	$0.50 \times 10^3 \pm 0.08 \times 10^3$	severe
SDS	131.50 ± 8.50	220.00 ± 12.00	$4.10 \times 10^3 \pm 0.12 \times 10^3$	severe-moderate
Tween 20	375.00 ± 9.80	1900.00 ± 100.00	$45.00 \times 10^3 \pm 1.14 \times 10^3$	mild

Results are expressed as mean values ± standard deviations.
[a] MTT_{50} concentration of surfactant (μg/ml) inhibiting 50% of control MTT conversion.
[b] Ref. [18] was used as the source for in vivo data.

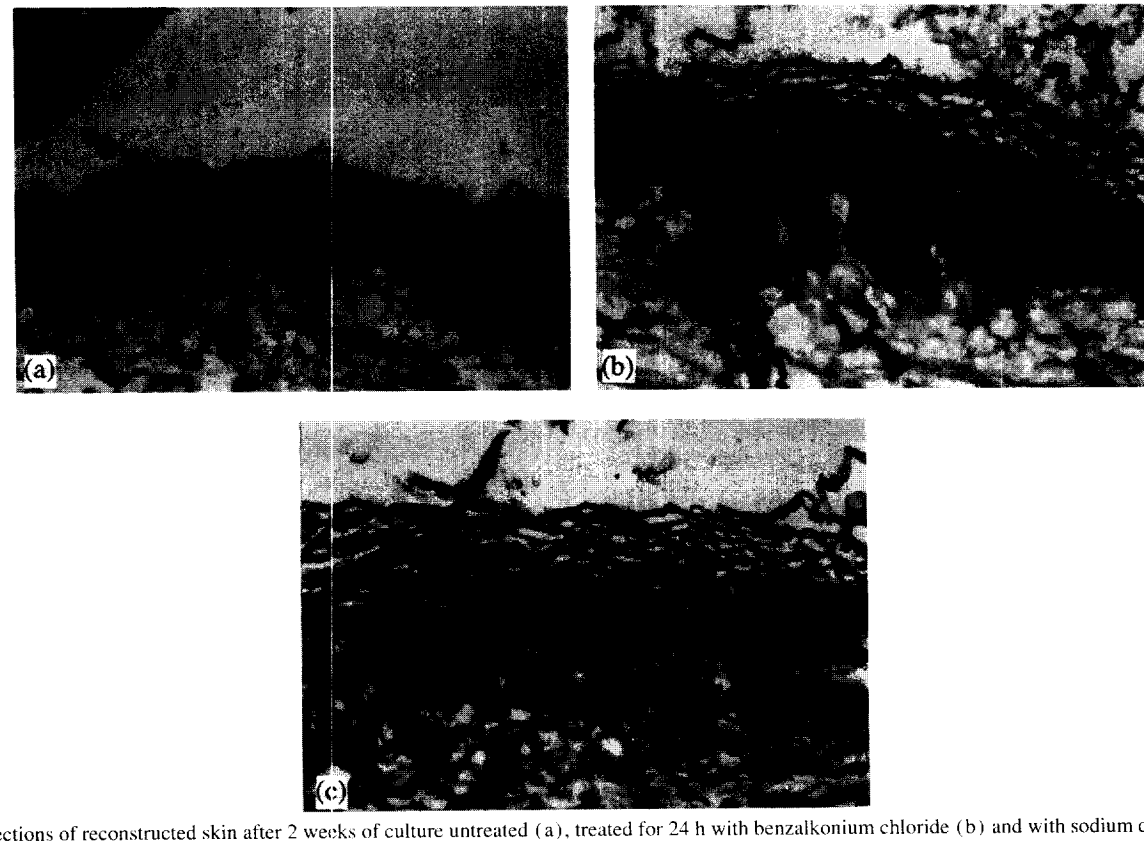

Fig. 4. Sections of reconstructed skin after 2 weeks of culture untreated (a), treated for 24 h with benzalkonium chloride (b) and with sodium dodecylsulfate (c) at MTT_{50} concentrations. Arrows indicate necrolysis (N) and parakeratosis (P).

obtained by Roguet and Régnier [22], who tested a series of compounds on submerged and exposed to the air keratinocyte cultures.

Nevertheless, epidermal differentiation could play an important role in cell responses to the surfactants. Considering that both submerged cultures and cultures exposed to the air are treated with chemicals, the former appear more sensitive than the latter, certainly because of the lack of stratum corneum [22]. Moreover, the submerged confluent cultures were less sensitive to surfactants than non-confluent ones which lacked cell stratification.

Since submerged keratinocyte cultures appeared more sensitive, less expensive and quicker than the air-exposed ones, we think they might be suitable for preliminary short-term toxicity screening having a limited lifespan. On the contrary, in vitro reconstituted human skin, which represents a complex model resembling the in vivo situation, could be employed to study the effects of long-term exposure to chemicals since reconstructed skin preserves for several days characteristics similar to those in vivo.

Actually, the concentrations of surfactants required to induce epidermal irritancy in air-exposed cultures were lower

than those inducing irritation in human skin in vivo [23]. This is probably due to a incomplete barrier capacity of the stratum corneum which regulated the amount of test agent reaching the proliferative layers of the epidermis. Thus further efforts to improve the in vitro model are necessary, changing culture media, growth factors and especially controlling humidity level.

Ponec and Kempenaar [7] reported that SDS concentrations, inducing cytotoxic effects in human skin reconstructed in vitro on DED, approximate those inducing irritation in human skin in vivo. The discrepancy between our data and theirs is probably due to the differences in growth media and in the treatments. In fact we treated air-exposed cultures with volumes (100 $\mu l/cm^2$) of surfactant solution 10-fold higher than those employed by Ponec and Kempenaar [7] (10 $\mu l/cm^2$).

Finally our results show that cultures exposed to the air could be used for histopathological in vitro studies resembling the in vivo ones. In fact, on reconstructed epidermis benzalkonium chloride and sodium dodecylsulfate induced stratum corneum scaling as well as the necrosis of suprabasal and basal layers as demonstrated in human skin in vivo by Willis et al. [23].

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References

[1] J.H. Draize, G. Woodard, H.O. Calvery, Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes, Pharmacol. Exp. Ther. 82 (1994) 377–390.

[2] M.F. Dickson, J.N. Lawrence, D.J. Benford, Cytotoxicity of 12 chemicals of known human and animal skin irritation potential in human keratinocyte cultures, Toxicol. in Vitro. 8 (1994) 661–663.

[3] G. Zamburino, M.L. Santantonio, A. Gannetti, Colture di cheratinociti umani normali, G. Ital. Dermatol. Venereol. 125 (1990) 59–77.

[4] M. Ponec, In vitro cultured human skin cells as alternatives to animals for skin irritancy screening, Int. J. Cosm. Sci. 14 (1992) 245–264.

[5] M. Régnier, D. Caron, U. Reichert, H. Schaefer, Reconstructed human epidermis: a model to study in vitro the barrier function of the skin, Skin Pharmacol. 5 (1992) 49–56.

[6] P.J. Dykes, M.J. Edwards, M.R. O'Donovan, V. Merrett, H.E. Morgan, R. Marks, In vitro reconstruction of human skin: the use of skin equivalents as potential indicators of cutaneous toxicity, Toxicol. in Vitro 5 (1991) 1–8.

[7] M. Ponec, J. Kempenaar, Use of human skin recombinants as an in vitro model for testing the irritation potential of cutaneous irritants, Skin Pharmacol. 8 (1995) 49–59.

[8] C. Cohen, K.G. Dossou, A. Rougier, R. Roguet, Episkin: an in vitro model for the evaluation of phototoxicity and sunscrin photoproctective properties, Toxicol. in Vitro 8/4 (1994) 669–671.

[9] E. Bell, N. Parenteau, R. Gay, C. Nolte, P. Kemp, P. Bilbo, B. Ekstein, The living skin equivalent: its manufacture, its organotypic properties and its responses to irritants, Toxicol. in Vitro 5/5-6 (1991) 591–596.

[10] M. Régnier, D. Asselineau, M.C. Lenoir, Human epidermis reconstructed on dermal substrates in vitro: an alternative to animals in skin pharmacology, Skin Pharmacol. 3 (1990) 70–85.

[11] M. Rosdy, L.C. Clauss, Terminal epidermal differentiation of human keratinocytes grown in chemically defined medium on inert filter substrates at the air liquid interface, J. Invest. Dermatol. 95 (1990) 409–412.

[12] J.G. Rheinwald, H. Green, Epidermal growth factor and the multiplication of cultured human epidermal keratinocytes, Nature 265 (1977) 421–424.

[13] J.G. Todaro, H. Green, Quantitative studies of the growth of the mouse embryo cells in culture and their development into established lines, Cell 19 (1963) 299.

[14] G. Grubauer, K.R. Feingold, P.M. Elias, Relationship of epidermal lipogenesis to cutaneous barrier function, J. Lip. Res. 28 (1987) 746–752.

[15] J. Brod, E. Bavelier, P. Justine, A. Weerheim, M. Ponec, Acylceramide and lanosterol-lipid markers of terminal differentiation in cultured human keratinocytes: modulating effect of retinoic acid, In Vitro Cell. Dev. Biol. 27A (1991) 163–168.

[16] P.W. Wertz, D.T. Downing, Covalently bound w-hydroxyacylsphingosine in the stratum corneum, Biochem. Biophys. Acta 917 (1987) 108–111.

[17] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay, J. Immunol. Methods 65 (1983) 55–63.

[18] H.E. Kennah, D. Albulescu, S. Hignet, S. Barrow, A critical evaluation of predicting ocular irritancy potential from an in vitro cytotoxicity assay, Fund. Appl. Toxicol. 12 (1989) 281–290.

[19] G. Santigny, M. Bonnard, O. Damour, Collombel, Reconstruction of epidermis on a chitosan cross-linked collagen-GAG lattice: effect of fibroblast, Acta Derm. Venereol. 73 (1993) 175–180.

[20] M.C. Lenoir-Viale, C. Galup, M. Darmon, B.A. Bernard, Epidermis reconstructed from the outer root sheath of human hair follicle. Effect of retinoic acid, Arch. Dermatol. Res. 285 (1993) 197–204.

[21] M. Ponec, A. Weerheim, J. Kempenaar, A.M. Mommaas, D.H. Nugteren, Lipid composition of cultured human keratinocytes in relation to their differentiation, J. Lipid Res. 29 (1988) 949–961.

[22] R. Roguet, M. Régnier, The use of reconstituted human skin in dermo toxicity testing, Toxicol. in Vitro 8 (1994) 635–639.

[23] C.M. Willis, J.M. Stephens, J.D. Wilkinson, Epidermal damage induced by irritants in man: a light and electron microscopic study, J. Invest. Dermatol. 93 (1989) 695–699.