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Epidermal-skin-test 1000 (EST-1000)—A new reconstructed epidermis for in vitro skin corrosivity testing

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Abstract

The determination of a possible corrosive or irritative potential of certain products and ingredients is necessary for their classification and labeling requirements. Reconstructed skin as a model system provides fundamental advantages to single cell culture testing and leads to promising results as shown by different validation studies (for review: Fentem, J.H., Botham, P.A., 2002. ECVAM's activities in validating alternative tests for skin corrosion and irritation. ATLA 30(Suppl. 2), 61–67). In this study we introduce our new reconstructed epidermis "Epidermal-Skin-Test" (EST-1000). This fully grown epidermis consists of proliferating as well as differentiating keratinocytes. EST-1000 shows a high comparability to normal human skin as shown by histological and immunohistochemical data. Characteristic markers (KI-67, CK 1/10/5/14, transglutaminase, collagen IV, involucrin, beta 1 integrin) can be identified easily. The main focus of this work was to characterize EST-1000 especially with respect to its barrier function by testing several substances of known corrosive potential. Skin corrosion was detected by the cytotoxic effect of the substances on a reconstructed epidermis after short-term application to the stratum corneum. The effect was determined by standard MTT assay and accompanying histological analysis. Hence EST-1000 shows a very high predictive potential and closes the gap between animal testing and the established full-thickness model Advanced-Skin-Test 2000 (AST-2000) (Noll, M., Merkle, M.-L., Kandsberger, M., Matthes, T., Fuchs, H., Graeve, T., 1999. Reconstructed human skin (AST-2000) as a tool for pharmaco-toxicology. ATLA 27, 302).

Keywords: Alternative method; EST-1000; In vitro human epidermis; In vitro testing; In vivo/in vitro comparison; Reconstructed skin equivalent; Skin corrosion; 3R

1. Introduction

As the skin is our cover against most environmental influences the protection of its integrity is of highest interest. Everything is done to reduce risks for health, especially from the growing number of synthetic compounds and new formulations. In default of alternative methods, animal testing has been the only way to evaluate the potential of substances and mixtures to harm the skin. For ethical reasons the European Coun-

Abbreviations: ET_{50}, effective time of exposure required to reduce the viability of treated cultures to 50% of controls; CK, cytokeratin; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; SDS, sodium dodecyl sulfate.

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cil decided in 1976 within the Cosmetic Directive 76/768/EEC to pursue the policy of reduction, refinement and replacement (3R) of animal testing. Meanwhile great efforts have been made to reach this goal at least for few applications (Fentem and Botham, 2002; Noll et al., 1999). With the Seventh Amendment to Directive 76/768/EEC the European Commission considers the scientific progress made so far and concluded that it is impossible to ban animal testing to a determined point of time. Nevertheless, this amendment obliges the use of alternative testing if a suitable method is developed and validated within the OECD. The new OECD Test Guideline 431 "In Vitro Skin Corrosion" (OECD, 2004) defines the requirements for in vitro skin models to be validated for skin corrosivity testing. Here we present data showing clearly that the new skin model EST-1000 Epidermal Skin Test fulfills the requirements of OECD TG 431.

2. Materials and methods

On receipt EST-1000 in vitro reconstructed human epidermis models (0.63 cm^2 surface, CellSystems, Germany) were adapted overnight to cell culture conditions (37 °C, 5% CO_2, saturated humidity) in 1 ml maintenance medium according to the provider's SOP. Before corrosivity testing, the medium was replaced by 1 ml equilibrated (37 °C) medium.

For embedding the epidermis models were fixed in PBS containing 200 mM HEPES and 8% neutralized formalin. After equilibration in PBS the tissues were completely cut out of the inserts and directly embedded with cornified surface up into Tissue Freezing Medium (Leica, Nußloch, Germany) without removing the carrier membrane. For preparation of vertical cryosections (7 μm) the embedded ESTs were frozen in the gaseous phase of liquid nitrogen.

Immunohistochemical analysis of epidermal markers was performed using monoclonal mouse anti-human antibodies (Monosan, Uden, The Netherlands) unless otherwise noted. FITC or Cy5 labelled goat anti-mouse antibodies were used for fluorescence detection. Immunohistochemical staining was performed according to standard procedures.

Lipid analysis was performed by a modified procedure according to Doering et al., 1999. In brief, the tissue samples were homogenized and epidermal lipids were extracted at 37 °C for 24 h in a solvent mixture (chloroform/methanol/water 1:2:0.6 (v/v/v). Total lipid extracts were applied to thin-layer Silica Gel 60 plates (Merck Darmstadt, Germany). Ceramides were resolved twice using chloroform/methanol/acetic acid (190:9:1, v/v/v) followed by diethylether/hexane/acetic acid (80:20:1.5, v/v/v) as developing solvent. For separation of glucosylceramides, the chromatograms were developed with chloroform/methanol/water (70:30:5, v/v/v). After development, plates were air-dried, sprayed with 8% (w/v) H_3PO_4 containing 10% (w/v) $CuSO_4$, and charred at 180 °C for 10 min. Subsequently lipids were quantified by photodensitometry (CAMAG, Berlin, Germany).

For skin corrosivity testing, EST-1000 epidermal models were exposed to 50 μl of liquid test item applied topically or 25 mg of solid compound spreaded homogenously over the surface by flotation with 25 μl PBS. Exposition to the substances was performed exactly for defined periods of time (3 and 60 min). Measurements for each time point and compound were done in triplicate. As negative control PBS was applied topically using the same procedure. After defined exposition the epidermal equivalents were washed three times in PBS by carefully dipping the whole insert. Determination of cell viability was done by standard MTT assay (Mosmann, 1983).

3. Results

The Epidermal-Skin-Test EST-1000 is provided in tissue culture well plate inserts. The growth area of each insert is covered completely. Therefore, the application of liquid test items is accomplished without leakage along the inner wall of the insert. The skin model shows a dry surface homogenously matt white in color. When detached from the membrane of the insert by dispase digestion it shows significant resistance to a tensile test.

Cryosections of EST-1000 (Fig. 1) show a proper epidermal morphology with high comparability to the native situation. A minimum of 5 viable cell layers exhibit a progressing differentiation from the bottom towards the stratum corneum. These viable layers are forming the stratum basale, spinosum and granulosum. On top at least 10 layers of finally differentiated keratinocytes are

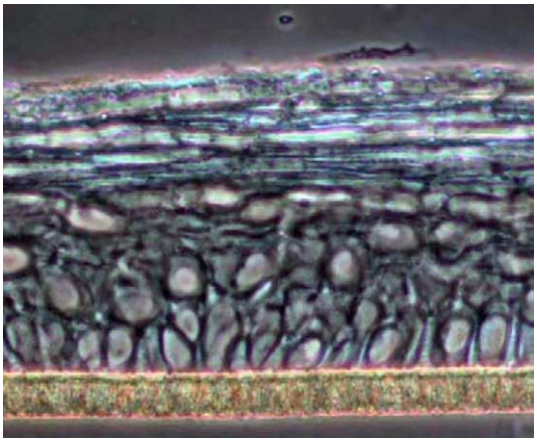

Fig. 1. Cross-section of EST-1000, phase contrast.