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Dose-response Evaluation Using an Epidermal Model, an Alternative to Skin Irritation Testing

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Abstract
Using 3-dimensional cultured human skin and epidermal models, an alternative to skin irritation testing have been developed. In the ECVAM (European Centre for the Validation of Alternative Methods) validation protocol, hazardous chemicals have been identified using these models. We considered it likely that these models would also be useful for examining the dose-response of the toxicity of a chemical. Therefore, we used the epidermal model (Labcyte™, Japan Tissue Engineering Co. Ltd.) to evaluate the dose-response of cytotoxicity and compared the findings with those for irritancy by human patch testing of 4 chemicals: sodium lauryl sulfate, benzethonium chloride, nonanoic acid and propylene glycol.

The cytotoxicity of the chemicals was found to be stronger than the irritancy shown by human patch data. Difference ratios ranging from 1.4 to 52.7 were found between irritancy and EC50(concentration causing a 50% reduction in the MTT assay compared to the untreated control value) in LabCyte™ for 4 chemicals and solvents.

We consider that these models are useful for evaluating the dose-response of skin irritancy, and could be used to establish a database for risk assessment of chemicals.

Key words: skin irritation, alternative, human patch test, risk assessment

Introduction
At an EU Council meeting held in 2004, the European Council Directive 76/768/EEC was amended for the 7th time, banning or restricting the marketing of products, whose ingredients have been tested on animals (European Commission, 2004a). According to the schedule announced then at the EU, skin irritation testing using animals will be completely phased out by March 11th, 2009, and so an alternative to testing methods is urgently needed. (European Commission, 2004b). Thus, 3-dimensional cultured human skin and epidermal models, continuously validated and peer-reviewed as they are progressively refined, are being developed as available alternatives to skin irritation testing. There have been many reports about investigations being conducted by ECVAM (European Centre for the Validation of Alternative Methods), but the final validation has not yet been obtained (Botham, 2004; Fentem and Botham, 2004).

Developing cosmetic products that do not cause skin disorders is crucial from the consumer safety viewpoint, and skin irritation testing is indispensable for this. Until now, skin irritation testing has been performed on both animals and humans for risk assessment (CTFA, 1991; JCIA, 2001). As alternative methods for assessing the risk of skin irritancy, we consider that skin and epidermal models may also be useful for screening to evaluate the dose-response of the toxicity of chemicals.

In this study, we investigated whether a model
system could be used to estimate the irritancy range of cosmetic ingredients through pre-clinical evaluation. To assess the risk of a chemical, we compared the dose-response obtained in a human irritancy testing with that by assessing cytotoxicity in an epidermal model (LabCyte\textsuperscript{TM}, Fig. 1). We tested 4 irritants: sodium lauryl sulfate, benzethonium chloride, nonanoic acid, and propylene glycol.

**Material and Methods**

**Materials**

We used the 3-dimensional cultured human epidermal model; LabCyte\textsuperscript{TM}, is produced by Japan Tissue Engineering Co. Ltd., Aichi, Japan. This model was fabricated by air-lift culturing of NHEK (normal human epidermal keratinocytes). Human epidermal cells were cultured in culture flasks by the methods of Green, using 3T3-J2 cells as the feeder layer (Rheinwald and Green, 1975). The medium was changed every 2 days until the cultures became subconfluent. These epidermal cells were collected with trypsin and seeded on the cell culture insert (12 mm diameter; culture area 1.1 cm\textsuperscript{2}) that contained a microporous membrane measuring 0.4 \( \mu \)m pore size in the well of a 12-well plate. Each culture was cultivated by the air-lift method to form a multilayered, highly skin-like structure. The cells were organized in basal, spinout, granular and cornfield layers with a high concentration of keratohyalin granules and desmosomes (Hamajima et al., 2005).

After the arrival of the kits in our laboratory, 1 mL of assay medium was added to each wells of a 12 well plate (Falcon, Becton Dickinson Labware) and these models were cultured for a few hr at 37\textdegree C in a 5 \% CO\textsubscript{2} environment (Tabai Espec, Osaka, Japan).

**Test chemicals**

Test chemicals included benzethonium chloride (BC), nonanoic acid (NAA), propylene glycol (PG) and sodium lauryl sulfate (SLS). BC and PG were obtained from Katayama Chemical Co., Ltd. (Osaka, Japan), and NAA and SLS obtain from Sigma (St. Louis, MO, USA). Distilled water (DW) from Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan) was used as diluent for BC, PG and SLS solutions. Olive oil from Mitsui Ebisu Co., Ltd. (Tokyo, Japan) was used to make BC, NAA and SLS stock preparations. NAA was solubilized in olive oil. BC and SLS were suspended in olive oil. Olive oil was also used as control material.

**Methods**

**Cytotoxicity test with LabCyte\textsuperscript{TM}**

In the pre-test and main trial, the models were treated with chemicals at 3 to 5 exposure doses and EC\textsubscript{50} values were obtained (concentration causing a 50\% reduction in the MTT assay compared to the untreated control value).

Test chemicals were dispersed in DW or olive oil and 100 \( \mu \)L of the test solution was applied to the model. Under each membrane filter, 1 mL of assay medium was then added to each well of a 12 well plate and the culture was incubated for 20-24 hours at 37\textdegree C in a 5 \% CO\textsubscript{2} environment. After this, the models were placed in culture medium containing 0.5 mg/ml MTT after washing twice with phosphated buffer saline (pH 6.8). Three hours later, the resultant MTT formazan crystals were solubilized in 500 \( \mu \)L of isopropanol and the absorbance was measured at 540 nm with a microplate reader (Emax, Molecular Devices, Menlo Park, CA, USA).

**Patch test procedures**

All studies were conducted according to the protocols supplied by Research Laboratories, Nippon Menard Cosmetic Co. Ltd (Nagoya, Japan). Approximately 40 subjects per study were exposed to each test chemical for 24-hour. In each study, DW or olive oil was tested as a negative control. Test chemicals were applied in 0.1 mL volumes, either neat or after suitable dilution in DW or olive oil, to Hay’s patch test chamber.

The upper arm was placed in the test chamber and a patch was applied. After removal of the patch, the skin sites were gently wiped with damp cotton to remove excess residual test chemical. The sites were graded at 1 and 24 hours after patch
removal using the accepted Japanese patch test reading criteria (JPTRC: Kawamura et al., 1970) as shown in Table 1. Observers were trained to use the standardization assessment criteria using the color atlas made by the Research Group for Skin Irritation, Japanese Society for Contact Dermatitis (Kawai, 2004).

The principals of the human patch testing procedure and the testing approach are intended to avoid the production of strong irritation responses. The procedures used in these studies were subjected to review and approval by local ethical review committee and all experiments were in accordance with the Helsinki Declaration of 2002. (Helsinki Declaration, 2002). All procedures were described in detail to all study subjects, who read and signed informed consent forms.

**Data analysis**

For the *in vitro* assay, the ratio of the MTT reading of treated vs. non-treated cells was determined and the EC\(_{50}\) value was calculated based on duplicate dose-response curves (MTT reduction assay, Mosmann, 1983).

As human patch testing, we obtained the average score and standard deviation after scoring the skin response as shown in Table 1. After that, we determined the dose of each chemical that caused a maximal average score of up to 0.2. These doses corresponded to 10% of the doses which caused a score of 2 or more in human patch testing.

### Table 1.

<table>
<thead>
<tr>
<th>Judge</th>
<th>Score</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>−</td>
<td>0</td>
<td>No reaction</td>
</tr>
<tr>
<td>±</td>
<td>0.5</td>
<td>Weak erythema</td>
</tr>
<tr>
<td>+</td>
<td>1.0</td>
<td>Erythema</td>
</tr>
<tr>
<td>++</td>
<td>2.0</td>
<td>Erythema + edema</td>
</tr>
<tr>
<td>+++</td>
<td>3.0</td>
<td>Erythema + edema, seropapules, vesicles</td>
</tr>
<tr>
<td>++++</td>
<td>4.0</td>
<td>Bulla</td>
</tr>
</tbody>
</table>

### Table 2.

Comparison between the dose of human patch irritancy and the cytotoxicity in LabCyte\(^{TM}\)

<table>
<thead>
<tr>
<th>Chemical</th>
<th>NA</th>
<th>PG</th>
<th>BC</th>
<th>SLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent</td>
<td></td>
<td></td>
<td>DW</td>
<td>Olive oil</td>
</tr>
<tr>
<td>Patch</td>
<td>15.8</td>
<td>100</td>
<td>0.1</td>
<td>1</td>
</tr>
<tr>
<td>&gt;0.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LabCyte</td>
<td>0.3</td>
<td>70</td>
<td>0.05</td>
<td>0.03</td>
</tr>
<tr>
<td>EC50</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Results

The dose-response curves of 4 chemicals tested here are shown in Fig.2. The cytotoxicity of the chemicals shown in the epidermal model was stronger than the irritancy shown by human patch testing. The rank of irritancy in human patch testing (in decreasing order) was BC, SLS, NAA and PG. The rank of cytotoxicity was similar, with the order BC, SLS=NAA, PG.

As shown in Table 2, there were the difference ratios from 1.4 to 52.7 between the human patch data and EC\(_{50}\) in LabCyte\(^{TM}\) for each chemical and solvent. When the solvent was DW, the difference ratios between the irritancy and cytotoxicity of LabCyte\(^{TM}\) were small (from 1.4 to 2.0). In contrast, when the solvent was olive oil, the difference ratios were large (from 10 to 52.7).
Discussion

Hazard identification or risk assessment

For identifying chemical hazards to human skin, OECD Test Guideline 404 was established in 1981. This guideline states that a chemical is applied at 100% concentration for 4-hour on rabbit skin, and whether it causes corrosivity or skin irritation is evaluated. This guideline was revised in 2002, and new assay methods were introduced for use as possible alternative testing methods (OECD 404, 2002). In Japan, this test using animals such as rabbits or guinea pigs was welcomed as a method.
of safely developing new cosmetics, as the ingredients require pre-screening in order to assess the health risks involved (Guide to Quasi-drugs and Cosmetics Regulation in Japan, 2001). According to the revised guidelines, a chemical is applied at several doses for 24 hours on animal skin, and its strength of skin irritancy is evaluated. For example, potassium hydrate (KOH) is corrosive at more than 10% concentration, but it is used widely for pH adjustment at low concentration, at which it does not cause irritation. For a primary skin irritation testing, chemicals have been evaluated using different methods such as hazard identification (OECD 404, 2002) or the dose-response of the toxicity (cosmetic guidance).

Today, it might be useful to identify corrosive hazards by comparing in vitro and animal results (OECD, 430, 431, 2004). In vitro skin irritation methods, continuously validated and peer-reviewed as they are progressively refined, are being developed as viable alternatives to skin irritation testing by ECVAM. In Japan, a validation committee of the Japanese Society for Alternatives to Animal Experiments (JSAAE) has been conducting its own validation study of alternative skin irritation testing since 1999. A few testing models already on the market in Japan have been validated repeatedly (Sonoda, et al., 2002). A comparison of the validation efforts by ECVAM and JSAAE reveals a large difference as to the purpose of validation. The ECVAM validation focuses on the identification of chemical hazards, as an alternative to the “acute dermal irritation/corrosion” of OECD Test Guideline 404, while the JSAAE validation is mainly concerned with the dose-response of the toxicity, i.e., the methods used to predict the effects of cosmetic ingredients through pre-clinical evaluation. In this paper, we investigated to utilize in vitro skin irritation testing for the dose-response of the toxicity.

Utilization of data of human skin irritation testing

For risk assessment of chemicals, the human patch testing is an important method of evaluating the sensitivity of the human skin to the chemicals; human volunteers are used for this test and it allows the prediction of the risks involved in the cosmetic use of certain chemicals through pre-clinical evaluations regarding dose, duration, and type of exposure; it also allows the development of new ingredients and products (Fregert, 1981; Guide to Quasi-drugs and Cosmetics Regulation in Japan, 2001; JCIA, 2001).

Lately, a few researchers (Augustin et al., 1998; Roguet et al., 1998; Perkins et al., 1999) have obtained good correlations in comparisons of data acquired from human patch testing and cultured-epidermis models (Episkin™ or EpiDerm™). In those studies, the factor of irritancy was unclear and problems with poor reproducibility were attributed to the cosmetic products used or unknown surfactants, except in the study reported by Lee et al.(2000). Lee et al. examined the correlation between the irritancy and the cytotoxicity of skin equivalents of typical surfactants at 1% concentration. However, their results do not show comparison between the dose-dependent of the responses to surfactants (Lee et. al, 2000). Accordingly, we used in vivo data obtained for the dose-dependent of the skin response in 24-hour human patch testing for risk assessment of 4 chemicals.

Recently, Basketter proposed a 4-hour closed human patch test for identifying skin hazards as an alternative to the testing prescribed by OECD Test Guideline 404 (OECD, 2002; Basketter et al., 2004). Because of experimental design differences, those data can not be used for comparison with the data obtained in vitro in the present study.

Chemicals used

In this study, we used 4 chemicals which are well known irritants. SLS is an anionic detergent, that is a typical irritant, and numerous researchers have reported studies on this substance. Though one study found no irritancy of a 1% solution (Nardo, et al., 1996), this substance was found in other studies to a distinct skin response at 1% concentration (Wahlberg et al., 1985, Reiche et al., 1998, Nicholson et al., 1999, Wahlberg and Lindberg, 2003). Our data are in accord with those found in the latter studies. BC is a cationic detergent with a similar structure to that of benzalkonium chloride, which is a typical strong irritant. Benzalkonium chloride causes a clear skin response at 0.5% concentration (Wills et al., 1988, Wahlberg et al., 1985, Wahlberg and Lindberg, 2003). These data are in agreement with our data for BC when tested as an irritant at concentrations up to 0.1%. NAA is a fatty acid containing 9 carbons and is a weak irritant. Some studies showed moderate irritancy of NAA at 5% in 1-propanol (Wahlberg and Maibach, 1980, Wahlberg et al., 1985), and this substance caused skin response at up to 10.0% concentration in our study (Wahlberg and Lindberg, 2003). PG is a glycol and a mild irritant. This substance was

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reported to cause a well-defined skin response at 100% concentration as shown in our study (Hannksela, et al., 1975, Hayakawa, 1984, Funk and Maibach 1994, Hosono, 1999).

As a solvent, we tested both DW and olive oil as solvents for SLS and BC. DW causes a high frequency of the skin response, and its effects depend on such factors as the individual and the season. Therefore, this solvent is difficult to use in human closed patch testing. In additions, surfactants are usually easily solubilized at low concentration in DW, but, they may not be easily solubilized in DW at high concentration. To rank the irritancy of chemicals, we should use the same solvents. Accordingly, we usually use olive oil or Vaseline as a solvent in this test regardless of the water solubility of the tested chemicals.

Selection of in vitro model

In order to develop an alternative to skin irritation testing, a trial using cultured monolayer cells was carried out (Konishi et al., 1977a, 1977b) but a skin equivalent or cultured skin is used by most researchers trying to develop alternative ways of testing skin irritants. The results obtained with monolayer cells revealed a significant correlation with those from the skin equivalent and cultured skin (Kojima et al., 1998, 2000; Faller et al., 2002).

In Japan, two epidermal models (Labcyte™ and EpiDerm™, Kurabo Co., Ltd.) and two skin models with a corneal layer (TESTSKIN™: TOYOBO Co. Ltd. and Vitrolife-Skin™: Gunze Co. Ltd.) are commercially available at the present time. Though the epidermal and skin models are clearly different in structure, two models show the same patterns of cytotoxicity (Kubo, 2005). Therefore, we used a representative model, LabCyte™, as an epidermal model in this study.

Utilization of in vitro model for risk assessment

To obtain data using LabCyte™, it is important to select a solvent to solubilize or suspend a chemical. We considered olive oil or Vaseline, non-DW solvents, to be useful just as they are in human patch testing. Though we tested SLS and BC at up to 10% concentration using Vaseline in LabCyte™, we did not observe distinct cytotoxicity and obtained no EC₅₀ (data not shown). Solubilizing or suspending them in olive oil resulted in severe cytotoxicity in this model, with an EC₅₀ similar to that found when they were tested in DW. Therefore, it may be most useful to use olive oil as a non-DW solvent for the cytotoxicity test in the epidermal model.

The difference rates between the irritancy and the cytotoxicity in the epidermal model were small in case solvent is DW. Though we tested only 4 chemicals, our data suggested the cytotoxic dose found in the epidermal model may predict the dose that would cause the irritancy in human patch testing.

In contrast, the difference ratios between the two tests were large when the solvent was olive oil. The irritancy-inducing dose of a chemical in olive oil is generally about 10 times higher than the dose considered safe. Making this assumption, we might be able to determine an appropriate dose for performing human patch testing. Sugibayashi reported that the skin models rapidly predicted simultaneous diffusion of a pro-drug, lactic acid, Triton X-100 and SLS through human skin (Sugibayashi et al., 2002, 2004). Therefore, this model is more sensitive than intact skin. These data were in accord with our data when the solvent is not DW.

To further test the usefulness of this method, we consider that these models are useful for evaluating the dose-response of skin irritancy, and could be used to establish a database for risk assessment of chemicals.

Acknowledgments

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