(Ashikaga et al., 2002; Yoshida et al., 2003), and we named this test the human cell line activation test (h-CLAT). In our previous study, we optimized the test conditions (Ashikaga et al., 2006) and confirmed good predictive performance using nine chemicals (Sakaguchi et al., 2006). When the criteria for positive response of CD86 and CD54 in h-CLAT were set at 150% and 200% respectively, the correspondence between in vivo and in vitro was more than 90% (Ashikaga et al., 2007). h-CLAT could predict the sensitization potential of preservatives, which are well-known sensitizers (Sakaguchi et al., 2007). These results suggested that h-CLAT could be a useful in vitro test system for predicting sensitizing properties of chemicals. Before submission of h-CLAT to a public center for validation of alternative methods, we required further data, especially on inter-laboratory reproducibility among multiple laboratories. Therefore, this inter-laboratory study was set up to confirm the transferability and reproducibility of the h-CLAT protocol. Seven Japanese laboratories participated in this study, with the support of the Ministry of Health, Labor and Welfare.

Materials and Methods

Study management and SOP

An initial test protocol was developed based on our previous study (Ashikaga et al., 2006). In the light of subsequent experiments, a refined and detailed standard operating procedure (SOP) was defined for conducting further study.

Cells and culture

THP-1 cells (ATCC No. TIB-202) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI 1640 medium (Invitrogen Corp., Carlsbad, CA USA) with 10% FBS (v/v) (MP Biomedicals, Morgan Irvine, CA, USA, Cat. No. 29165, Lot. No. 2688H), 0.05 mM 2-mercaptoethanol and 1% Antibiotic-Antimycotic (Invitrogen Corp., Carlsbad, CA USA).

h-CLAT procedure

THP-1 cells were seeded at between $0.1x10^6$ and $0.2x10^6$ cells/mL, and pre-cultured for 48 h or 72 h. After the incubation, THP-1 cells were plated at 1 x 10^6 cells/ml in a 24-well plate and treated for 24 h with test chemical. The final concentration of DMSO, when this was used as a solvent, in culture media was less than 0.2%. Chemical-treated cells were washed twice with PBS(-) containing 0.1% BSA. Then, the cells were treated with 0.01% globulins, Cohn fraction II, III (Sigma-Aldrich) for FcR blocking, for 10 min at 4℃. Cell staining was done at 4° C for 30 min. Anti-human CD86 antibody was obtained from BD-PharMingen (Clone: Fun-1, San Diego, CA, USA). Anti-human CD54 antibody was obtained from DAKO (Clone: 6.5B5, Glostrup, Denmark). FITC labeled-mouse IgG1 was purchased from DAKO (Clone; DAK-G01, Glostrup, Denmark) and used as an isotype control. Cells were washed once with PBS(-) containing 0.1% BSA, and expression of cell surface antigens was analyzed by flow cytometry. Dead cells were gated out by staining with propidium iodide (PI, 0.625 μg/ml). In total, 10,000 living cells were analyzed. When the cell viability was less than 50%, Relative Fluorescence Intensity (RFI) was not calculated because of diffuse labeling of cytoplasmic structures due to cell membrane destruction (Becker et al., 1992). RFI was used as an in-

Table 1 Test chemicals and common dose setting (ND= No data).

Test chemicals	LLNA EC3(%)	Potency category by LLNA	Common CV75 (μg/mL)	vehicle for h-CLAT
p-Benzoquinone (BQ)	0.0099	Extreme	3.5	DMSO
1-Chloro-2,4-dinitrobenzene (DNCB)	0.05	Extreme	6.0	DMSO
Glutaraldehyde (GA)	0.1	Strong	8.0	Saline
Ethylene diamine (ED)	2.2	Moderate	250	Saline
Nickel sulfate (Ni)	4.8	Moderate	150	Saline
Eugenol (EU)	13	Weak	150	DMSO
Lactic acid (LA)	Not calculated	Non-sensitizer	2800	Saline
Sodium lauryl sulfate (SLS)	N.D.	False potitive	60	Saline

dicator of CD86 and CD54 expression and was calculated as follows:

$$\text{RFI (\%)} = \frac{\text{(MFI of chemical-treated cells} - \text{MFI of chemical-treated Isotype control cells)}}{\text{(MFI of vehicle control cells} - \text{MFI of vehicle Isotype control cells)}} \times 100$$

MFI = (Geometric) Mean fluorescence intensity

Test chemicals and application doses

Eight test chemicals are shown in Table 1. All chemicals have been evaluated and classified with the LLNA (Gerberick et al., 2005). Six sensitizers were evaluated: two extreme, one strong, two moderate, and one weak allergens, as classified by LLNA. Two non-sensitizers were also evaluated: one non-classified allergenic chemical and the other false positive by LLNA. All chemicals were purchased from Sigma-Aldrich. In first and second trials, application doses were determined from the results of cytotoxicity tests conducted at two laboratories. Cytotoxicity was evaluated by flow cytometry with propidium iodide (PI) (PI assay). From the PI assay data, eight doses based on the dose estimated to give 75% cell viability (CV75) were used [1.2 x CV75, 1 x CV75, 1/1.2xCV75 (or 0.8333xCV75), $1/1.2^2$xCV75 (or 0.6944xCV75), $1/1.2^3$xCV75 (or 0.5787xCV75), $1/1.2^4$xCV75 (or 0.4822xCV75), $1/1.2^5$xCV75 (or 0.4019xCV75) and $1/1.2^6$x CV75 (or 0.3349xCV75)]. The appropriateness of this dose setting was confirmed by the evaluation of more than 60 chemicals (Ashikaga et al., 2007). All CV75 doses of test chemicals used in this study are shown in Table 1. The vehicle was saline or DMSO (SIGMA-ALDRICH, Cat. No. 154938, purity ≧99.9%). In the third trial, each laboratory individually conducted cytotoxicity testing for determination of the application doses.

Data analysis

Tests were performed three times with each chemical. The values of cell viability and CD86/54 expression were calculated as the mean of the three tests. The average of three experiments at any dose should exceed the positive criterion ("CD86 ≧ 150 or CD54 ≧ 200") in order for the test chemical to be considered as 'positive'.

Cell culture conditions

THP-1 cells cultured in Lab "F" showed unacceptably low viability (less than 50%) when treated with 5 μg/mL DNCB (CV75/1.2), which was used as a positive control in every experiment. For that reason, the dose-response of DNCB in Lab "F" was different from the results in other laboratories (Fig. 1-a). However, when freshly cultured THP-1 cells were introduced in Lab "F", the results were very similar to those in the other laboratories (Fig. 1-b). Both the firstly tested cell and the freshly cultured cell originated from a same lot of THP-1.

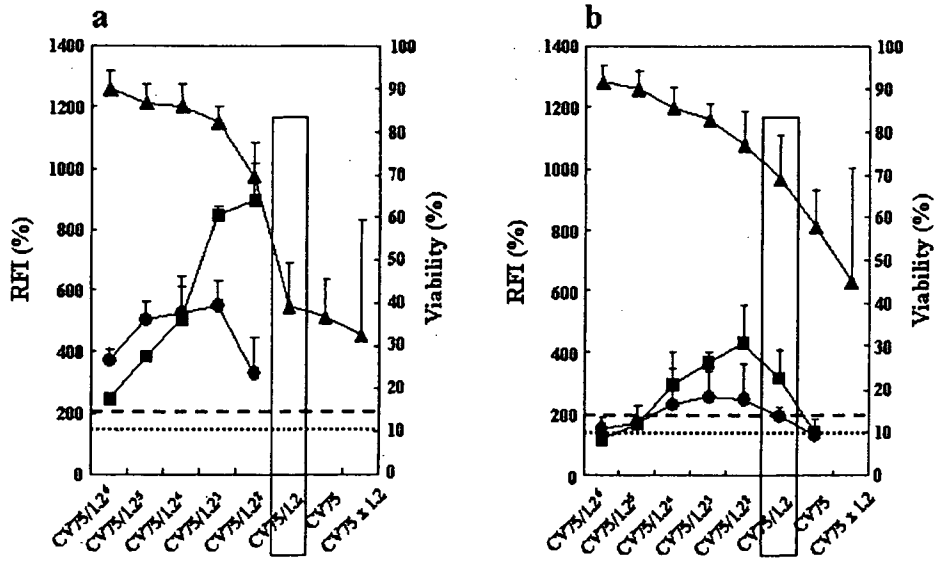

Fig. 1 Improvement of reproducibility (example 1)
At laboratory "F", the cell viability at 5 μg/mL DNCB (CV75/1.2) was improved when DNCB was re-evaluated with freshly cultured THP-1 cells. a=First experiment; b=Second experiment. ▲= Viability; ●= CD86; ■= CD54. Small dotted line: criterion for CD86 positivity; dashed line: criterion for CD200 positivity.

The viability of the freshly cultured cell treated with 5mg/mL of DNCB (CV75/1.2) was about 70%. On the other hand, that of the firstly tested cell was less than 40%, and the value meant condition of the cell was not good. Condition of the firstly tested cell could have decreased due to wrong operation such as over-growth during cell culture. Moreover, in Lab "D" the dose-response of Ni was initially different from those in the other laboratories (Fig. 2-a). Cell viability of control cells at this time was less than 90%, which was unusual. When Lab "D" re-evaluated Ni with freshly cultured THP-1 cells, the viability of control cells was over 90% and both CD86 and CD54 were enhanced by treatment of the test cells with Ni (Fig. 2-b). These results suggested that tight control of cell culture conditions is important for good reproducibility in the test.

Inter-laboratory reproducibility with three well-known chemicals

As a first trial, seven laboratories tested two well-known sensitizers (DNCB and Ni) and one non-sensitizer (SLS), after the introduction of tighter control of cell culture conditions. Fig. 3 shows the inter-laboratory reproducibility for

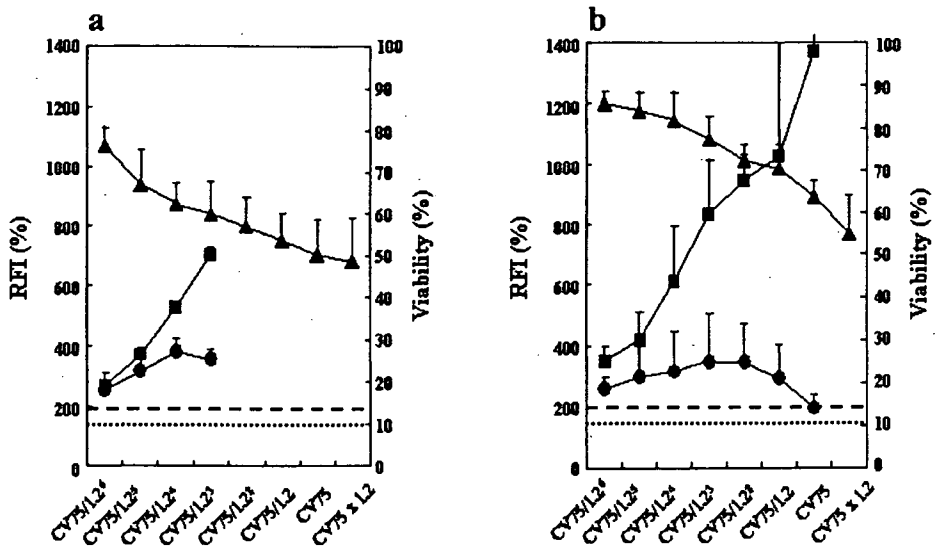

Fig. 2 Improvement of reproducibility (example 2)
At laboratory "D", the cell viability of control cells was improved when freshly cultured THP-1 cells were used, and the dose-response curve of Ni was similar to those in the other laboratories. a=First experiment; b=Second experiment. ▲= Viability; ●= CD86; ■= CD54. Small dotted line: criterion for CD86 positivity; dashed line: criterion for CD200 positivity.

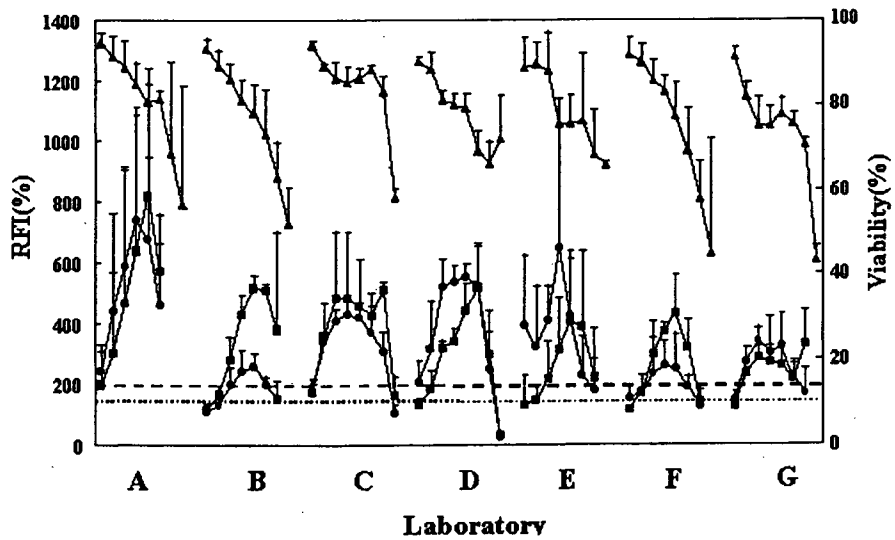

Fig. 3 Inter-laboratory reproducibility of prediction for DNCB
▲= Viability; ●= CD86; ■= CD54. Small dotted line: criterion for CD86 positivity; dashed line: criterion for CD200 positivity.

DNCB. In all laboratories, DNCB clearly enhanced both CD86 and CD54 at several doses and the dose-response relationships were basically similar. Both CD86 and CD54 were augmented dose-dependently at lower doses and their expression was suppressed due to cytotoxicity at higher doses. Ni also enhanced both CD86 and CD54 of THP-1 cells in all laboratories (Fig. 4). In particular, CD54 expression was remarkably induced by the Ni treatment in a dose-dependent manner. On the other hand, SLS, a non-sensitizer, did not affect CD86 or CD54 expression at any dose, including higher "subtoxic doses", in all laboratories (Fig. 5). All seven laboratories correctly evaluated the sensitizing potential of these three chemicals. The reproducibility of dose-response relationships among laboratories was excellent.

Inter-laboratory reproducibility of five additional chemicals

Next, four sensitizers, covering diverse sensitizing potentials, and one non-sensitizer were tested as a

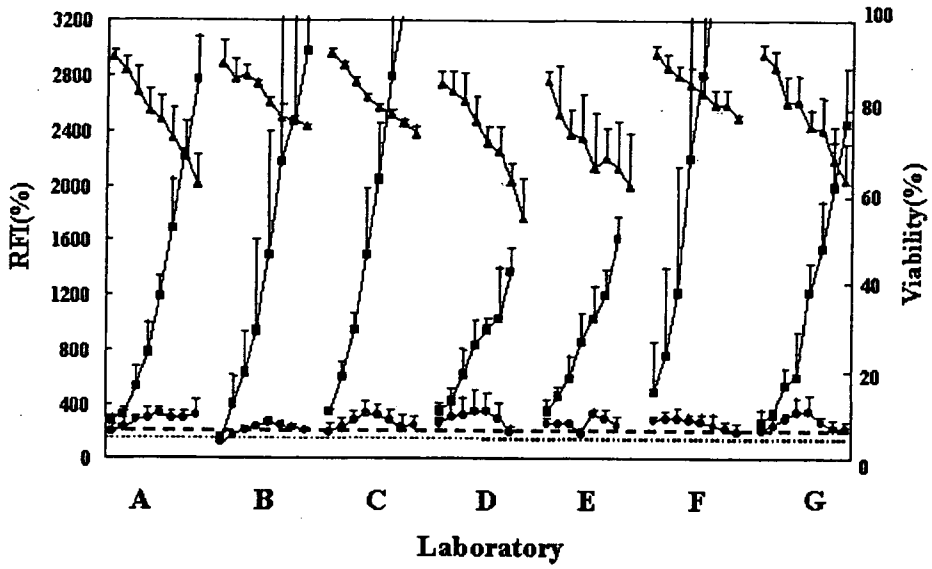

Fig. 4 Inter-laboratory reproducibility of prediction for Ni
▲= Viability; ●= CD86; ■= CD54. Small dotted line= criterion for CD86 positivity; dashed line: criterion for CD200 positivity.

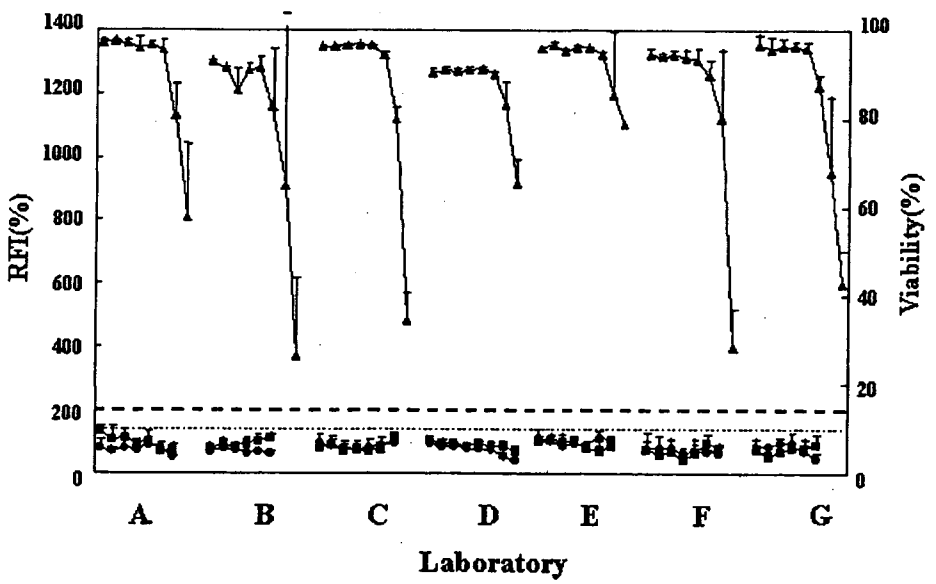

Fig. 5 Inter laboratory reproducibility of SLS
▲= Viability; ●= CD86; ■= CD54. Small dotted line= criterion for CD86 positivity; dashed line: criterion for CD200 positivity.

second trial. In the second trial, all five chemicals were tested based on common CV75 values. The results for the five chemicals in seven laboratories are summarized in Table 2. Thirty-three out of 35 tests corresponded with LLNA. There were two false-negatives (ethylene diamine and eugenol), but no false-positives. The overall accuracy of the 1st and 2nd trials was about 96%. In summary, the reproducibility of h-CLAT was basically good. Either CD86 or CD54 was slightly enhanced in the two false-negative cases (data not shown), but the increases did not meet the criteria for positivity.

Inter-laboratory reproducibility, including dose finding

In order to further assure of the performance of the assay, chemicals tested in the second trial were evaluated again in a third trial. In this trial, each laboratory individually performed cytotoxicity assay, and determined the application doses based on their own results. Table 3 shows CV75 values (estimated dose affording 75% cell viability) at each

Table 2 Summary of the first and second trials
The results of evaluation of eight chemicals in the seven laboratories are summarized.
Results; += positive; -= negative. Battery (CD86/CD54). *; Laboratory "D" judged BQ positive as a result of eight experiments. Shaded cell= LLAN and h-CLAT predictions differ.

	Laboratory						
Test chemical	**A**	**B**	**C**	**D**	**E**	**F**	**G**
p-Benzoquinone (BQ)	+ (+/+)	+ (+/+)	+ (+/+)	+ (+*/-)	+ (+/-)	+ (+/+)	+ (+/-)
Glutaraldehyde (GA)	+ (+/+)	+ (+/+)	+ (+/+)	+ (+/-)	+ (+/-)	+ (+/+)	+ (-/+)
Ethylene diamine (ED)	+ (+/-)	+ (+/-)	- (-/-)	+ (+/-)	+ (+/-)	+ (+/-)	+ (+/-)
Eugenol (EU)	+ (+/+)	+ (-/+)	+ (+/+)	+ (+/-)	+ (+/+)	+ (+/+)	(-/-)
Lactic acid (LA)	- (-/-)	- (-/-)	- (-/-)	- (-/-)	- (-/-)	- (-/-)	- (-/-)

Table 3 Values of CV75 at each laboratory in the third trial
Grand mean= mean value of all seven laboratories; SD= standard deviation; CV= coefficient of variation.

Test chemical	Common CV75 in the second trial	CV75s (μg/mL) in the third trial									
		A	B	C	D	E	F	G	Grand mean	SD	CV
DNCB	6.0	4.3	4.2	5.0	4.6	3.6	4.6	6.4	4.7	0.9	0.19
pBQ	3.5	2.6	5.5	3.2	7.3	5.4	2.8	3.7	4.3	1.7	0.40
GA	8.0	9.7	12	12	20	7.5	8.2	9.2	11	4.3	0.38
ED	250	267	256	274	278	248	370	277	281	41	0.14
EU	150	153	161	155	202	177	190	120	165	27	0.17
LA	2800	2730	2754	3045	3055	2997	3300	2920	2972	195	0.07

Table 4 Summary of the third trial
The results of re-evaluation of five chemicals in the seven laboratories are summarized.
Results; += positive; -= negative. Battery (CD86/CD54). Hatched cell= LLAN and h-CLAT predictions differ.

	Laboratory						
Test chemical	**A**	**B**	**C**	**D**	**E**	**F**	**G**
BQ	+ (+/+)	+ (+/+)	+ (+/+)	+ (+/-)	+ (+/-)	+ (+/+)	+ (+/-)
GA	+ (+/+)	+ (+/+)	+ (+/+)	+ (+/-)	+ (+/+)	+ (+/+)	+ (+/+)
ED	+ (+/-)	+ (+/-)	- (-/-)	- (-/-)	+ (+/-)	+ (+/-)	+ (+/+)
EU	+ (+/+)	- (-/-)	+ (+/-)	+ (+/-)	+ (+/-)	+ (+/+)	+ (+/+)
LA	- (-/-)	- (-/-)	- (-/-)	- (-/-)	- (-/-)	- (-/-)	- (-/-)

laboratory. There was some variation of CV75 among laboratories. This might have been caused by differences of culture conditions between laboratories, because the same lots of serum and the same cell line were used at all laboratories. However, the CV75s at the individual laboratory were very close to the common CV75s used in the second trial. The coefficient of variation for each test chemical was between 0.07 and 0.4, and the range of CV value was good compared with that in another inter-laboratory study on cytotoxicity assay (Tani et al., 1999). The results of the third trial are summarized in Table 4. Among the five test chemicals, p-benzoquinone, glutaraldehyde and lactic acid were correctly evaluated at all laboratories. On the other hand, two laboratories missed the sensitizing potential of ethylene diamine or eugenol. These results are almost the same as those in the second trial.

Discussion

Several in vitro skin sensitization methods using cell lines have been reported in response to current trends in animal welfare and regulatory opinion (Casati et al., 2005), but final validation and regulatory acceptance have not yet been achieved. We have reported that h-CLAT using THP-1 cells was useful for predicting skin sensitization in vitro (Ashikaga et al., 2007; Sakaguchi et al., 2007). However, more data were needed, especially about the transferability of the protocol, and the inter-laboratory reproducibility of the test, in order to support formal validation activities (Hartung et al., 2004). Therefore, we organized a multi-laboratory study involving seven laboratories. As a first step, two well-known sensitizers (DNCB and Ni) and one non-sensitizer (SLS) were evaluated. The purpose of the first trial was to establish technology transfer of the h-CLAT protocol. Because all laboratories correctly evaluated the sensitization potentials of these three chemicals, transferability of the assay was judged to be basically good. However, some differences in dose-response relationship were observed. The reproducibility improved when re-evaluation was conducted with freshly cultured THP-1 cells. These results suggest that tight control of cell culture conditions is important, especially for good reproducibility of cell-based assay in which protein expression is used as an indicator.

Based on these results, we refined the standard operating procedure (SOP). We introduced the requirements that the viability of control cells should be more than 90 %, and that the viability in the positive control should be more than 60%. After the introduction of tighter control of cell culture conditions, the reproducibility of the dose-response relationship was improved. From the result of the first trial, we concluded that the h-CLAT protocol is easy to transfer, and to further confirm the reproducibility with various kinds of chemicals, we tested four sensitizers and one non-sensitizer in a second trial. In the total of 35 tests (seven laboratories, five chemicals), there were two false-negatives (ethylene diamine and eugenol). Therefore, inter-laboratory reproducibility of the assay was basically good. Ethylene diamine is known to be very reactive with organic compounds (Agius et al., 1991), and it evaporates at room temperature. Further, eugenol showed poor water solubility at the application doses, because oil drops were observed in the cell culture medium. It would be difficult for h-CLAT to evaluate the sensitization potential of such chemicals, so the false negative results may simply reflect the particular characteristics of these two chemicals. It will be necessary to clarify the extent of applicability of h-CLAT, particularly in relation to the physicochemical properties of target molecules. Some differences in CD86/CD54 expression pattern were also observed among laboratories. This confirms the importance of predicting sensitizing potential not just with one marker, but with two or more markers. Python et al. (2007) reported that a combination of at least two markers was needed to establish a reliable evaluation of dendritic cell activation potential. We also should mention problems of h-CLAT. Test chemicals are treated with THP-1 cells in cell culture medium. Therefore, if test chemical disperse non-equally in cell culture medium (e.g., sticky, water-proof particle, oil spill, etc.), h-CLAT may not evaluate these potential correctly. In addition, THP-1 is thought to almost not have metabolic enzymes such as P-450 (Prof. Yoshida, Showa Univ., personal communication). Therefore, h-CLAT might not be able to evaluate a potential of chemical that can be changed by metabolism. Study on the applicability domain of h-CLAT remains to be done.

Finally, chemicals tested in the second trial were re-evaluated with doses determined at each individual laboratory as a third trial, to see whether more appropriate application doses could be selected, depending on the precise test conditions. However, differences of the values of CV75 between laboratories were not large. Furthermore, the results (positive/negative judgment) were almost

the same as in the second trial with common application doses. In conclusion, further study is necessary, especially to clarify the limitations of the assay. Finally, all laboratories correctly judged the sensitization potential of six test chemicals among eight chemicals. These results suggest that the h-CLAT protocol is easy to transfer, and that inter-laboratory reproducibility is basically good. We consider that h-CLAT will be ready for formal pre-validation study after further minor improvements of the method.

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Corresponding author:
Takao Ashikaga
Shiseido Co., Ltd., Quality Assurance Center,
2-12-1, Fukuura, Kanazawa-ku, Yokohama,
236-8643, Japan
Tel: +81-45-788-7308
Fax: + +81-45-788-7295
E-mail: takao.ashikaga@to.shiseido.co.jp

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日本動物実験代替法学会

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WC6を終えて

第6回国際動物実験代替法会議　会長　大野泰雄

1. 謝辞

第6回国際動物実験代替法会議は日本動物実験代替法学会，日本学術会議，Alternative Congress Trust (ACT，日本語では国際動物実験代替法連合と訳した)の主催で，平成19年8月21日(火)から8月25日(土)までの5日間，東京都江東区のホテルイースト21で，34ヵ国および1地域（台湾）より1,036人（国外440人，国内596人）の参加者を得て，開催され，成功裏に終了することができた．これは，表1に示したように，日本動物実験代替法学会を中心とする多数の機関および方々の協力のおかげです．ここに深くお礼を申し上げるとともに，以下に，内容を報告させていただきます．

2. 会議の目的と経緯

ACTは1) 教育，研究および試験における生命科学研究における動物福祉の向上と動物実験代替法開発を促進すること，2) 科学の進展や生物や疾患への理解を深めるために動物実験が必要であるとの認識を醸成すること，また，3) 科学者と社会とのコミュニケーションを促進することを目的に設立された基金です．ACTは，1) 教育，研究，試験分野における3Rs (Reduction, Refinement, Replacement)の実現に向けての進展を概観し，2) 動物実験代替法の状況に対する現実的な理解を深め，3)動物を用いる研究が臨床研究やin vitro試験法とともに，科学の発展をもたらすものであるという理解を醸成し，4) 生物学や疾患に対する我々の基本的な理解に貢献し，並びに5) 動物保護グループと科学者との間に建設的な議論を行うことを奨励することを目的に，国際動物実験代替法学会（World Congress on Alternatives and Animal Use in the Life Sciences）を開催してきた．第1回は1993年にボルチモア（米国）で開催され，それ以来，1996年にユトレヒト（オランダ），1999年にボロニア（イタリア），2002年にニューオーリンズ(米国)，2005年にベルリン(ドイツ）と回を重ねてきた．

日本動物実験代替法学会は，第1回会議から日本からの参加や発表の呼びかけを行い，参加者への旅費支援，また，運営委員会への参画等を通じて積極的に協力するとともに，外国の関係者との交流を進めてきた．また，日本開催に向けて準備金を積み立ててきた．これらの基盤の上で，日本開催に向けて立候補し，平成15年11月開催のACT会議で2007年に東京で開催することが認められた．それ以来，学会では開催の準備を進めてきた．本会議の開催はアジアでは初めてであったことから，今回の会議では上記の目的を達成するとともに，アジアにおける代替法研究の発展とアジアからの代替法発信をめざし，韓国ならびに中国の関係者にも協力を求めた．日本動物実験代替法学会は以前より市民との対話を重視しており，動物福祉団体や動物実験に反対する団体の代表者をシンポジウムに呼び，対話を行って来た．今回の国際会議においても市民を対象としてセッションを市民公開講座として設けた．

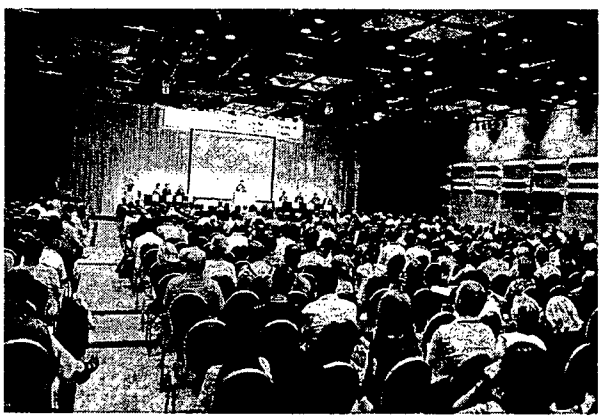
3. 会議の内容

今回の会議では「動物実験代替法開発の促進，3Rs [Reduction (動物実験の削減)，Refinment (動物の苦痛軽減)，Replacemnet (動物を用いない方法への置き換え)]のグローバリゼーション並びに科学者と動物福祉活動者との対話」をメインテーマに，プレナリーレクチャーや特別講演(表3)，また，表4に示したように，3Rsの原則に関係する多岐にわたる分野についてシンポジウムが開催された．即ち，動物福祉(Theme 1)，動物使用の道徳，倫理および文化(Theme 2)，3Rs教育(Theme 3)，知識管理と情報サービス(Theme 4)，トキシコロジーとバリデーション(Theme 5)，環境毒性(Theme 6)，バイオロジクスの開発・生産・品質管理における3Rs (Theme 7)，新しい科学と技術の3Rsへの応用(Theme 8)，3Rsのグローバリゼーション(Theme 9)，リスクアセスメントと規制(Theme 10)の分野で最新の情報交換が行われた．これらとは別に，メインテーマに掲げた「科学者と動物福祉活動者との対話」のための特別シンポジウムを実施した．レクチャーの総数は10，シンポジウムの総数は47であった．多数のシンポジウムを通して，多岐な分野に研鑽を積むことがで

きた．また，総数256のポスター発表や，若手研究者の一般演題 20 など多数の発表を見聞きすることができた．講演会場は8会場に分かれていたが，1階のメイン会場以外はすべて3階に集中しており，参加者は興味あるシンポジウムを渡り歩き，時間を有効に使うことができた．また，会場のあちらこちらで参加者が熱い討論を繰り返していた．現在，会議の成果は吉村出版委員長を中心に，プロシーディングとしてまとめられている．

会議初日のウェルカムパーティでは動物慰霊祭，会議3日目の夕刻の都内観光，4 日目夕刻の晩餐会，その他，同伴者のためのエクスカーションなど，学術面以外のイベントについても，数多くのボランティアの方々のご協力により，楽しんでいただいた．

なお，会議では外国からの多数のシンポジストに旅費の補助を行うとともに，国内外の若手研究者（約 70 名）に渡航補助を行うとともに，彼らの中から優れた演題に賞を送った．科学委員の投票により 11 名の受賞者が選ばれ，若手研究者にとっては励みになったと思われる．



開会式の会場風景

若手研究者の授賞式の一場面

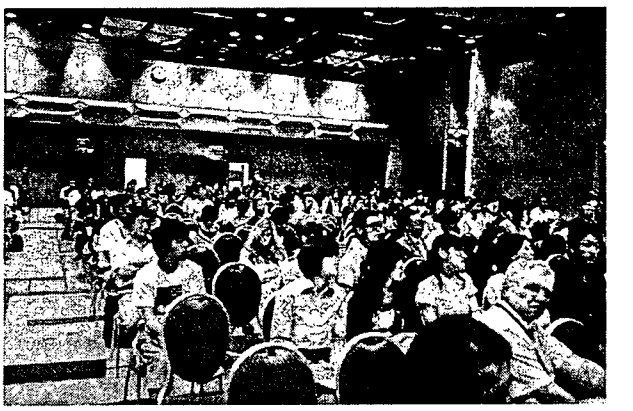
4. 会議の成果

会議では世界の方々が動物実験代替法に関する最先端の研究成果を発表するとともに，倫理的な動物実験についての発表があった．国内はもとより，海外の参加者からも会議の質が高く，満足したとの声が多く聞かれたのは，大変嬉しかった．我が国の動物実験代替法に向けた熱意を感じ取って頂け，国際社会の中で日本の存在感を示すことができたと感じている．また，我が国のこの分野の科学者が世界の多くの科学者と直接交流することができ，今後の我が国における動物福祉と動物実験代替法開発に関する研究を更に発展させる契機となったと思われる．このような成果は，やむを得ず行う動物実験を用いた医薬品の有効性や安全性の評価，また，生産等に対する社会の同意を得ることに資するものと期待される．

動物福祉や動物実験代替法という分野は日本においてはマイナーであるが，欧米においてはきわめて大きな課題となっている．OECD における安全性試験法ガイドラインの作成においても，動物福祉活動団体が参加するようになっている．今回の会議を日本が主催し，成功させることができたことは今後の我が国の研究者の国際的な活動に資するものと思われる．

5. 市民公開講座

会議最終日の8月25日(土)午後2時から5時半まで，ホテルイースト21東京の1階ホールで，「実験動物のためにできること一研究者の立場から一」というテーマで市民公開講座を開催した．これには，総数214名(会議に参加していない一般市民が約8割を占めた)の方が参加してくれた．これには動物福祉団体のご協力が大きかったと考えま



市民公開講座の会場風景

ま．そこでは，実験動物や動物実験の意義や役割について研究者の意見を聞いて頂くとともに，参加者からの意見や質問に多数答えることができた．質疑応答は予定の時間を大幅に上回り，参加者に満足して頂ける内容となったと考えている．メインテーマの一つである科学者と動物福祉活動者との対話を実現する意義深い内容であったと考える．

6. 会計

先に述べたように日本動物実験代替法学会は会議を招請するにあたり，多額の準備金を用意した．また，ACT は本会議の計画と運営について全面的な協力を行うとともに，極東地域以外の参加者への支援等のため 15 万ドルの支援してくれた．本会議の企画に際しては，会場費や招待者の旅費，警備費等に多くの費用がかかり，収支が懸念された．国内組織委員会では赤字が出たときには，幹事が連帯して責任を負うとの誓約書を作成し，不退転の決意で準備をすすめた．しかし，我が国内外の多くの機関から多額の寄付をいただいたこと，主催者である日本学術会議から多額の補助金を得たこと，また，予想以上の参加者を得たことから，どうやら赤字を出さずにすんだ．集計結果については，公認会計士の監査を受けた上で，別途報告する予定である．

7. サテライトシンポジウム

本会議とは別に，会議の前に北京とソウルで，会議の後では京都でサテライトシンポジウムが開催された．それぞれは独立採算で，北京は National Institute for the Control of Pharmaceutical and Biological Products (NICPBP)の Xing Ruichang 実験動物センター長の主催で「Welfare and Alternatives in Animal Experiments」について，ソウルはソウル大学獣医学部教授で韓国動物実験代替法学会長である Jae-Hak Park ソウル大学獣医学部教授の主催で「New Era of Korean Alternative Research for World Harmonization」について，京都は大阪歯科大学の今井弘一博士の主催で「New Bioscience for 3Rs Research」について，開催された．北京とソウルは約 100 名，京都は約 50 名の参加者を集め，会議が催された．

8. 次回会議への動き

次回会議は，2009 年に ECVAM の Thomas Hartung 所長および European Commision の Herman Koetter 博士が会長となり，イタリアのローマにおいて開催される．今回のメインテーマの一つである「動物実験代替法開発の促進，3Rs のグローバリゼーション」は次回会議に引き継がれ，議論が深まると思われる．

9. 最後に

私にとって初めての国際会議の主催であり，多くの不手際があったにも関わらず，会議の準備と当日の運営，その後のフォローアップに多大な時間と労力をかけてご協力いただいたすべての方々に感謝します．特に，国内組織委員会の幹事の方々には大変お世話になりました．厚くお礼申し上げます．また，皇族をお呼びすることにより，日本における動物福祉への熱意を世界に伝えたいと考えましたが，いろいろな行き違いがあり，実らせることができなかったことが残念でした．なお，日本学術会議におかれては会議の結果に満足していただき，更に，フォローアップシンポジウムを開催しないかとの提案を受けた．そこで，よりよい動物実験を目指すとともに，市民との交流を深めるため，現在，2 月 23 日の朝 10 時より午後 5 時半頃まで，六本木ヒルズ(仮押さえ)で「3Rs に**基づく動物実験の規制と第三者認証**」のタイトルでシンポジウムを開催する予定です．厚生労働省傘下のヒューマンサイエンス財団は動物実験の第三者認証機関としての準備を進めており，それについての情報も得られることになっています．参加費は無料です．多数の参加者をお待ちしておりますので，希望者は代替法学会のホームページを見て，お申し込みください．

ORIGINAL ARTICLE

Validation of human skin models for skin corrosivity tests in Japan

Hajime Kojima[1,6], Tomoko Ando[2], Katsuhiro Inagaki[3], Mahito Ohhira[4], Tadashi Kosaka[5], Yosuke Nakamura[7], Hisashi Torishima[8], Noriyuki Morikawa[9], Jun Kanno[2], Mami Kuboki[4], Michiru Genno[8], Masaru Nokata[3], Takanori Harada[5], Takashi Morimoto[7], Isao Yoshimura[10] and Yasuo Ohno[11]

[1]Japanese Center for the Validation of Alternative Methods (JaCVAM), National Institute of Health Sciences (NIHS), Japan,
[2]Div. Cellular and Molecular Toxicol. NIHS, Japan,
[3]Res. & Develop., Div., Product Safety & Pharmaceutical Research Unit, Nihon Nohyaku Co., Ltd., Japan,
[4]Toxicol. Res. Dep.,ODAWARA Res. Center, Nippon Soda Co.,Ltd., Japan,
[5]Toxicol. Div. The Inst. Environ. Toxicol., Japan,
[6]Res.Lab., Nippon Menard Cosmetic Co., Ltd., Japan,
[7]Environ. Health Sci. Lab., Sumitomo Chemical Co., Ltd., Japan,
[8]Bio-Medical Dep., Kurabo Industries Ltd., Japan,
[9]Div. R&D, Gunze Ltd., Japan, [10]Fac. Eng. Tokyo Univ.Science, Japan, [11]NIHS Japan

Abstract
As shown in OECD test guidelines 430 and 431, the human skin epidermal assay and Transcutaneous Electrical Resistance Test (TER) were validated and peer reviewed as an alternative method to corrosivity testing; however, these methods have not been used widely in Japan. The problems related to techniques and evaluation are not clear. Therefore, we performed a validation study of EPI-200 (EpiDerm™), a 3-dimensional cultured epidermal model and Vitrolife-Skin™, a 3-dimensional cultured skin model made in Japan as a catch-up validation trial of alternatives for skin corrosivity testing using 13 chemicals including a positive control: 10% potassium hydroxide solution in Japan. From the obtained data, we identified the potential of utilizing these models to evaluate the corrosivity of a chemical.

Key words: Skin corrosivity, cultured epidermal model, cultured skin model, validation

Introduction

Over the last decade, the European Centre for the Validation of Alternative Methods (ECVAM) has supported formal validation studies using *in vitro* tests as a replacement for the *in vivo* rabbit test for predicting skin corrosivity (Botham, et al.,1995, Barratt, et al., 1998, Fentem et al., 1998, Liebsch et al., 2000). As a result, two new test methods for skin corrosion, which incorporates a rat skin transcutaneous electrical resistance assay (TER) and two human skin epidermal assays, were included in Annex V of Directive 67/548/EEC in mid-2000, thereby making the use of *in vitro* alternatives for skin corrosivity testing of chemicals mandatory in the European Union (EC, 2000). As human epidermal model assays, two methods based on commercial human epidermal models, EPISKIN™ (EPISKIN, Chaponost, France) and

EpiDerm™ (MatTek, Ashford, MA, USA), were also endorsed.

Meanwhile, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) in the USA prepared final recommendations on these methods for their consideration and acceptance where appropriate (NIH Publication No.02-4502; ICCVAM, 2002). As a result, these assays were published as an alternative method to corrosivity testing as shown in the OECD test guidelines 430(OECD 430; 2004), and 431(OECD 431; 2004).

In Japan, these methods have not been widely used. The problems related to techniques and evaluation are not clear. In the present study, therefore we performed a catch-up validation trial to evaluate skin corrosivity using the human epidermal and skin models, that is, evaluations were made based on the ECVAM experimental protocol.

We performed a validation study of EPI-200 (EpiDerm™), a 3-dimensional cultured epidermal model and Vitrolife-Skin™, a 3-dimensional cultured skin model as validation trials of alternative for skin corrosivity testing in Japan. From the obtained data, we investigated the possibility of utilizing these models to evaluate the corrosivity of a chemical. We may suggest using these models to the ad hoc. committee of toxicology at MHLW in Japan.

Materials and Methods

Study management and organization

The study was performed according to the Japanese Society for the Alternative to Animal Testing Experiments (JSAAE) validation scheme as shown in Fig.1. The chairman was Dr. I Yoshimura at the Fac. Eng. Tokyo Univ. Science, who is head of the validation committee in JSAAE. Dr. Ohno at the National Institute of Health Sciences (NIHS) prepared the protocol and supported this validation with a grant from MHLW. Six Laboratories joined the study as shown in Table 1, and a blind trial with 13 chemicals including a positive control (10% potassium hydroxide solution) was performed using the protocol. In addition, Dr. Y. Ohno, the chemical distributor, coded and distributed the test chemicals to be used in the blind trial. After submission of all coded data to biostaticians, an independent biostatistical analysis of the blind trial was performed at the Fac. Med. Kyoto Univ. and Fac. Eng. Tokyo Univ. Science. The study director at each laboratory, a chemical distributor, biostaticians and kit suppliers were organized into study management teams in this validation assay as shown in Fig. 1. Finally, the chairman reported the outcome of this validation and forwarded this report to JSAAE.

Technical transfer and preliminary tests

The management team performed the technical transfer by kit suppliers at NIHS, Tokyo on January 28, 2004. After that, technicians performed the preliminary test using 10% potassium hydroxide solution and benzalkonium chloride 10 % solution. A qualified technician from each laboratory participated in the technical transfer and the preliminary

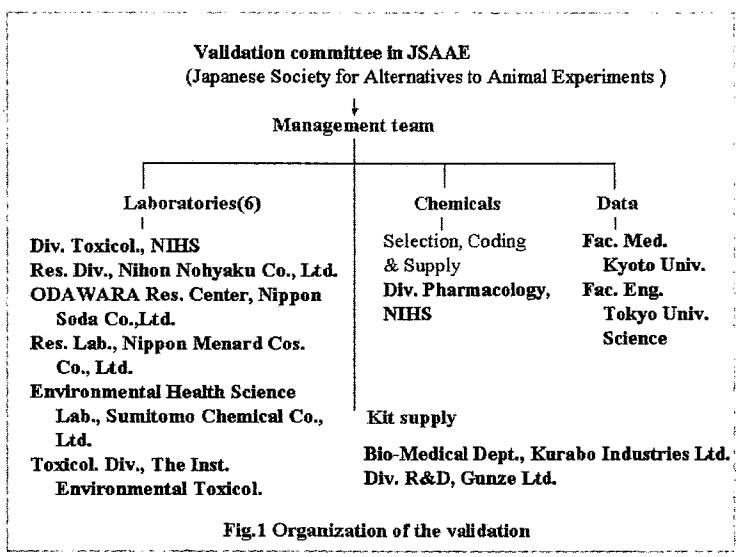
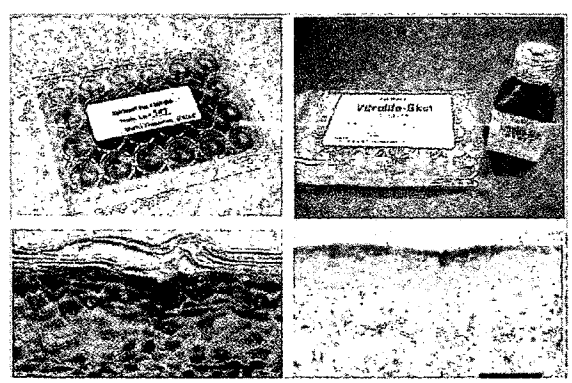


Fig.1 Organization of the validation



400μm 100μm

Fig.2 EpiDerm™ Fig.3 Vitrolife-Skin™

test. All technicians obtained good results in this test.

Cultured epidermal and skin models

EpiDerm™ (EPI-200) models were purchased from KURABO Corporation (Osaka, Japan) as kits containing 24 models as shown in Fig.2, with sufficient amounts of Dulbecco's modified Eagle's medium (DMEM)-based assay medium, and phosphate-buffered saline (PBS) solution. These kits are made by MatTek Corporation (Ashland, MA, USA). The human epidermal model consisting of an epidermis with cornified layers was prepared as previously described (Liebsch et al., 2000).

Vitrolife-Skin™ models were supplied from Gunze Corporation Ltd. (Kyoto, Japan) as kits containing 24 models, collagen sponges without cells and sufficient amounts of DMEM-based assay medium, as shown in Fig.3. The human skin model consisting of a dermis and epidermis with cornified layers was prepared as previously described (Morikawa et al., 2002; Morota et al., 1998; Morota et al., 1999).

Materials

A total of 13 test chemicals including a positive control (10 % potassium hydroxide solution) were selected from the chemicals tested in the ECVAM skin corrosive validation study (Fentem et al., 1998, Liebsch et al., 2000). The chemical distributors selected test chemicals considering a balanced representation of the chemical classes, rate of corrosion or non-corrosion, solubility etc. from the total 60 chemicals tested in the ECVAM validation study. Test chemicals included six of which are known to be corrosive *in vivo*, six which are non-corrosive, six liquids, four solids and two powders, excluding the positive control. Each laboratory was sent the rotated 11 chemicals, including the positive control, in 13 test chemicals as shown in Table 2. Therefore, five data items from each laboratory for each chemical were obtained. All blinded test chemicals were treated as powerful drugs or poisons in each laboratory. The management team considered the minimum appropriate number of chemicals for catch up validation.

All test chemicals used were from the same batch and were purchased from Sigma Aldrich(Milwaukee, USA) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and were supplied to each laboratory by the chemical distributors. Phosphate-buffered saline (PBS) and isopropanol were obtained from Wako Pure Chemical Industries,

Table 1 List of members in skin corrosivity validation assay

Japanese Society for Alternative to Animal Experiments Validation Executive Committee

	Organization	Name
Chairman	Tokyo University of Science, Faculty of Engineering, Dept. Management Science	Isao Yoshimura
	National Institute of Health Science, Biological Safety Research Center, Division of Pharmacology	Yasuo Ohno

Study Director

	Organization	Name
	National Institute of Health Sciences, Biological Safety Research Center, Division of Toxicology	Tomoko Ando
	Nihon Nohyaku Co., Ltd., Research Division, Toxicological & Pharmaceutical Research Center	Katsuhiro Inagaki
	Nippon Soda Co., Ltd., Odawara Research Center, Toxicological Research Department	Mami Kuboki
	Nippon Menard Cosmetic Co., Ltd., Research Laboratories	Hajime Kojima
	Sumitomo Chemical Co., Ltd., Environmental Health Science Laboratory, Biochemistry Group	Yosuke Nakamura
	The Institute of Environmental Toxicology, Toxicology Division II, Laboratory of Immunotoxicology	Tadashi Kosaka

Kit supplier

	Organization	Name
	Kurabo Industries Ltd., Bio-medical Department	Hisashi Torishima
	Kurabo Industries Ltd., Biomedical Department	Michiru Genno
	Gunze Limited, Division of Research & Development	Noriyuki Morikawa

Coordinator

	Organization	Name
	Sumitomo Chemical Co., Ltd., Environmental Health Science Laboratory, Biochemistry Group	Naohiko Isobe
	Nippon Soda Co., Ltd., Agro Product Division, Regulatory Affairs Group	Yukihiro Kanaguchi
	National Institute of Health Sciences, Biological Safety Research Center, Division of Toxicology	Jun Kanno
	The Institute of Environmental Toxicology, Toxicology Division II	Takanori Harada
	Nihon Nohyaku Co., Ltd., Research Division, Toxicological & Pharmaceutical Research Center	Masaru Nogata
	Nippon Soda Co., Ltd., Agro Product Division, Regulatory Affairs Group	Mitsuo Hattori
	Nippon Soda Co., Ltd., Odawara Research Center, Toxicological Research Department	Yoshinobu Fujii
	The Institute of Environmental Toxicology, Toxicology Division II, Laboratory of Neurotoxicology	Sayaka Ishimine
	Sumitomo Chemical Co., Ltd., Environmental Health Science Laboratory, Biochemistry Group	Takashi Morimoto

Ltd. and 3-(4,5-dimethylthiazol-2-yl)- 2,5- diphenyltetrazolium bromide (MTT) and MTT formazan were obtained from Sigma Aldrich. They were supplied by the management team.

Methods

Chemical application procedure according to the ECVAM validation study.

The experimental steps of the method were performed according to the protocol used in phase III of the EpiDerm™ skin corrosivity test (Liebsch et al., 2000) with slight modifications. The EpiDerm™ models were equilibrated at 37°C and 5% CO_2 within one hour after receiving a kit and placed in 1 mL of DMEM-based assay medium in 6-well plates before use. If kept for a few days, it was preserved in a refrigerator. The Vitrolife-Skin™ models were placed in 250 µL of DMEM-based assay medium in 24-well plates and equilibrated for several hours' incubation (37°C, 5% CO_2) within a few days after receiving a kit. One hour before dosing, the models were transferred in 1 mL of DMEM-based assay medium to 6-well plates. Test chemicals were applied directly to the stratum corneum of two replicate models per chemical. Liquids (50 µL) were applied using a positive displacement pipette. Solids were crushed to a powder, if necessary, and 25 mg was applied using a spatula with the addition of 25µL of distilled water to ensure good contact with the surface. Two models were dosed with 100 µL distilled water as a negative control. After exposure for three or 60 min. at room temperature (15-25°C), two replicate models for each exposure time were rinsed thoroughly with PBS to remove the test chemical from the surface.

Table 2 Test chemicals

No.	Name	C/NC	Comments
1	Potassium hydroxide(10%aq)	C	Positive control
2	Sulfuric acid(10% wt)	C	
3	Octanoic (Caprylic) acid	C	
4	Sodium hydroxide(4.88%)	C	
5	Phenol	C	
6	Chromium trioxide	C	
7	Phosphoric acid	C	
8	Sodium perborate	NC	
9	Tetrachloroethylene	NC	
10	Potassium hydroxide(5% aq)	NC	
11	4-Amino-1,2,4-triazole	NC	
12	L-Lactic acid	NC	
13	Isopropanol (2-propanol)	NC	

Calculation of cell viability

The effects of the test chemicals on cell viability were determined using an MTT reduction assay. After blotting, the models were incubated in 0.3 mL (EpiDerm™) or 1 mL (Vitrolife-Skin™) of each DMEM-based assay medium containing 0.5 mg of MTT for an additional three hours at 37°C and 5% CO_2. Living cells were dyed dark-violet by the MTT reagents. After the models were washed with PBS, biopsies of Vitrolife-Skin™ models were taken using a biopsy punch (6 mm diameter), although this operation is not used in EpiDerm™ models. The biopsies were separated from the models using forceps, and placed into acidified isopropanol (2.0mL: EpiDerm™,1.0 mL: Vitrolife-Skin™), after removing excess water by placing the samples on absorbent paper. Precipitated formazan was extracted overnight at room temperature with protection from light. The absorbance of the extracts was measured at 570 nm using a UV-VIS spectrophotometer. Adequate absorbance of spectrophotometers was checked using 0.1mg/mL solution of MTT formazan prior to the validation study. Cell viability of EpiDerm™ models determined by the MTT reduction assay method was expressed as follows:

$$\text{Cell viability} = \frac{A_t}{A_c} \times 100\,(\%), \qquad (1)$$

where *At* and *Ac* are the absorbancies of the extracts when test chemicals and a negative control, respectively, are applied to the cultured skin model.

In case of Vitrolife-Skin™, additional tests using collagen sponges without cells were performed, with the potential to interfere with the MTT assay, and thus cell viability was expressed as follows:

$$\text{Cell viability} = \left(\frac{A_t - A_{bt}}{A_c - A_{bc}} \right) \times 100\,(\%), \qquad (2)$$

where *At* and *Ac* are absorbancies of the extracts

when test chemicals and a negative control, respectively, are applied to the viable Vitrolife-Skin™ model, and A_{bt} and A_{bc} are the values obtained for a blank test using a test chemical and the negative control, respectively, with a collagen sponge without cells.

Prediction models

Predictions of *in vitro* corrosiveness/non- corrosiveness were made according to the refined final prediction model (PM2) used in phase III of the EpiDerm™ skin corrosivity test (Liebsch *et al.*, 2000). Hence, chemicals that reduced cell viability to less than 50% upon exposure to the Vitrolife-Skin™ model for three min. were predicted to be 'corrosive' *in vivo*. If 3 min. exposure produced cell viability of ≥ 50%, the chemical was classified as 'non-corrosive' after a 3 min. exposure, but the same chemical was still be classified as 'corrosive' if viability after a 60 min. exposure was below 15%. The results obtained using the EpiDerm™ and Vitrolife-Skin™ models in this study were compared with the results of ECVAM validation studies using EpiDerm™ (Liebsch *et al.*, 2000) and EPISKIN ™ (Fentem *et al.*, 1998) for skin corrosivity testing.

This test was repeated twice. If different results from the two tests were obtained, a third test was performed at each laboratory and used for final judgment.

Table 3 Data from each laboratory

Chemical / Lab.	Potassium hydroxide (10%). Corrosive						Sulfuric acid (10%). Corrosive					Tetrachloroethylene. Non-Corro				
	NIHS	NN	NS	NM	SC	IET	NIHS	NN	NS	NM	SC	NIHS	NN	NS	NM	IET
Blind No.	1	2	3	4	5	6	13	14	15	16	17	18	19	20	21	22
EpiDerm -test 1-	C	C	C	C	C	C	NC	NC	NC	C	C	NC	NC	NC	NC	NC
EpiDerm -test 2-	C	C	C	C	C	C	NC	C	C	C	C	NC	NC	NC	NC	NC
EpiDerm re-trial	C	C	C	C	C			C	C		C					
Judge	C	C	C	C	C	C	NC	C	C	C	C	NC	NC	NC	NC	NC
Vitrolife-Skin -	C	C	C	C	C	C	C	C	C	C	C	NC	NC	NC	NC	NC
Vitrolife-Skin -	C	C	C	C	C	C	C	C	NC	C	C	NC	NC	NC	NC	NC
Vitrolife-Skin re-	C	C	C						C							
Judge	C	C	C	C	C	C	C	C	C	C	C	NC	NC	NC	NC	NC

Chemical / Lab.	Octanoic acid. Corrosive					Potassium hydroxide(5%). Non-					Sodium hydroxide(4.88%) Corrosive				
	NIHS	NN	NS	NM	IET	NIHS	NN	NS	SC	IET	NIHS	NN	NS	SC	IET
Blind No.	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
EpiDerm -test 1-	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
EpiDerm -test 2-	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
EpiDerm re-trial		C	C				C	C							
Judge	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
Vitrolife-Skin -	C	C	NC	C	C	C	C	C	C	C	C	NC	C	C	C
Vitrolife-Skin -	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
Vitrolife-Skin re-			C				C	C				C			
Judge	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C

Chemical / Lab.	4-Amino-1.2.4-triazole. Non-Corro.					Phosphoric acid. Corrosive					L-Lactic acid. Non-Corro.				
	NIHS	NN	NM	SC	IET	NN	NS	NM	SC	IET	NIHS	NS	NM	SC	IET
Blind No.	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52
EpiDerm -test 1-	NC	NC	NC	NC	NC	C	C	C	C	C	NC	C	C	NC	C
EpiDerm -test 2-	NC	NC	NC	NC	NC	C	C	C	C	C	C	C	C	C	C
EpiDerm re-trial											C	C	C	C	
Judge	NC	NC	NC	NC	NC	C	C	C	C	C	C	C	C	C	C
Vitrolife-Skin -	NC	NC	NC	NC	NC	C	C	C	C	C	C	C	C	C	C
Vitrolife-Skin -	NC	NC	NC	NC	NC	C	C	C	C	C	C	C	C	C	C
Vitrolife-Skin re-											C	C	C		
Judge	NC	NC	NC	NC	NC	C	C	C	C	C	C	C	C	C	C

Chemical / Lab.	Isopropanol. Non-Corro.					Phenol. Corrosive					Sodium perborate. Non-Corro.					Chromium trioxide. Corrosive				
	NN	NS	NM	SC	IET	NIHS	NN	NM	SC	IET	NIHS	NN	NS	NM	SC	NIHS	NS	NM	SC	IET
Blind No.	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72
EpiDerm -test 1-	NC	NC	NC	NC	NC	C	C	C	C	C	NC	NC	NC	NC	NC	C	C	C	C	C
EpiDerm -test 2-	NC	NC	NC	NC	NC	C	C	C	C	C	NC	NC	NC	NC	NC	C	C	C	C	C
EpiDerm re-trial							C													
Judge	NC	NC	NC	NC	NC	C	C	C	C	C	NC	NC	NC	NC	NC	C	C	C	C	C
Vitrolife-Skin -	NC	NC	NC	NC	NC	C	C	C	C	C	NC	NC	NC	NC	NC	C	C	C	C	C
Vitrolife-Skin -	NC	NC	NC	NC	NC	C	C	C	C	C	NC	NC	NC	NC	NC	C	C	C	C	C
Vitrolife-Skin re-	NC											NC								
Judge	NC	NC	NC	NC	NC	C	C	C	C	C	NC	NC	NC	NC	NC	C	C	C	C	C

Laboratory:
NIHS: Div. Toxicol., NIHS
NS: ODAWARA Res. Center, Nippon Soda Co., Ltd.
SC: Environmental Health Science Lab., Sumitomo Chemical Co., Ltd.
NN: Res. Div., Nihon Nohyaku Co., Ltd.
NM: Res. Lab., Nippon Menard Cosmetic Co., Ltd.
IET: Toxicol. Div., The Inst.Environmental Toxicol.

C: Corrosive, NC: Non-Corrosive

Results

This validation study was not performed under GLP. However, all data obtained in each laboratory followed GLP compliance and spirit. Their records (data and detailed documents) could be checked after the assays, and raw data was sent to Tokyo Univ. of Science for analysis by biostatisticians. All documents were checked by the chairperson, biostatisticians and chemical distributors, and are stored in the NIHS.

Predictivity

Using cell viability after exposure to test chemicals for three or 60 min., the chemical classifications according to the EpiDerm™ prediction model are shown in Table 3. Data for positive controls in the two models were evaluated correctly at all laboratories. The EpiDerm™ data summarized in Table 4 excluded the positive control data. Of 30 classifications of six chemicals in the corrosive class, 29 classifications of EpiDerm™ were correctly predicted to be corrosive, and sensitivity was 96.7%. All six chemicals in the corrosive class were correctly predicted excluding one laboratory. Lab.1 gave a negative classification of sulfuric acid from two data sets, but this chemical is corrosive. Cell viability values after expose to sulfuric acid for 60 min. were 18.54% and 38.80%, and these values

Table 4 Contigency table for EpiDerm™ predictions

Vitro / *Vivo*	Corrosive	Non-Corrosive
Corrosive	29	1
Non-Corrosive	10 (5% KOH, Lactic Acid)	20

Table 5 Contingency table for Vitrolife-Skin™ predictions

Vitro / *Vivo*	Corrosive	Non-Corrosive
Corrosive	30	0
Non-Corrosive	10 (5% KOH, Lactic Acid)	20

Table 6 Key statistical parameters for the four tests

	EpiDerm™	Vitrolife-Skin™	EpiDerm™ (ECVAM)	EPISKIN™ (ECVAM)
No. of Chemicals	12	12	24	60
Sensitivity	100% (12/12)	100% (12/12)	92%	82%
Specificity	66.7% (4/6)	66.7% (4/6)	83%	84%
Accuracy	83.3% (10/12)	83.3% (10/12)	92%	83%
False positive rate	16.7% (2/12)	16.7% (2/12)	17%	19%
False negative rate	0% (0/12)	0% (0/12)	8%	14%

were slightly high compared to 15%, which is the border line. On the other hand, of 30 classification of six chemicals in the non-corrosive class, 20 classifications of EpiDerm™ were correctly predicted to be non-corrosive, and specificity was 66.7%, but two were false positives. There were 5% potassium hydroxide and lactic acid. All the laboratories gave them positive classifications from two data sets, which is a non-corrosive chemical. Positive predictivity was 74.4% (29 true corrosive classifications / 39 corrosive classifications in this assay). Negative predictivity was 95.2% (20 true non-corrosive classifications / 21 non-corrosive classifications in this assay). The total consistency rate was 81.7% (49 true classifications / 60 classifications in this assay).

The Vitrolife-Skin™ data are summarized in Table 5, excluding the positive control data. Of 30 classifications of six chemicals in the corrosive class, 30 of Vitrolife-Skin™ were correctly predicted to be corrosive, and sensitivity was 100%. All six chemicals in the corrosive class were correctly predicted.

On the other hand, of 30 classification of six chemicals in the non-corrosive class, 20 of Vitrolife-Skin™ were correctly predicted to be non-corrosive, and specificity was 66.7%, but two were false positives. They were 5% potassium hydroxide and lactic acid, which all laboratories gave a positive classification from two data sets. This chemical is non-corrosive. Positive predictivity was 75% (30 true corrosive classifications / 40 corrosive classifications in this assay). Negative predictivity was 100% (20 true non-corrosive classifications / 20 non-corrosive classifications in this assay). The total consistency rate was 83.8% (50 true classifications / 60 classifications in this assay).

Predictability of these two models was similar to the results obtained by the ECVAM validation study.

Intralaboratory variation

Most chemicals did not show any great differences in scores on tests repeated at each laboratory. Different classifications of EpiDerm™ accounted for 6.66% (4/60). These data are not shown in the Tables. Cell viabilities of sulfuric acid after exposure for 60 min. in Lab. 2 were 17.26%, 9.46% and 12.02%, and those in Lab.3 were 15.72%, 10.58% and 9.01%, respectively. On the other hand, cell viabilities of lactic acid after exposure for 60 min. in Lab. 1 were 16.55%, 13.39% and 7.19%, while those in Lab.5 were 15.85%, 12.01% and 15.89%, respectively. These cell viabilities were around 15% after exposure for 60 min. (the success criteria).

Different classifications of Vitrolife-Skin™ accounted for 5.0% (3/60). Cell viabilities of sulfuric acid after exposure for 60 min. in Lab. 3 were 5.90%, 16.09% and 6.34%, while after exposure to octanoic acid for 60 min in Lab.3 were 21.37%, 11.77% and 10.71%. These cell viabilities were around 15% after exposure for 60 min (the success criteria). Meanwhile, cell viabilities of sodium hydroxide (4.88%) after exposure for 3 min. in Lab. 2 were also 55.12%, 15.41% and 17.51%. These cell viabilities were around 50% after exposure for 3 min. (the success criteria).

These cell viabilities were in an extremely narrow range despite the different classifications. Therefore, intralaboratory variation between the two models is presumed to be small.

Interlaboratory variation

In EpiDerm™, inter-laboratory variation was significant for only sulfuric acid. The classification of sulfuric acid in Lab. 1 was different from the data in the other four laboratories. In the data of Lab.1, not shown in the Tables, cell viabilities after exposure for 60 min. were 18.54% and 38.80%, and these values were almost the same as the positive classification. For Vitrolife-Skin™, inter- laboratory variation was not significant. From these results, the feasibility of using EpiDerm™ and Vitrolife-Skin™ was suggested by the experiment.

Discussion

From the obtained data, we confirmed the potential of using EpiDerm™ and Vitrolife-Skin™ as methods to evaluate the corrosivity of a chemical. We consider the data form these models has high predictivity, and low intra- and inter-laboratory variation.

With Vitrolife-Skin™, however, it is necessary to use limited blank data using collagen sponges without cells.

Modified points of Vitrolife-Skin™ from the ECVAM skin corrosivity validation study

Application volume

Although the surface of the Vitrolife-skin™ model (0.5 cm^2) is similar to that of EpiDerm™ (0.63cm^2), 50 μL of Liquid chemical was often insufficient for the surface. In this study, therefore,

the application volume of liquids was increased from 50 μL, the volume used in the phase III protocol in the EpiDerm™ skin corrosivity test, to 100 μL. For the same reason, 50 mg of solid chemical was applied and 50 μL of water was added to ensure good contact with the surface (in contrast to the Phase III protocol, in which 25mg of solid and an additional 25 μL of water were applied. Additional tests using collagen sponges without cells, the Vitrolife-Skin™ model uses a collagen sponge without cells to construct the dermal layer, and this allows test chemicals to be easily absorbed and bound, compared with epidermal models consisting of only an epidermal layer and supporting material. In a previous study, tests using collagen sponges without cells, instead of non-viable Vitrolife-Skin™ models, were performed for several test chemicals with the potential to interfere with the MTT assay (Mirokawa, 2006). For 3- methoxypropylamine and n-heptylamine, these experiments suggested about 50-60% and 80% "viability", respectively, due to a chemical reaction with the MTT medium. Hence, the 70-80% viability obtained for 3-methoxypropylamine with the Vitrolife-Skin™ model should be corrected to about 20%. In the same way, the 120% viability obtained for n-heptylamine should be decreased to about 40%. Therefore, these two chemicals, which were incorrectly classified as negatives by testing without using blank collagen sponges, should correctly be classified as corrosive by adding blank collagen sponges, in agreement with the results from the EpiDerm™ model. The additional test for the other six chemicals gave results of around 15% "viability", such that the Vitrolife-Skin™ *in vitro* prediction of corrosivity was not changed.

Therefore, we obtained blank data using collagen sponges without cells in the validation of Vitrolife-Skin™. In this validation study, we detected solibilization, swelling and color change after exposure to chemicals, and the need to use blank collagen sponges without cells.

Comparison of skin models

As shown in Table 6, there was no difference in sensitivity, specificity, accuracy, false positive rate or false negative rate between EpiDerm™ and Vitrolife-Skin™ in this validation study. The result in this validation study may be due to no difference in structure between a two-layer skin model consisting of a dermis and epidermis (Vitrolife-Skin™) and epidermal models (EpiDerm™). The barrier function of cornified layers of the cultured epidermal and skin model is less effective compared with human skin tissue (Kojima *et al.*, 2000). In addition, as chemical exposure times become longer, stronger cytotoxicity occurs due to the accumulation of chemicals which permeate the cornified layer of the skin model. However, it is considered the barrier function of these model is similar.

The sensitivity was 92% in phase III of the EpiDerm™ study and 82% in EPISKIN™ study, and the present values (100%) were higher than data of these previous validation assays. The specificity, however, was 83% in phase III of the EpiDerm™ study and 84% in the EPISKIN™ study, and the present ones (66.7%) were lower than those. We consider these accuracy and false positive rates to be no different between the present validation and previous validation study. On the other hand, none of the false negative rates in present validation study were lower than data from previous validation studys. This issue must be handled carefully, because this assay is a catch-up validation trial, and the number of chemicals and classes is small.

Though peer review of these models is in progress, the ad hoc. committee of toxicology at MHLW in Japan has approved the utilization of these models to evaluate the corrosivity of a chemical.

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