第２表　製造／輸入量に応じた環境影響評価[3改変]

番号	試験項目	製造／輸入量（t／年）			
		1 － 10	10 － 100	100 － 1000	1000 以上
9.1	水生毒性				
9.1.1	無脊椎動物（ミジンコ）に対する急性毒性	○	○	○	○
9.1.2	水生生物（藻類）生長阻害試験	○	○	○	○
9.1.3	魚類急性毒性試験		○	○	○
9.1.4	活性汚泥呼吸阻害試験		○	○	○
9.1.5	無脊椎動物（ミジンコ）に対する長期毒性			○	○
9.1.6	魚類長期毒性試験（どれか一つ）				
9.1.6.1	魚類初期生活段階毒性試験			○	○
9.1.6.2	魚類の胚・仔魚類における短期毒性試験			○	○
9.1.6.3	魚類稚魚成長毒性試験			○	○
9.2	分解				
9.2.1	生物的				
9.2.1.1	生物的分解性 - 易分解性	○	○	○	○
9.2.1.2	表層中での究極分解シミュレーション試験			○	○
9.2.1.3	土壌シミュレーション試験			○	○
9.2.1.4	底質シミュレーション試験			○	○
9.2.2	非生物的				
9.2.2.1	非生物的分解性－ pH に伴う加水分解		○	○	○
9.2.3	分解性生物の特定			○	○
9.3	環境中運命および挙動				
9.3.1	吸着／脱着スクリーニング試験		○	○	○
9.3.2	水生種（魚）生物濃縮度			○	○
9.3.3	吸着／脱着の追加試験			○	○
9.3.4	環境運命、分解生成物に関する挙動の追加試験				○
9.4	陸生生物への影響				
9.4.1	無脊椎動物への短期毒性試験			○	○
9.4.2	土壌微生物への影響			○	○
9.4.3	植物短期毒性試験			○	○
9.4.4	無脊椎動物への長期毒性試験				○
9.4.5	植物の長期毒性試験				○
9.5	底生生物への長期毒性試験				○
9.6	鳥類への長期毒性または生殖毒性試験				○

これによる影響分析で代替法で50％および定量構造活性相関（QSAR：Quantitative Structure-Activity Relationship）で20％の動物数の削減を専門家が分析している[5]。

ただし、新規試験法が開発され、行政的に受け入れられるためには10年がかかると言われており、これまで通りの方法では2011年には多くの試験法を用意できない。そこで、バリデーションの短期化、バリデーションが終了したものと同等のものを揃える、バリデーションを実施せずにREACHのために“適切な”方法の選択をする方策が検討されている。

以下にECVAMでREACHに対する代替法の利用についての目処がたった試験法について説明するとともに、日本の対応状況を追記する。

1）皮膚刺激性

2007年4月、欧州の代替法の認証機関であるESAC（ECVAM Scientific Advisory Committee）が培養表皮モデルEPISKINを認証した[6]。EPISKINに化学物質を15分間処理し、48時間後にMTT法による細胞毒性とインターロイキン1αを評価指標として測定するものである。これを受け、他の培養表皮モデルとしてSkin Ethicsの評価が始まろうとしている。

日本でもこれまで培養皮膚・表皮モデルの利用について手をこまねいていた訳ではない。東洋紡績株式会社製のTESTSKIN、グンゼ株式会社製のVitrolife-Skin、MatTek製でクラボウが販売しているEpiDermを用いて、プレバリデーションを実施し、良好な結果を得ている[7]。

さらに、化粧品原料の使用濃度における動物を用いる皮膚刺激性試験代替を目的に、TESTSKIN[8]およびVitrolife-Skinでバリデーションを実施した[9,10]。得られた結果とパッチテストのデータを比較した場合、動物を用いる皮膚刺激性試験の予測率と同程度であったことから、バリデーションとしてはある程度の成果を残したと考えている。ただし、まだ専門家による第三者評価（以後、第三者評価と記す）に至っておらず、日本の中でのコンセンサスは得られていない。来年、日本代替法バリデーションセンター（JaCVAM）における第三者評価を計画中である。

EPISKINのESACによる認証を受け、国内モデルにおいても補完バリデーションを行う希望があり、日本製のモデルがEPISKINと比較して化学物質の安全性評価に用いる試験法として認められるのか、日本動物実験代替法学会で検討される予定である。

2）眼刺激性試験

米国の代替法に関する評価を行う複数省庁の合同委員会（ICCVAM：Interagency Coordinating Committee on the Validation of Alternative Methods）では摘出眼球試験や摘出角膜試験の第三者評価が終了し[11]、強刺激性物質のスクリーニングとしての有用性が指摘されている。ESACでもこれを評価し、同様の結論を導いている[6]。さらにESACでウサギへの少容量法において第三者評価が行われており[6]、一方、ECVAMでバリデーションが実施された細胞毒性試験（ニュートラルレッド放出試験、赤血球試験、フルオレッセン放出試験、サイトセンサーマイクロフィジオメーター試験や受精鶏卵試験）の評価がESACで始まるとともに、EpiOcularやSkin Ethicsというヒト再構築モデルにおいてバリデーション計画が進みつつある。

日本では1998年に厚生労働科学研究補助金を得て作成された細胞毒性試験による眼刺激性試験代替法のガイダンスが中々普及していない[12]。もう一度、JaCVAMとして第三者評価を行い、細胞毒性試験における眼刺激性試験代替法としての有用性を検討する予定である。この資料には日本動物実験代替法学会で実施された細胞毒性試験[13]や、厚生労働科学研究の細胞毒性試験[14]を用いることになろう。

3）感作性試験

OECDガイドライン429として認証されているLocal Lymph Node Assay（LLNA）が代替法として利用されている[15]。LLNAに関しては一濃度のみで評価するreduced-LLNAがESACの認証を得るとともに[6]、評価基準や非放射線同位元素による試験法の確立が欧米で検討されるなど、現在もっとも議論が盛んな分野である。

非放射線同位元素による方法は、日本では実施できる施設が限られる。そこで、ATP量を指標としたLLNA-DA法[16]、ブロモデオ

キシウリジン（BrdU）の取り込みを指標としたLLNA-BrdU法のバリデーションおよび第三者評価が進んでいる[17]。LLNA-DA法のバリデーションは良好な結果を得て、現在、JaCVAMにて第三者評価を実施中である。一方、LLNA-BrdU法に関しては、第一期のバリデーション結果を受けてプロトコールを改良した第二期バリデーションを実施中である。これらが将来的には日本で汎用される日は近いと考えている。

4）環境毒性

2年間のECVAMおよびEuropean工業会のバリデーションにおいて、60%の魚の削減が見込まれている[5]。REACHへの適用により、魚170,000匹および23millionユーロの費用削減になると予想されている。日本ではこの分野の代替法研究は進んでいない。

4．おわりに

適切な資金·資源の提供を通して、代替法の開発やバリデーションを加速し、安全性評価ための代替法の行政による承認の迅速化を目指すため、欧州ではEPAA（European Partnership for Alternative Approaches to Animal Testing）が設立され、Enterprise, Research, Health & Consumer Protection, Environmentなどの省庁、AISE（石鹸洗剤協会）、CEFIC（欧州化学品工業会）、COLIPA（欧州化粧品工業会）、ECPA（欧州農薬工業会）、EFPIA（欧州製薬団体連合会）、EuropaBIO（欧州バイオテクノロジー工業会）、IFAH（欧州動物愛護協会）などの産業界が協力している[18]。

さらに、化粧品の国際規制に対応すべく日米欧カナダによる国際協調組織ICCR（International Cooperation on Cosmetics Regulations）が2007年9月に開催された。その中で、代替法がテーマの一つとして選択され、試験のデザインや、実施、評価についての協力を進めていくための方向性を示すよう要請されている[19]。

以上のような代替法を巡る国際動向から目が離せない状況になりつつある。日本でも各社においてREACHの対策が急がれている。REACHの目的は化学物質の安全性の確保であるが、その中心は代替法である。しかし、すでに確立された代替法は少ない。日本でも化学物質の安全性評価の為に代替法研究が厚生労働省や経済産業省で勧められているが、国際的にも日本にさらなる支援が求められつつあることをよく認識しなければいけないと考えている。

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皮膚感作性試験代替法の現状

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1．なぜ動物実験代替法が必要か？

欧州における化学物質の安全性再評価（REACH：Registration, Evaluation and Authorization of CHemicals）[1]および化粧品の製造・販売に動物実験を用いないという2009年と2013年に施行される予定の化粧品規制により[2]，動物実験代替法（代替法）を巡る議論が欧米では盛んである．日本では他人事のように捉えられているが，経済のグローバリゼーションを考えれば，欧州の規制に従わない化学物質や化粧品は少なくとも欧州では販売できない，欧州から輸入される化学物質や薬用化粧品（医薬部外品）は日本の規制に合わず売れないことにより，経済的損失も大きくなり傍観しているわけにはいかない．

このような状況から，皮膚の安全性評価でもっとも大きなウエイトを占める皮膚感作性についても代替法の開発が盛んである．具体的には，既存のモルモットを用いた動物実験に代わりうる，後述するマウスを用いたLocal Lymph node Assay（LLNA）を *in silico*（コンピュータを用いた手法）や *in vitro* 試験に置き換えるための検討が国際的に進んでいる．

ただし，すでに開発された方法が多数あったとしても，ことはそう簡単ではない．たとえば，これまで汎用されているモルモットを用いるMaximization testやBuehler testを新しい感作性試験の代替法に置き換え，安全性が担保できるか検証するためには，図1に示すように[3]，バリデーションや専門家による第三者評価，行政的な受け入れのための評価という何段階にもわたるチェックが必要である．これは2005年に認証されたOECDガイダンス文書34に定められており[4]，この過程を経ないと公的な試験法として国際的に認められない．

これに対応する部署として，筆者が室長を務める新規試験法評価室が国立医薬品食品衛生研究所 安全性生物試験研究センター 薬理部内に2005年11月に設立された[5～7]．この部署の業務の一つが新規代替法のバリデーションと第三者評価であり，この活動をJaCVAM（Japanese Center for the Validation of Alternative Methods）と名づけている．

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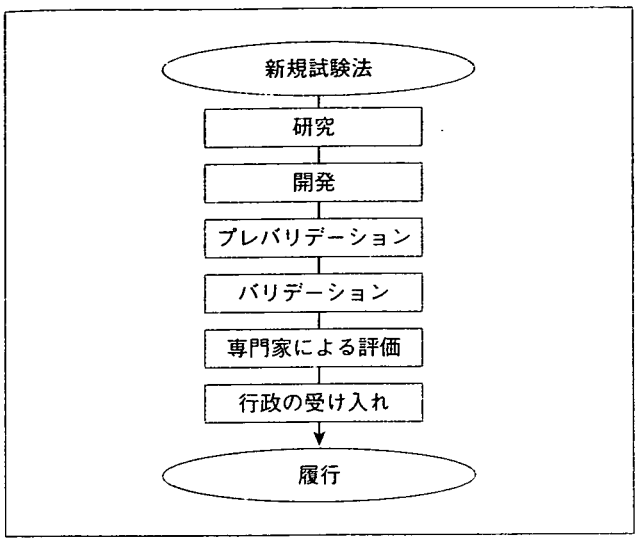

図1　試験法バリデーションのプロセス[3]

2．感作性試験代替法 LLNA

さて，以上の国際的な潮流を念頭に，本章のタイトルである感作性試験代替法の現状として，まずLLNAについて触れておきたい．

2002年，モルモットを用いる方法に代わり，LLNAがOECDガイドライン429として認証された[8]．この方法は動物の完全置き換えができた代替法ではないが，中動物から小動物へ動物種を変更，動物数の削減が可能になった動物実験の3Rs（Redection，Refinement，Replacement）のうち，ReductionやRefinementを可能にした試験方法である．感作性の成立までの複雑なシステムをリンパ球の増殖で補うという実験規模と実験期間を短縮した試験法である．ただし，トリチウムチミジンの取り込みを評価指標とするため，日本では実験できる施設が限られ，普及度は高くなかった．そこで，放射性物質を使わない方法の検討が進んでいる．

たとえば，日本ではダイセル化学工業（株）によりATPの取り込みを指標としたLLNA-DA法が開発され[9]，すでにバリデーション研究が終了して，専門家による第三者評価が進んでいる[10]．BrdUの取り込みを指標としたLLNA-BrdU法も（財）化学物質評価研究機構によって開発され，日本動物実験代替法学会にてバリデーション研究が実施されている．

欧米では，規制に用いるための予測性の検証や[11～13]，放射性物質を用いない試験法の開発はもちろんのこと，改良試験法の追加バリデーションを実施するための試験法基準の作成が議論されており，日本のバリデーションにも影響を及ぼしている．さらに，動物数を削減するため，1濃度のみで試験を行うreduced LLNAについても検討が進んでいる[14]．欧州ではこの方法がREACHのためにスクリーニングとして認められている．以上のようなLLNAを巡る動向は2007～2008年にかけて活発に議論されている．

ところで，安全性評価はhazard identification（有害性の同定）とrisk assessment（リスク評価）とに大別されており，リスク評価は有害性の同定，用量反応性，曝露評価などを合わせてなされる[15]．従来のモルモットを用いるBuehler assayにおいてはリスク評価が可能であったが，reduced LLNAでは有害性の同定しかできない．LLNAでは用量反応性が捉えられるが，曝露評価，感作性の成立まで考慮に入れた研究や議論がもっとなされなければならない．ともかく，規制当局がモルモットの試験結果を採用しないとなれば，LLNAだけで新たな医薬品や農薬の開発に支障を来さないことを示す検討を必要とする．

化粧品の場合はまず安全性ありきの製品であることから，有害性の同定を第一に考えればよいかもしれない．よって，LLNAの導入に異論はない．後述する動物実験を用いない試験法でも公式に認められれば置き換えていけばよいと考えている．ただし，医薬品や農薬の場合にはリスク評価と薬効を天秤に掛けねばならないことから，LLNAによるリスク評価が未完全な場合，Human Repeated Insult Test（HPIT）のようなヒトによる連続適用試験が確認試験でなく，リスク評価に用いられることになろう．これはボランティアの人権，倫理的な面からみて許されるものではない．真の実用化にはまだ課題も多い．

3．*In vitro* 感作性試験の開発

さて，どの試験法もバリデーションが実施されておらず，すでに2009年には間に合わないが，2013年の化粧品規制までの設立を目指している*in silico*および*in vitro*感作性試験代替法の現状に触れておきたい．

代替法としては，①*in silico*における評価，②*in vitro*試験としてペプチド結合試験，③培養細胞を用いる試験，④培養皮膚モデルを用いる試験に大別される．これらは図2に示すような，感作誘導に重要な作用機構に立脚した試験法であり，動物を使わないで評価する

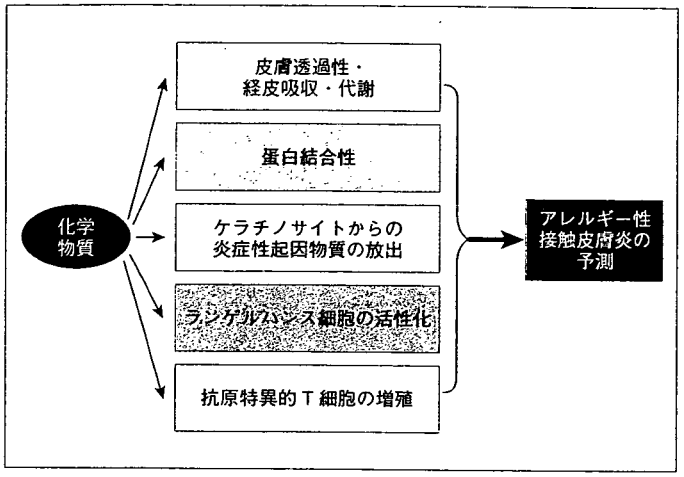

図2　アレルギー性接触皮膚炎の感作誘導における重要な作用機構

ならば，これらの試験を組み合わせたスキームの構築が重要であると考えている[16, 17]．

① *in silico*

感作性物質の構造活性部位はほぼ明らかになってきており，専門家であれば，構造をみればある程度の感作の有無を予測できよう．Aptula らはこれを統計学的な手法などによって検討を行い[18]，Enslein らもデータベースを活かして感作性強度を判断してきた[19]．これらの文献を数値化したソフト（DEREK[20]や TOPKAT[21]など）が多数販売されており，かなりの精度で感作性の強度予測が可能である．ただし，データベース数（ヒトやモルモットの結果が揃っているデータ）がこれ以上増える可能性が低いこと，すべての化学物質が純品でなく，不純物が感作性を示す場合もあり，ここで陰性の結果を得たからといって以下の試験を省略できるわけではない．あくまでも有用な第一次スクリーニングである．

② ペプチド結合試験

感作性物質がハプテンに結合すること，特定のアミノ酸構造に結合することを利用して，ペプチド，とくにシステインをもつグルタチオンや合成ペプチドとの結合による挙動の変化を HPLC（高速液体クロマトグラフィ）で測定するものである．Kato ら[22]，Gerberick ら[23]，中村[24]の検討がなされている．Gerberick らの検討によれば[25]，システインやリジンペプチドとの反応性で得られた結果と LLNA の一致率は 89％であり，4 段階の強度分類も可能とされており，スクリーニング法として有用であるとされている．

③ 培養細胞を用いる試験

皮膚のなかで最初に感作性物質に曝露させる細胞はケラチノサイトである．皮膚に浸透された感作性物質はハプテンとしてケラチノサイトに結合し，サイトカインやケモカインを産生して感作源となる．ケラチノサイトを用いた感作性のスクリーニングは Vandebriel らの検討があるが，あまり多くない[26, 27]．

むしろ，アレルギーといえばランゲルハンス細胞の挙動であろう．以前から，ランゲルハンス細胞の活性に着目した検討がなされたが[28, 29]，試験法を開発するうえで十分な細胞を確保できないという課題があった[30]．そこで，ランゲルハンス細胞起源であるヒト末梢血や臍帯血の細胞を樹状細胞に分化させる方法が検討されてきた．バイオマーカーとしては，MHC Class Ⅱ，CD86 などの表面抗原[31～33]，TNF-α[34]，IL-1β[35]，IL-8[36]などのサイトカイン，CXCR4[37]，CCR7[38]，CCL2[39]などのケモカインおよびケモカインレセプターなどが報告されている．

しかし，細胞誘導に時間を要することや経費の問題などに加え，同一ドナーから入手できる細胞数に限界があり，かつドナー間の反応性に相違があることから，試験法の構築には支障が大きかった[31, 35, 40]．そこで，安定供給や個体差のない単球系の細胞株（CD14 陽性の THP-1 細胞[33, 41]，U937 細胞[42, 43]，CD34 陽性の KG-1 細胞[44]，MUTZ-3 細胞[45]など）を用いた検討が主流となっている．

これらのなかで，資生堂と花王のグループが THP-1 細胞に着目し，その表面抗原のなかで，CD86 および CD54 の変化を指標とした試験法を確立した[46, 47]．この試験法は 96 well プレートを用いてフローサイトメトリーで解析を行う簡便な方法であり，ヒト細胞株活性化試験（human Cell Line Activation Test：h-CLAT）と命名されている．2 施設間の比較試験では，高い一致性が報告されており，LLNA との一致率も 62 物質で 90％ときわめて高い．そこで，厚生労働科学研究の支援を受けた化粧品企業や欧州化粧品工業会との間で共同研究が実施され，技術移転の容易性や施設間再現性が検討されている状況である．本試験方法はバリデーションに移行すべき段階に来ていると考えられる．

④ 培養皮膚モデルを用いる試験

Coquette らは，3 次元培養表皮モデルに感作性物質を適用して，ケラチノサイトの放出する IL-1 α や IL-8

を調べ，感作性物質とIL-8の関係を報告している[48]．

また，樹状細胞を導入した3次元培養表皮モデルによる感作性物質の挙動の報告もある[49,50]．ランゲルハンス細胞の培養がむずかしく，免疫担当細胞を導入したモデルの開発は進んでいなかったが，幹細胞研究やヒト細胞の利用が進む昨今，このモデルの研究がより進むと考えられる．h-CLATなどのヒト樹状細胞株を用いたこれまでの成果が本モデルの構築に反映されれば，より精度の高いモデルの開発が期待される．本モデルは，種々の濃度や製剤の形態で感作性物質を適用し，その後のサイトカインやケモカインの挙動を検索できるという点で用量反応性や曝露評価に利用できる可能性が高く，リスク評価モデルとして期待されている．

おわりに

JaCVAMの立場は欧米との国際協調を重視して新規の動物実験代替法を確立することである．これはグローバリゼーションが進む昨今，避けては通れない課題である．だからといって，化粧品や化学物質の安全性評価の質を下げるべきではない．安全性の担保が第一優先である．昔のような化粧品による黒皮症を再燃させるわけにはいかない．黒皮症は原因究明に当たられた皮膚科医の努力と[51]，その後行政や業界が導入したモルモットを用いた試験法により激減したわけであり[52]，この安全性の質を担保しなければならい．安全性を担保しながら代替法の国際協調を進める，"出口があって実はないかもしれない"この問題を解く鍵は，「これまでにない技術」による新試験法の開発であると信じている．そのためには，基礎研究の充実が欠かせない．専門家の研究から素晴しい成果が得られることを期待してやまない．

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Perspectives on Validation and Regulatory Acceptance of Animal Alternative Testings in Japan

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From the Editor

Recent scientific advancement in understanding of toxic mechanisms enables risk assessors to develop new and revised toxicological test methods that better predict potential toxic effects of chemicals. In this issue, we update current status of research activities in the development of animal alternative methods that may refine, reduce, or replace animal use for toxicity testing. Contributors to this issue include Dr. Kojima at NIHS, Japan, and Dr. Scott Belanger, environmental toxicologist and Dr. Pauline McNamee, human safety toxicologist at P&G.

Seok (Soga) Kwon, Ph.D.

Inside this issue:

In November 2005, Japanese Center for the Validation of Alternative Methods (JaCVAM) was established in the Division of Pharmacology at the National Center for Biological Safety and Research affiliated to National Institute of Health Sciences (NIHS) in Japan. JaCVAM facilitates the validation, peer-review, and international harmonization of alternative methods. Key objectives of JaCVAM are: 1) to facilitate 3R's* with Reduction and Replacement prioritized and 2) to ensure new test methods are validated, peer reviewed, officially accepted by the regulatory agencies, and internationally harmonized. JaCVAM has its own steering committee which is in charge of JaCVAM activities. JaCVAM facilitates validation studies and peer-review process for new methods. JaCVAM also proposes a validated method to the regulatory agencies for their acceptance.

JaCVAM's current activities and future directions on the validation and peer review of alternative to animal testings will be discussed in this paper. JaCVAM plays an important role in evaluating alternative test methods to be used in the safety assessment of cosmetic products. A new EU directive prohibiting *in vivo* tests on cosmetic ingredients (the 7th Amendment, Council Directive 2003/15/EC) [1] also engaged JaCVAM to actively support the validation of test methods.

The major activities of JaCVAM include:

1) Coordinate the technical evaluation and regulatory acceptance of new and revised test methods
2) Delegate validation work for new and revised test methods
3) Promote 3R's and facilitate international harmonization of alternative to animal testings

JaCVAM is currently coordinating validation studies and peer-review for several test methods (shown in Table 1). Most of the test methods are to be used in the safety assessment of cosmetic products. In Japan, there is a unique cosmetic product category called "Quasi-Drug (QD)" which requires pre-market registration approved by Japan government prior to their introduction to markets. Thus, JaCVAM is also involved in the

*3R's : Refinement, Reduction, Replacement

technical review of the overall safety evaluation process for QD products, which utilizes alternative test methods. In 2007, a new committee called "Review committee on the safety assessment for QD products" was established with participation of representatives including dermatologists, experts from cosmetic industries and Japanese Society for Alternatives to Animal Experiments (JSAAE). This committee is conducting a 3-year research project funded by Ministry of Health, Labor, and Welfare (MHLW) to provide technical guidance on the safety evaluation of QD products. The project progress is to be updated in 2009 when the 7th Amendment in EU becomes effective. Specific activities on the validation of alternative test methods on major toxicity endpoints related to cosmetic products in Japan will be discussed.

1) Skin Irritation

In April 2007, the European Center for the Validation of Alternative Methods (ECVAM) Scientific Advisory Committee [ESAC] endorsed the EPSKIN method employing cultured epidermis models (EPISKIN™)[2]. This endorsement triggered Japanese researchers to accelerate their validation activities. Japanese researchers have been using commercially available cultured skin/epidermis models such as TESTSKIN™ (Toyobo), Vitrolife-Skin™ (Gunze), and EpiDerm™ (MatTek and Kurabo). Pre-validation studies have been completed for these models with favorable results[3]. TESTSKIN™ model[4] and Vitrolife-Skin™ model[5-6] were further assessed for validation studies to be considered as alternative test methods for predicting skin irritation of cosmetic ingredients at their usage levels. These two skin models successfully completed validation studies. The results showed that their predictive capability of skin irritation is equivalent to those of Human Patch Test (HPT) and *in vivo* primary skin irritation test. However, these methods have not completed the peer-review process in Japan. Japanese manufacturers are planning to validate the Japanese skin models in the spring of 2008 by employing reference substances used by EU for the EPISKIN™ and currently seeking technical collaboration with JSAAE.

2) Eye Irritation

In 1998, the guidance for alternative methods on eye irritation utilizing a cytotoxicity method[7] was established[8]. This work was funded by MHLW. However, this guidance has not been widely utilized in Japan. For example, this guidance was not referred in "Questions and Answers (Q&A) attached to the QD manufacturing and marketing approval application and cosmetics criteria amendment request" issued in 2006[9]. This suggests that the cytotoxicity method has not been recognized as a validated alternative test method by the regulatory agencies. Therefore, JaCVAM is to conduct the technical evaluation of a cytotoxicity method by reviewing validation study results generated by

Toxicological endpoint	Test methods	Current activities
Phototoxicity	Testing Battery (Yeast-RBC): Yeast Growth Inhibition Phototoxicity Assay Red Blood Cell Photochemolysis Assay	Peer review in progress
Skin sensitization	Local Lymph Node Assay using ATP contact measurement (LLNA-DA)	Peer review in progress
	Local Lymph Node Assay using BrdU uptake measurement (LLNA-BrdU)	Validation in progress
	Human Cell Line Activation Test (h-CLAT)	Planning
Corrosiveness	Culture model	Regulatory acceptance in progress
Skin irritation	Culture model	Planning on peer review
Endocrine disrupter	Lumi-cell, CER-estrogen reporter assay	Validation in progress
Mutagenicity	Comet assay (*in vivo* or *in vitro*)	Validation in progress

Table 1. Current status of validation and peer review for animal alternative methods coordinated by JaCVAM

JSAAE, MHLW Scientific Research, EU and USA. In EU and USA, however, the review panels have completed the peer-review process for Isolated Eye Test methods, Isolated Corneal Test method, and Hen's Egg Test[10]. And the panels concluded that these methods could be used for screening purpose. JaCVAM is also planning to conduct the peer-review of these test methods.

3) Phototoxicity

In vitro 3T3 Neutral Red Uptake (NRU) photo-toxicity test has been peer reviewed and accepted as a reliable alternative method in Japan[11]. This method had been previously adopted by OECD[12]. On the other hand, Shiseido developed a test battery including Yeast Membrane Disruption assay and Red Blood Cell Hemolysis test[13-15]. At Shiseido's request, a review committee of MHLW Scientific Research assessed the validity of these test methods. The test methods were further refined in response to results of the initial validation, and they have undergone the 2nd validation process. The peer-review of these methods is in progress and their regulatory acceptance will be assessed in 2008.

4) Skin Sensitization

The Local Lymph Node Assay (LLNA) can be used as an alternative method because it has been already adopted by OECD[16]. However, the use of radioisotopes (RI) limits the practical application of LLNA by Japanese laboratories. Thus, other methods involving no RI are currently under the validation process. They include LLNA-DA method (ATP content index)[17] and LLNA-BrdU method (BrdU uptake index)[18]. The LLNA-DA method showed favorable results of validation studies and was further progressed to the peer review process. The LLNA-BrdU method is in the process of 2nd validation with protocol modification in response to the 1st validation. In future, these test methods are expected to be widely utilized in Japan. New test methods are also continuously being developed for the safety assessment of cosmetic products because LLNA still requires animal use. Therefore, Shiseido and Kao developed h-CLAT (human Cell Line Activation Test) method[19,20] in collaboration with industry associations such as Japan Cosmetic Industry Association (JCIA) and the European Cosmetic Toiletry and Perfumery Association (COLIPA). The scientific validation for this test method is expected to occur. The Structural Activity Relationships (SAR) analysis could be used for predicting skin sensitization in future considering significant technical advancement in computer modeling areas. In addition to h-CLAT method, other new test methods are also being developed. They include peptide binding assay and cell culture methods. JaCVAM will coordinate validation studies and peer-review of these new test methods.

This paper described current status and future directions of the validation and regulatory acceptance of alternative to animal testings focusing on major toxicity endpoints related to cosmetic products in Japan. JaCVAM also coordinates technical evaluation of alternative methods for other toxicological endpoints such as acute toxicity and mutagenicity. Detailed information on these endpoints will be discussed in future. In collaboration with international organizations, significant progress has been made in the validation of alternative methods. JaCVAM's mission is to facilitate the validation of alternative methods while domestically developed test methods are prioritized.

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JaCVAM: An organization supporting the validation and peer review of new alternatives to animal testing

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Abstract
In November 2005, the Japanese Center for the Validation of Alternative Methods (JaCVAM) was established as part of the Division of Pharmacology at the National Center for Biological Safety and Research, affiliated with the National Institute of Health Sciences (NIHS) in Japan. The key objectives of JaCVAM are: 1) to ensure that new or revised test methods are validated, peer reviewed, and officially accepted by regulatory agencies, and 2) to expand international cooperation on alternatives to animal testing. This paper describes in further detail JaCVAM's current activities and future direction.

Keywords: validation, peer review, alternative, JaCVAM

1. Introduction

In November 2005, the Japanese Center for the Validation of Alternative Methods (JaCVAM) was established as part of the Division of Pharmacology at the National Center for Biological Safety and Research, affiliated with the National Institute of Health Sciences (NIHS) in Japan. One mission of JaCVAM is to promote practice of the 3Rs (Reduction, Refinement and Replacement) in the area of animal testing, with Reduction and Replacement prioritized in Japan. Key objectives of JaCVAM are: 1) to ensure that new or revised test methods are validated, peer reviewed, and officially accepted by the regulatory agencies, and 2) to expand international cooperation on alternatives to animal testing. The main activities of JaCVAM will focus on the following missions and objectives: 1) coordination of peer review and regulatory acceptance of new and revised test methods; 2) support of validation work for new and revised test methods; and 3) promotion of the 3Rs and 4) working to promote international partnership surrounding the issue of alternative methods.

2. Organization of JaCVAM and supporting groups

JaCVAM has five members: a director, Dr. Hajime Kojima; a visiting researcher, Dr. Mitsuteru Masuda; a researcher, Miss Shoko Arai; and two secretaries. Though JaCVAM is a small organization, it is supported by several groups, as shown in Fig. 1. Firstly, JaCVAM has its own steering committee, which is charge of JaCVAM activities. This committee is comprised of six members: Dr. Toru Inoue (Chair: Director of the National Center for Biological Safety and Research in NIHS), Dr. Yasuo Ohno (Vice President of NIHS, and a founder of JaCVAM), Dr. Kenichi Nakazawa (Head of the Division of Pharmacology), Dr. Mitsuteru Masuda (JaCVAM), Dr. Hiroshi Itagaki (Shiseido Co., Ltd., President of Japanese Society for Alternative to Animal Experiments: JSAAE) and Dr. Hajime Kojima (JaCVAM).

The roles of the JaCVAM steering committee are:

- To prepare the validation or peer review process of new or revised test methods.
- To evaluate international cooperation.
- To select advisory board members.
- To select peer review panel members.
- To select regulatory acceptance board members.
- To consult with NIHS support members.
- To check JaCVAM plans and reports.

Secondly, JaCVAM (and its steering committee) have an advisory board which monitors its activities. This board is comprised of eight members, including one dermatologist and other individuals belonging to the Japanese Society of Toxicology (JST), the Japanese Association for Laboratory Animal Science, the Japanese Society for Alternative Animal Experiments (JSAAE), the Japanese Pharmaceutical Manufacturers Association (JPMA), the Japanese Cosmetic Industry Association (JCIA), the Ministry of Health, Labour and Welfare (MHLW), and the Animal Welfare Network. These members are reelected every two years. The meeting of the advisory board is held at NIHS twice per year. JaCVAM reports on its activities

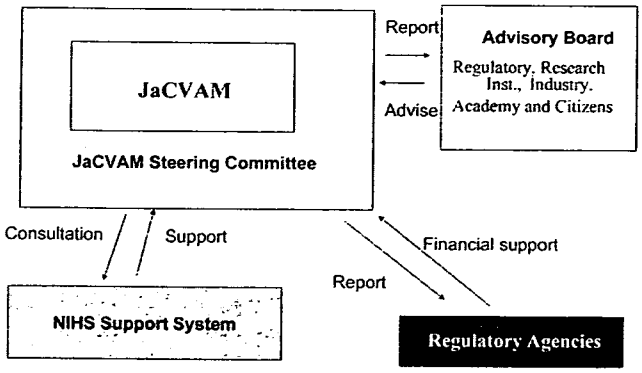

Fig. 1. Organization of JaCVAM

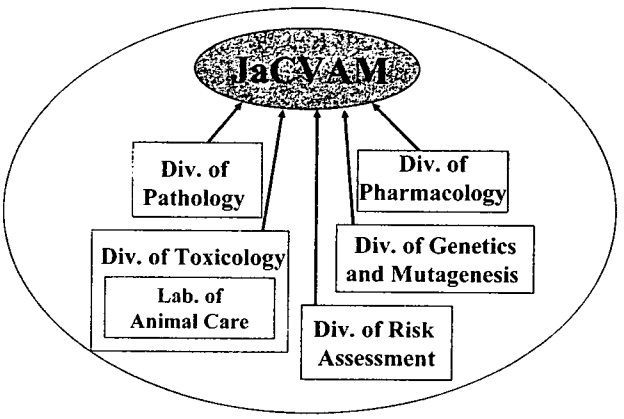

Fig. 2. NIHS Support System

and future programs to board members, and in return receives advice on JaCVAM's methods of operation.

Thirdly, JaCVAM is supported financially by the MHLW. The specific source of funding for JaCVAM's development and validation of alternative methods is the MHLW's Government Pension Investment Fund.

Finally, many laboratories at the NIHS, as shown in Fig. 2, assist the JaCVAM with scientific consultations. NIHS conducts research on the quality, safety, and efficacy of pharmaceutical products, foods, and chemicals in the environment. JaCVAM benefits from the large number of specialists working at the NIHS.

3. Organization for validation studies

In Japan, new or revised test methods that are recommended by domestic researchers or developers are validated by an applied science society like the JSAAE, the Japanese Environmental Mutagen Society (JEMS), or the Japanese Society for Dermatoallergology and Contact Dermatitis (JSDCD). A Validation Management Team, which consists of researcher of a particular toxicity, a specialist on validation studies, a biostatistician, and a study manager from each participating laboratory, validates the repeatability and reliability of the new or revised

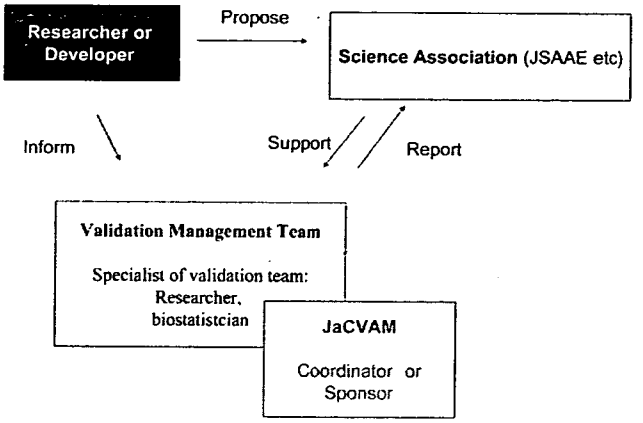

Fig. 3. Framework for Validating Alternative Methods

method according to OECD Guidance No.34 (OECD,2005) and the National Institute Health (NIH) report (ICCVAM, 1997). JaCVAM participates in this process as a specialist in validation studies, and is responsible for selecting participating laboratories, managing test materials (initial selection, blinding of test substances, and distribution to each laboratory), managing data (data sheet preparation and data collection) and providing financial support. The above details are illustrated graphically in Fig. 3.

4. Organization for peer review and regulatory acceptance

As shown in Fig. 4, JaCVAM has a framework for peer review and regulatory acceptance of alternative methods. After JaCVAM has received a request for peer review from a researcher or developer, the JaCVAM steering committee meets to deliberate on the proposal methods; this should take no longer than three months. Upon the receipt of permission for peer review, JaCVAM organizes the oversight committee in order to evaluate a new test method. The roles of this ad hoc committee are to collect references on the toxicity involved in a new test method, to prepare a draft report, to propose a new validation study, and to provide advice on further research related to the proposed test method. Based on the report and

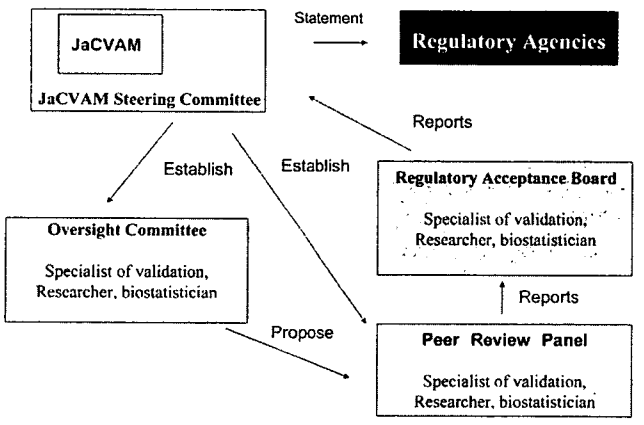

Fig. 4. Framework for Peer Review and Regulatory Acceptance of Alternative Methods

references prepared by the oversight committee, a peer review panel evaluates a new or revised test method. The roles of this ad hoc panel consist of the principles and criteria as laid out in OECD guideline No. 34. The members of the oversight committee and the peer review panel assigned to evaluate a new test method are selected by the JaCVAM steering committee. They include five or six specialists selected from academia, industry, the JaCVAM regulatory acceptance board, and the NIHS supporting system.

JaCVAM and its steering committee have a regulatory acceptance board for new or revised methods. This board is comprised of ten members who are either biostatisticians or dermatologists or who are delegates of JST, JSAAE, JPMA, JCIA, the Pharmaceuticals and Medical Devices Agency (PMDA), or the NIHS. These members are reelected every two years. The meeting of this board is held at the NIHS on a case-by-case basis if necessary. This board reviews new or revised test methods based on the reports of the peer review panel and prepares a report and statement on the test method for regulatory agency.

5. International coordination

Significant progress has been made in validating alternatives to animal testing in collaboration with international organizations. Particularly important is the JaCVAM cooperation with the European Center for the Validation of Alternative Methods (ECVAM), and NICEATM (NTP Interagency Center for the Evaluation of Alternative Toxicological Methods)/ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods). These three centers are closely united and this may increase international cooperation. Together, our three organizations validated and peer reviewed a new test method which has been approved by OECD guidelines.

Table 1. Current validation and peer review conducted by JaCVAM

Test method	Material	Current activities
Photoxicity	Yeast-RBC	Peer Review in progress
Skin sensitization	LLNA-DA	Peer Review in progress
	LLNA-BrdU	Validation in progress
	h-CLAT	Planning
Corrosivity	Culture model	Regulatory acceptance in progress
Skin irritation	Culture model	Planning on Peer Review
Endocrine disrupter	Lumi-cell, CER-estrogen reporter assay	Validation in progress
Mutagenicity	Comet assay (in vivo or in vitro)	Validation in progress

6. Conclusion

JaCVAM is currently coordinating validation studies and peer review for several test methods (shown in Table 1). Most of the test methods are to be used in the safety assessment of cosmetic products. JaCVAM's goals are to facilitate the validation of alternative methods with domestically developed test methods and to conduct peer review of these and internationally certified test methods, and promote practice of the 3Rs in the area of animal testing to accomplish our mission.

References

1. OECD (2005) OECD Series on testing and assessment Number 34. Guidance document on the validation and international acceptance of new or updated test methods for hazard assessment. ENJ/JM/MONO 14.
2. ICCVAM (1997) Validation and regulatory acceptance of toxicological test methods: a report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods. NIH Publication No: 97-3981, National Institute of Environmental Health Sciences (NIEHS).

ORIGINAL ARTICLE

Validation of human skin models for skin corrosivity tests in Japan

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[8]Bio-Medical Dep., Kurabo Industries Ltd., Japan,
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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

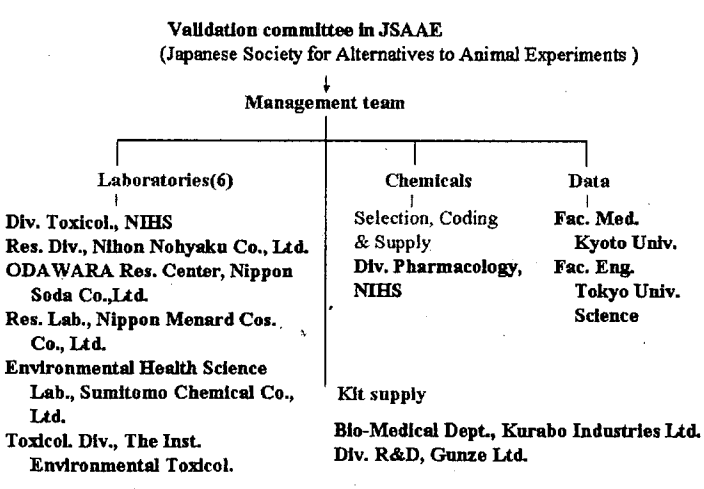
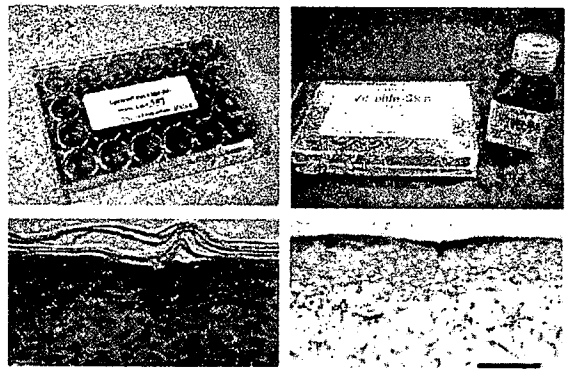


Fig.1 Organization of the validation



400μm

Fig.2 EpiDerm™

100μm

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