5. 日本の動向

日本でも眼刺激性、皮膚刺激性、感作性、光毒性試験などの代替法において日本動物実験代替法学会を中心にバリデーションや第三者評価が行われきた[22]。しかし、内容的に優れているものの、国際的には認知度が低かった。

幸い、昨年8月に第6回国際動物実験代替法会議(WC6)[23]、本年2月にWC6フォローアップがいずれも東京で開催され[24]、日本国内での盛り上がり以上に日本の活動に対する国際的な評価が高まっている。また、図 2 に示すように、JaCVAM を中心に日本でも国内の協力体制が整い、OECD、ICH(日米 EU 医薬品規制調和国際会議)、ICCR(化粧品国際規制会議)、ISO(国際標準化機構)などに関わる動物実験の3Rs 問題に対する国際協力体制が整うとともに、一昨年から、遺伝毒性、内分泌かく乱物質スクリーニングなどの分野で国際的な共同研究も始まっている。

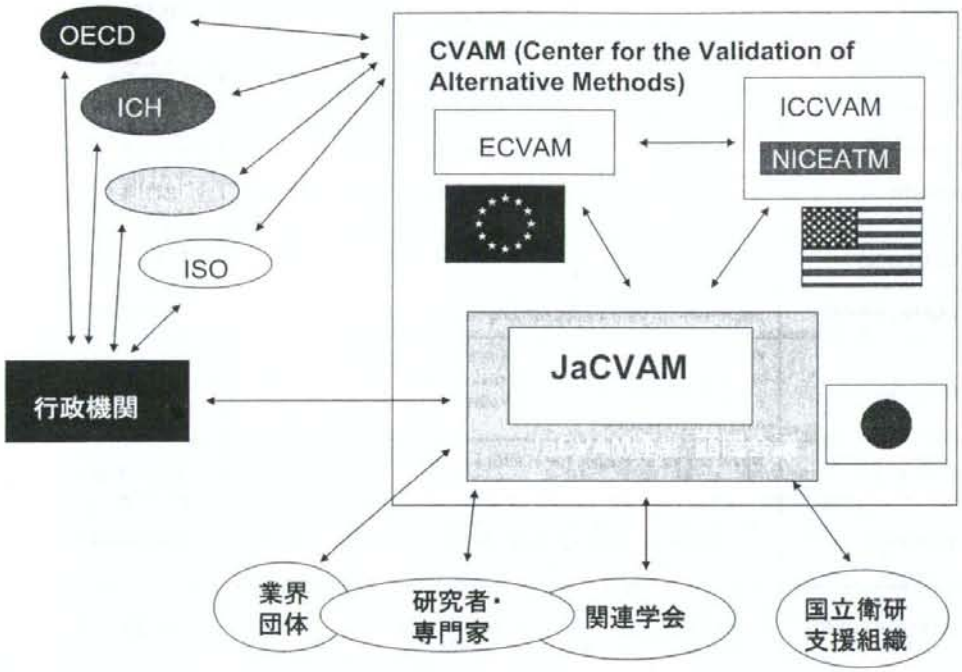

図2. JaCVAMの国際協力概要

以下に 2008 年 8 月時点で JaCVAM が関わる試験法の進捗についてまとめた。この概要は表 3 にまとめている。

1)皮膚刺激性試験

2007 年 4 月、ESAC (ECVAM Scientific Advisory Committee)が皮膚刺激性試験代替法として、培養表皮モデル EPISKIN を認証した[25]。EPISKIN に被験物質を 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。

表3. 2008年8月の日本におけるバリデーション、第三者評価の進捗状況

No.	試験法	試験法の概要	目標	現状
1	腐食性試験	培養皮膚モデルを用いた方法	化学物質の腐食性評価に代替法を利用するための公定化	評価会議で最終評価終了
2	光毒性試験	酵母膜破壊と赤血球の溶血試験	日本の医薬部外品ガイドラインへの収載	評価委員会(Peer Review Panel)にて評価中
3	LLNA-DA	マウスﾘﾝﾊﾟ節中のATP量の変化を指標とする方法	OECDガイドライン現行法の改変	評価会議にて評価中
4	LLNA-BrdU	マウスﾘﾝﾊﾟ節中のBrdUの取り込みを指標とする方法	OECDガイドライン現行法の改変	評価委員会にて評価開始予定
5	LLNA	LLNA改良法	日本の医薬部外品ガイドラインへの収載	評価委員会にて評価開始予定
6	h-CLAT	培養細胞を用いた感作性物質のｽｸﾘｰﾆﾝｸﾞ	OECDガイドライン	ECVAM、ICCVAMとの共同バリデーション決定
7	皮膚刺激性試験	培養皮膚モデルを用いた方法	OECDガイドライン	評価委員会にてEPISKIN法について評価中
8	皮膚刺激性試験	培養皮膚モデルを用いた方法	日本の医薬部外品ガイドラインへの収載	日本動物実験代替法学会によるバリデーション研究実施中
9	眼刺激性試験	細胞毒性試験、摘出眼球試験、摘出角膜試験	OECDガイドライン	評価委員会にて摘出眼球、摘出角膜試験の検証、細胞毒性試験の評価中
10	眼刺激性試験	細胞毒性試験(短時間処理法)	OECDガイドライン	日本動物実験代替法学会によるバリデーション研究実施中
11	コメットアッセイ	*in vitro*試験法	OECDガイドライン	ECVAM、ICCVAMとの共同バリデーションPhase Ⅱ実施中
12	コメットアッセイ	*in vivo*試験法	OECDガイドライン	ECVAM、ICCVAMとの共同バリデーションPhaseⅢ実施中
13	内分分泌かく乱物質スクリーニング	HeLaレポーター遺伝子アッセイ	OECDガイドライン	ECVAMおよび韓国とのantagonistバリデーション実施中
14	内分分泌かく乱物質スクリーニング	Lumi-cellアッセイ	OECDガイドライン	ECVAM、ICCVAMとの共同バリデーションPhase Ⅱ～Ⅲ実施中
15	Bhras assay	培養細胞を用いた発がん性物質のｽｸﾘｰﾆﾝｸﾞ	OECDガイドライン	ECVAM、ICCVAMとの共同バリデーション決定

日本でもこれまで培養皮膚や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。これらモデルが日本で製造・販売されており、多くのユーザーがいる。2000～2002 年にかけて東洋紡績株式会社製の TESTSKIN、グンゼ株式会社製の Vitrolife-Skin、MatTek 製でクラボウ株式会社が販売している 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 [26]。さらに、化粧品原料の使用濃度における皮膚刺激性試験代替を目的に、2002～2004 年にかけて TESTSKIN[27]および Vitrolife-Skin でバリデーションを実施した [28,29]。得られた結果が、当初からの評価基準であるパッチテストと動物試験における皮膚刺激性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、まだ第三者評価に至っておらず、国内でのコンセンサスは得られていない。一方、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が日本動物実験代替法学会の主催で開始されている。EPISKIN と比較し、化学物質の皮膚刺激性試験代替法として日本製のモデルの有用性の検証は重要であると考えている。

2）眼刺激性試験

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」は日本においても中々普及していない [30]。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の有用性を検討中である。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や [31]、厚生労働科学研究の細胞毒性試験に加え [32]、最近 ECVAM で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、サイトセンサーマイクロフィジオメーター試験）やバリデーションが計画されている EpiOcular というヒト再構築モデルの結果が用いられることになろう。

一方、欧米では強い眼刺激性評価のために摘出眼球試験、摘出角膜試験、受精鶏卵試験等の第三者評価が終了し [25, 33]、スクリーニングとしての有用性が指摘されている。前述した JaCVAM 第三者評価委員会において、これら試験についても評価を実施している。

3）光毒性試験

OECD ガイドラインとして認証されているニュートラルレッド取り込みによる細胞毒性試験については [34]、日本でも独自に第三者評価を行い、代替法としての有用性を評価した [35]。一方、資生堂は酵母膜破壊試験と赤血球溶血試験のバッテリーを用いる光毒性試験の検討を進め、厚生労働科学研究に第三者評価を依頼した [36-38]。評価の過程でバリデーション結果が不足しているとの指摘を受け、二期に渡るバリデーション研究が実施された。これらのバリデーション結果を用いた第三者評価を実施中である。

4) 感作性試験

OECD ガイドラインとして認証されている Local Lymph Node Assay (LLNA) がモルモットを用いた従来の試験の代替法として利用されている [39]。ただ、この試験法は放射線同位元素を用いることから日本では実施できる施設が限られる。そこで、ATP の取り込みを指標とした LLNA-DA 法 [40]、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 [41]。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。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 [25]、改良試験法の評価基準が欧米で検討されるなど現在、議論が盛んな分野である。

ただし、化粧品の安全性評価のためにマウスを用いる本試験を使い続けることは、完全な代替法とはいえない。国際的にも新たに種々の in vitro 試験法の開発が進んでおり、日本としては株式会社資生堂および花王株式会社が日本化粧品工業連合会の有志や欧州化粧品工業会 (COLIPA) の協力を得て開発を進めているヒト細胞株活性化試験(human Cell Line Activation Test: h-CLAT) に注目し [42,43]、バリデーションを計画している。ただし、この分野は構造活性相関のプログラム [44]、ペプチド結合試験の開発 [45]、h-CLAT 以外のヒト細胞株を用いた試験法などの開発・検討が盛んな分野であり、これらを使いこなして感作性を評価するシステムの検討が必要である。

5) 急性毒性試験

OECD に掲載されている評価法 [46-48] で動物数の削減が一般的になっている昨今、さらに 2005 年 ICCVAM にて細胞毒性試験を用いた代替法の第三者評価が行われた [33]。この評価の結果、ヒト正常角質細胞や 3T3 細胞によるニュートラルレッド法を用いて、非毒性物質の検出が可能とされている。さらに、「Human endpoint」に配慮した新たなワークショップも 2008 年 2 月に開かれ [33]、JaCVAM としても ICCVAM の第三者評価に協力している状況である。

6) 変異原性試験

エイムス試験、染色体異常試験、動物を用いた小核試験という 3 点セット [49] を補う試験法である肝臓の不定期 DNA 合成の代替法として、日本環境変異原学会 哺乳類変異原性試験研究グループを中心に、ECVAM、NICEATM の協力を得たコメットアッセイ [50] の国際バリデーション研究が実施されている。この試験法は *in vivo* 試験だけでなく、*in vitro* 試験のバリデーションをも進めている。本試験法は開発からかなりの時間がたっていることもあり、方法の統一化がこの国際バリデーションの課題である。

In vivo 試験としては、マウスの肝臓、胃を標的臓器として、日米欧 5 施設によるブラインド化した被験物質を用いた Phase Ⅲのバリデーションを実施中である。本バリデーションは、最終的なプロトコールを確定するための実験であり、2009 年からは、PhaseⅣの本バリデーションが開始される。

一方、*in vitro* 試験のバリデーションはやや遅れて、Phase I バリデーションを開始した。ブラインド化した6物質を用いプロトコール確定のための Phase II バリデーションを実施中である。

7) 内分泌かく乱物質のスクリーニング

財団法人 化学物質評価研究機構(CERI)の開発した HeLa-9903 細胞を用いたレポーター遺伝子アッセイは、OECD の定める内分泌かく乱物質評価のレベル 2 にあたるエンドクラインレセプターαへの結合を評価指標とする試験法である[51]。バリデーション終了後、OECD による第三者評価がなされ、評価基準の明確化などでプロトコールの改訂が求められるとともに、antagonist について検討がなされていないとのが指摘あった。そこで、JaCVAM では ECVAM の協力を得て、2008 年春よりバリデーション研究を開始している。

一方、米国 XDS 社で開発されたエンドクラインレセプターαへの結合を評価指標とするレポーター遺伝子アッセイ Lumi-cell アッセイについても[33]、JaCVAM では国際バリデーション研究を進めている。このバリデーション研究は ICCVAM が主催する ECVAM、JaCVAM との共同バリデーションである。日本では日吉株式会社が実験を担当しており、agonist および antagonist の試験結果を求めている。2008 年末までに Phase III まで進める予定である。この試験法も OECD ガイドラインを目指している。

6. 終わりに

動愛法の動物愛護管理基本指針には、毎年度達成状況を点検し、その結果を施策に反映させることや、策定5年後に当たる平成 24 年度を目処とした見直しが記載されている。また、本年から始まった第三者認証システムの普及など、実験動物の福祉問題はまだ完成された仕組みではない。

代替法においても、JaCVAM を中心に、当面は化粧品や化学物質の安全性確保のため、国際協調を進めていくことになると考えている。

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薬学研究における動物実験代替法研究の重要性とその問題点

大野泰雄

Importance of Research on Alternatives to Animal Experiments in Pharmaceutical Sciences

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The Japanese animal protection law was amended in 2005 to include the 3Rs principle in animal experiments. According to this new law, the Ministry of Education, Culture, Sports, Science and Technology, the Ministry of Health, Labor and Welfare, and the Ministry of Agriculture, Forestry and Fisheries developed announced several guidelines in 2006. These guidelines indicated responsibility of the president of each research institute conducting animal experiments to meet obligating of the animal experiment committee (AEC) and the education to be provided to scientists. About half a year after this notification, I conducted a survey on how these guidelines were put into practice in the pharmaceutical colleges and universities. I received 29 answers from 24 institutes. It seemed that every institute was following, the guidelines, however, there were many institutes where the details were inadequate. For example, questions on the existence of alternative methods and degree of distress and pain were not asked in some questionnaires sent to the AEC. Education on proper conduct of animal experiments (3Rs, methods to evaluate and decrease distress and pain, and methods of euthanasia) was not conducted in many institutes. Further improvement seems necessary.

Key words——animal experiment; alternative; japanese animal protection law; guideline, ethical committee

1. 序言

生物現象を明らかにし，医薬品や化学物質等のヒトへの影響を研究する薬学において，動物実験は基本的研究材料として欠かすことはできない（Table 1）．その結果得られた知見を基に医薬品開発を行う際にも，その有効性と安全性の確認には動物実験に頼らざるを得ないところが多い．また，生命現象を理解することなく，医薬品の適切な使用は行えないが，薬剤師教育の有効な手段としても，動物実験は重要である．

一方，動物実験における動物福祉に関する社会の関心は極めて高く，動物愛護や動物の権利を擁護するため多くの関連団体が設立され，活発な活動が行われている．しかし，Figs. 1-3 に示したように，動物実験への反感の程度はその目的や使用する動物の種類，また，実験の行い方により異なる．適切な説明が行われることにより，必要かつ適切な動物実験に対する同意が増加すると思われる．すなわち，動物実験に係わるすべての研究者及び教育者は，そ

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本総説は，日本薬学会第 127 年会シンポジウム S40 で発表したものを中心に記述したものである．

Table 1. Sale of Experimental Animals (JSLAS, 2004)

Species	Conventional	Clean	SPF	Sum
Mouse	232589	1285337	4751033	6268959
Rat	43839	449854	2062193	2555886
Other rodents	48		7468	7516
Guinea-pig	11514	227718	66293	305525
Hamsters	4486	963	32744	38193
Rabbit	23015	69746	29300	122061
Dog	12689	70	0	12759
Cat	260	0	626	886
Monkeys	2248	0	0	2248
Pig	1228	130	0	1358
Goat	0	0	0	0
Sheep	35	0	0	35
Birds	5094	0	12199	17293
Total*	352467	2033893	6961860	9348220

* Include Suncus, ferret, amphibians, and fishs.

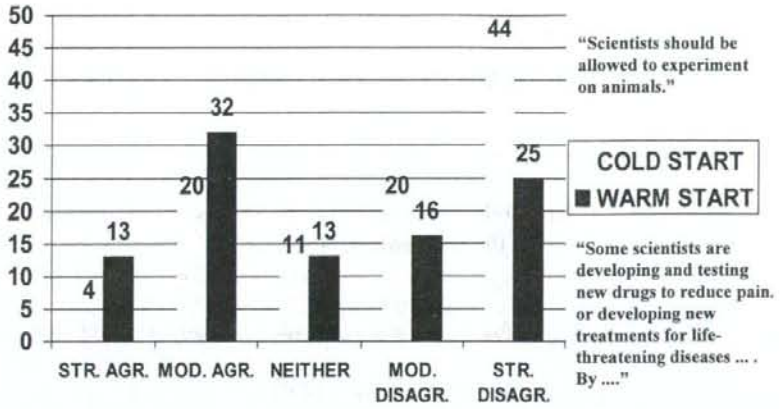

Fig. 1. Agreement on Animal Experiments (Difference Caused by Explanation)

(Aldhous P. *et al.*, New Scientist, 22 May, 1999, pp. 26–31), Light blue column: Explained as "Scientists should be allowed to experiment on animals." Blue column: Explained as "Some scientists are developing and testing new drugs to reduce pain or developing new treatments for life-threatening diseases.... by....", Ordinate: Answer (%), Abscissa: STR. AGR.: strong agreement, MOD. AGR.: moderate agreement, NEITHER: neither, MOD. DISAGR: moderate disagreement, STR. DISAGR.: strong disagreement.

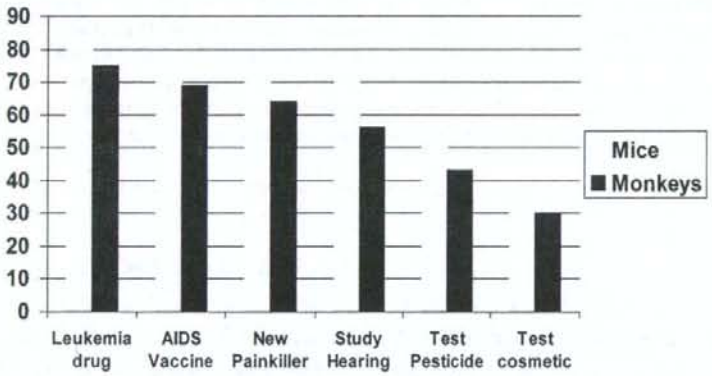

Fig. 2. Response to Animal Experiments (Difference Caused by Animalspecies and Object of Research)

(Aldhous P. *et al.*, New Scientist, 22 May, 1999, pp. 26–31), Yellow column: mouse, Blue column: monkey, Ordinate: Rate of agreement (%), Abscissa: Object of research.

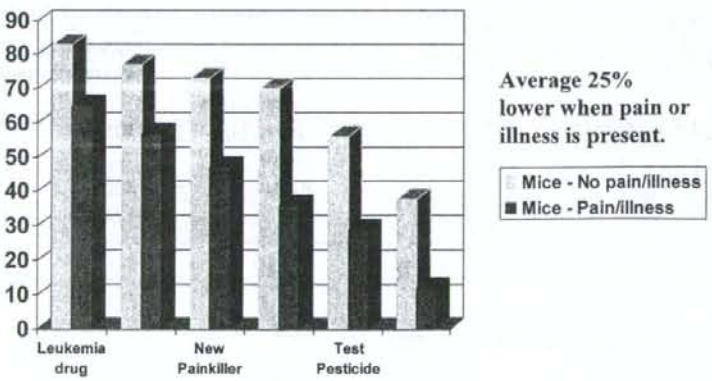

Fig. 3. Response to animal experiments (Difference by pain and illness)

(Aldhous P. *et al.*, New Scientist, 22 May, 1999, pp. 26–31), Yellow green column: Research without pain and illness, Red column: Research with pain and illness, Ordinate: Rate of agreement (%), Abscissa: Object of research.

の活動が社会の同意により許容され，支持されることにより，初めて可能となっていることを認識し，このような社会の動静に敏感に対応し，科学的・法的・倫理的に適切な研究を行わなくてはならない．

2. 動物実験の実施に関する指針

わが国においても科学研究においても動物福祉の尊重の必要性が認識され，平成17年6月の「動物の愛護及び管理に関する法律」（動愛法）の改定[1]で動物実験代替法に関する3R（Replacement，Reduction，Refinement）の原則が法に組み込まれた．また，平成18年6月までに，法令の目的を達成するため，環境省（環境省告示　第88号「実験動物の飼育及び保管並びに苦痛の軽減に関する基準」），[2] 文部科学省（文部科学省告示　第71号「研究機関等における動物実験等の実施に関する基本指針」），[3] 厚生労働省（厚生労働省通知　科発0601002号「厚生労働省の所管する動物実験等の実施に関する基本指針」）[4] 及び農林水産省（農林水産省通知「農林水産省の所管する研究機関における動物実験等の実施に関する基本指針」）[5] から実験動物の飼育・管理や動物実験についての指針が示された．日本学術会議からも動物実験に関する詳細指針「動物実験の適正な実施に向けたガイドライン」[6] が示された．

3Rの達成は一研究者の努力だけでは不可能であり，研究機関全体としての対応が不可欠である．そこで，上記指針には実施機関（研究機関）の長の責任が明確に示された（文部科学省告示　第71号「研究機関等における動物実験等の実施に関する基本指針」）．すなわち，実施機関の長は当該機関における動物実験等の実施に関する最終的な責任を有し，本指針に定める措置その他動物実験等の適正な実施のために必要な措置を講じること，と定められている．必要な措置とは，1）動物の愛護及び管理に関する法律（昭和48年法律第105号．以下「動物愛護管理法」という）や実験動物の飼養及び保管並びに苦痛の軽減に関する基準（平成18年環境省告示第88号以下「飼養保管基準」という．）並びに各省庁からの指針その他の動物実験等に関する法令等の規定を踏まえ，動物実験等の施設等の整備及び管理の方法並びに動物実験等の具体的な実施方法等を定めた規程を策定すること，2）動物実験委員会の設置，3）動物実験計画の承認，4）動物実験計画の実施結果の把握，5）教育訓練等の実施，6）自己点検及び評価，並びに7）適切な方法で動物実験等に関する情報公開を行うこと，と定められている．動物実験委員会は実施機関の長の諮問を受け，動物実験計画が本指針及び機関内規程等に適合しているか否かの審査を行い，その結果を実施機関の長に報告するものであり，また，動物実験計画の実施結果について，必要に応じ助言を行うものとされている．この委員会は1）動物実験等に関して優れた識見を有する者，2）実験動物に関して優れた識見を有する者，3）その他学識経験を有する者で構成される．また，動物実験実施に際しては，科学的合理性を確保し，目的を達成するに必要な適正な動物実験等の方法の選択や代替法の選択，苦痛の軽減について考慮するとともに，適切に維持管理された施設及び設備において動物実験等を実施するよう定められている．

3. 薬学会及び薬学系研究機関における動物実験への対応

日本薬理学会では以前より動物実験を巡る社会状況に対応するために動物実験指針の改訂作業を行い，法や通知の改訂の結果も取り入れ，これに反する研究結果を学会誌から排除することが明示された（大野泰雄 2007）．[7] しかしながら，薬学会では薬学雑誌の投稿規定に「ヒトならびに動物実験に関する倫理基準として，――，動物実験に関する報告も所属機関の定める動物実験ガイドラインに基づいて行われるのみならず，文部省（現文部省科学省）の策定したガイドライン，No. 141，1987年：“大学などにおける動物実験”に従って下さい」と古い指針を引用している．J. Health Sci. の投稿規定では「2」動物を対象とした論文は，所属機関の定める動物実験ガイドラインのみならず文部科学省など公的機関の策定したガイドラインに従って実施されたものに限り投稿を受け付けます．なお，当該論文はこれらのことを本文中に明記してください」とされているのみである．

一方，平成17年の日本薬理学会で動物実験結果をポスター発表した研究者にインタビューした結果では，医学部関係者の多くが，動物実験についての教育や動物実験委員会での審議について，明確に回答したのに対し，薬学系大学からの発表者の中には曖昧な回答者が多くいた．中には動物実験委員会が

Table 2. Suvey on the Existence of Committee for Animal Experiment in 2004

Institution		Exist	Not exist	%
Medical school	*	9	0	100
Pharmaceutical school	*	4	2	67
Agricultural school	*	1	0	100
CRO	*	1	0	100
Research institute	*	1	0	100
Private company	**	59	10	86

* Result of question to young scientists presenting posters at the annual meeting of The Japanese Pharmacological Society in 2004. ** Mutai *et al* (2004) Iyakuhin Kenkyu 35, 196–201.

存在しないとの回答もあった（Table 2：大野泰雄 2005）.[8] 平成6年に改訂された文部科学省の通知「大学等における動物実験について（文部省国際学術局長通知）」[9] では動物実験委員会の設置が明確に求められていたことから，動物実験委員会が存在しないとは考えられない．実際は存在しているが，適切に機能していないか，あるいは動物実験についての教育が若手研究者に十分に行われていないと推定された．今回，新たな動物愛護と管理に関する法令が改正され，省レベルの動物実験指針が示されたことから，薬学部における動物実験がどのように変わ

Table 3. Results of Survey on Animal Experiments in Pharmaceutical Schools

Number of answers 29
Number of Institutes 24

1) Type of Institutes

	Yes	Number of answers	%
State university	9	24	37.50
with Medical School	9	24	100.00
with veterinary School	3	24	33.33
Private University			
with Medical School	3	24	20.00
with veterinary School	0	24	0.00
Pharmaceutical College	7	24	29.17

2) Position in the Pharmaceutical School

	Yes	Number of answers	%
students	0	29	0.00
Research assistant	2	29	6.90
Associate professor	7	29	24.14
Porfessor	20	29	68.97

3) Dose your school conduct animal experiments?

Yes	Number of answers	%
28	28	100.0

4) Approximate number of animals used in a year

	Sum	Number of answers	Mean
Mouse	119033	17	7049
Rat	39610	18	2201
Hamster	72	12	6.0
Rabbit	424	14	30.3
Dog	20	13	1.5
Monnky	0	13	0.0
Others	810	10	81.0

5) Do you conduct animal experiment?

Did you conduct animal experiment recently?

Yes	Number of answers	%
27	29	93.1

6) Do you know guideline notified by the Ministry?

Yes	Number of answers	%
27	29	93.1

If you knows,

	Yes	Number of answers	%
7) Do you have the guideline?	23	28	82.1
8) Can you acces the guideline?	24	28	85.7

9) Do you know the guideline issued by Japanese Solence Council?

Yes	Number of answers	%
27	29	93.1

If you knows,

	Yes	Number of answers	%
10) Do you have the guideline?	21	27	77.8
11) Can you acces the guideline?	23	27	85.2

12) Does your institute have institutional guideline on animal experiments?

Yes	Number of answers	%
28	29	96.6

If there is,

	Yes	Number of answers	%
13) Do you have the guideline	24	27	88.9
14) Can you acces the guideline?	24	26	92.3

15) Is there Animal Experiment Committee in you institute?

Yes	Number of answers	%
29	29	100.0

If there is,

	Sum	Number of answers	Numbers/Committee
16) number of committee members	259	27	9.59
members from own institute	216	26	8.31

17) Members?

	Sum	Number of answers	施設当たりの人数
General public	20	16	1.25
Veterinarian	5	12	0.42
Expert of animal care*	10	14	0.71
Expert of animal experiment*	117	21	5.57
Expert of alternatives	3	10	0.30
Others	71	18	3.94

*: Other than veterinarian

18) How often do the committee meeting held?

	Sum	Number of answers	平均
Number of times in a year	60.5	23	2.63 回

19) How often do you submit application of animal experiment to the committee?

	Yes	Number of answers	%
At each purchase	3	23	13.0
At each planning	19	23	82.6
Once in a year	13	23	56.5
twice in a year	1	23	4.35

20) Descriptions of application form

	Yes	Number of answers	%
Purpose of experiments	27	28	96.4
Study director	28	28	100.0
Study practitioner	28	28	100.0
Animal species	28	28	100.0
Animal number	26	28	92.9
Alternatives	20	28	71.4
Pain	21	28	75.0
Method to decrease pain	25	28	89.3
Reason not to decrease pain	21	28	75.0

21) Does your institute educate ethics on animal experiment?

Yes	Number of answers	%*1
26	29	92.86

If education is conducted,

22) To whom and how many hours do you educate?

	Total hours	Number of answers	average (h)	target of education (%)*2
Students	60	24	2.50	92.3
Post graduate students	41.5	22	1.89	84.6
Research stuffs	27.5	17	1.62	65.4
Others	2.5	2	1.25	7.7

23) Caliculum?

	Yes	Number of answers	%*3
Animal protection law	21	24	80.8
Physiology of experimental animals	17	24	65.4
Methods of animal care	22	24	84.6
3Rs principles	18	23	69.2
Evaluation of pain	18	23	69.2
Methods to decrease pain	19	23	73.1
Method of euthanasia	21	23	80.8
Alternative methods	15	23	57.7
Others	11	17	42.3

24) Have your institute received inspection on animal experiments by third party.

Yes	Number of answers	%
3	28	0.11

If you have,

25) By whom the inspection was conducted?

	Yes	Number of answers	%*4
Stuff	2	2	0.67
Outsiders	2	2	0.67

*1: calcuated on the number of institutes that conducted animal experiments.
*2: calculated on the total number of answers (26).
*3: calculated on the number of instititues that conducted education on animal experiments.
*4: calculated on the number of inspected institutes.

ったかを知るため，平成19年の2–3月にかけてe-mailによるアンケート調査を行った．その結果は平成19年の薬学会年会でのシンポジウムで発表した．以下はその結果を示したものである．

アンケート調査の内容はTable 3に回答とともに示した．アンケートは筆者の友人である薬学部職員に直接e-mailで行い，29人（重複があったため24施設）から回答を得た．その結果をTable 3に示す．

動物実験委員会の設置状況を示したものであるが，今回はすべての動物実験実施施設に設置されていた．委員の数は6–10人が最も多かったが，16–20人というのも2施設あった．なお，委員の構成は動物実験の専門家が中心で一般人とその他が加わっているところが多く，獣医師や動物飼育及び動物実験代替法の専門家を委員としているところは少なかった．また，動物実験届けが年に1回のみの施設が56.5%を占めており，包括的な内容での審議が行われているものと推定される．一方，動物実験届けへ

Table 4. Classification of Pain and Distress Caused by Biomedical Experiments

Category A: Experiments involving either no living materials or use of plants, bacteria, protozoa, or invertebrate animal species.	Biochemical, botanical, bacteriological, microbiological, or invertebrate animal studies, tissue cultures, studies on tissues obtained from autopsy or from slaughterhouse, studies on embryonated eggs. Invertebrate animals have nervous systems and respond to noxious stimuli, and therefore must also be treated humanely.
Category B: Experiments on vertebrate animal species that are expected to produce little or no discomfort.	Mere holding of animals captive for experimental purposes; simple procedures such as injections of relatively harmless substances and blood sampling; physical examinations; experiments on completely anesthetized animals which do not regain consciousness; food/water deprivation for short periods (a few hours); standard methods of euthanasia that induce rapid unconsciousness, such as anesthetic overdose or decapitation preceded by sedation or light anesthesia.
Category C: Experiments that involve some minor stress or pain (short-duration pain) to vertebrate animal species.	Exposure of blood vessels or implantation of chronic catheters with anesthesia; behavioral experiments on awake animals that involve short-term stressful restraint; immunization employing Freund's adjuvant; noxious stimuli from which escape is possible; surgical procedures under anesthesia that may result in some minor post-surgical discomfort. Category C procedures incur additional concern in proportion to the degree and duration of unavoidable stress or discomfort.
Category D: Experiments that involve significant but unavoidable stress or pain to vertebrate animal species.	Deliberate induction of behavioral stress in order to test its effect; major surgical procedures under anesthesia that result in significant post-operative discomfort; induction of an anatomical or physiological deficit that will result in pain or distress; application of noxious stimuli from which escape is impossible; prolonged periods (up to several hours or more) of physical restraint; maternal deprivation with substitution of punitive surrogates; induction of aggressive behavior leading to self-mutilation or intra-species aggression; procedures that produce pain in which anesthetics are not used, such as toxicity testing with death as an end point; production of radiation sickness, certain injections, and stress and shock research that would result in pain approaching the pain tolerance threshold, *i.e.* the point at which intense emotional reactions occur. Category D experiments present an explicit responsibility on the investigator to explore alternative designs to ensure that animal distress is minimized or eliminated.
Category E: Procedures that involve inflicting severe pain near, at, or above the pain tolerance threshold of unanesthetized, conscious animals.	Use of muscle relaxants or paralytic drugs such as succinyl choline or other curariform drugs used alone for surgical restraint without the use of anesthetics; severe burn or trauma infliction on unanesthetized animals; attempts to induce psychotic-like behavior; killing by use of microwave ovens designed for domestic kitchens or by strychnine; inescapably severe stress or terminal stress. Category E experiments are considered highly questionable or unacceptable irrespective of the significance of anticipated results. Many of these procedures are specifically prohibited in national policies and therefore may result in withdrawal of federal funds and/or institutional USDA registration.

From guidance on the classification of pain and distress (2004) by The Japanese Association of Laboratory Animal Facilities of National University Cooperations (http://www.med.akita-u.ac.jp/~doubutu/kokudou/rinri/pain.pdf)

Table 5. Methods of Euthanasia (US Assoc. Veterinarians)

Species	Acceptable	Conditional acceptable
Cat	Barbiturates, inhalant anesthetics, CO_2, CO, potassium chloride in conjunction with general anesthesia.	N_2, Ar.
Dog	Same as above.	N_2, Ar, penetrating captive bolt, electrocution.
Non-human primates	Barbiturates.	Inhalant anesthetics, CO_2, CO, N_2, Ar.
Rabbit	Barbiturates, inhalant anesthetics, CO_2, CO, potassium chloride in conjunction with general anesthesia.	N_2, Ar, cervical dislocation (<1 kg), decapitation, penetrating captive bolt.
Rodents and other small manmals	Barbiturates, inhalant anesthetics, CO_2, CO, potassium chloride in conjunction with general anesthesia, microwave irradiation.	Methoxyflurane, ether, N_2, Ar, cervical dislocation (rats <200 g), decapitation.
Ruminants	Barbiturates, inhalant anesthetics, potassium chloride in conjunction with general anesthesia, penetrating captive bolt.	Chloral hydrate (IV, after sedation), gunshot, electrocution.
Swine	Barbiturates, CO_2, potassium chloride in conjunction with general anesthesia, penetrating captive bolt.	Inhalant anesthetics, CO, chloral hydrate (IV, after sedation), gunshot, electrocution, blow tothe head (<3 weeks of age).

AVMA Guidelines on Euthanasia (2007).

の記載内容については，代替法の有無，動物に与える苦痛の程度，及び苦痛軽減措置を取らない理由について記載しないところが25％以上あった．また，実験の目的そのものを記載しないとする回答が1件あった．動物実験倫理に関する教育については，92％の施設で行っており，行っていないところは29件の回答の内2件であった．教育内容については，3Rの原則や動物の苦痛の評価，苦痛の軽減方法，安楽死の方法，また，動物実験代替法について教えていないところが多くあった．なお，第3者による査察・調査が行われている施設が3施設あり，その内2施設では外部の者に依頼していた．

4. 結論

薬学部における動物実験実施状況と教育に関する今回のアンケート調査結果はすべての薬学部を網羅しているものではないが，大まかな傾向はつかめたものと思われる．動愛法の改定により3Rの原則が盛り込まれ，それを実行あるものとするため，指針が文部科学省をはじめとする行政機関から通知され，多くの施設で指針に基づき適正に行われていた．しかし，指針に示された動物実験委員会での適正な審議や研究者教育の内容に不十分なところもあると考えられた．それらの施設では今後の改善が望まれる．なお，倫理的な動物実験を行うための参考として，国立大学法人動物実験施設協議会が作成した苦痛の分類と米国獣医師会編が作成した安楽死の方法について，それぞれTable 4とTable 5に示した．

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ORIGINAL ARTICLE

A Study of the Criteria for Selection of THP-1 Cells in the Human Cell Line Activation Test (h-CLAT): Results of 2nd Japanese Inter-laboratory Study

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Abstract

The human Cell Line Activation Test (h-CLAT) is an in vitro skin sensitization method based on augmentation of CD86 and CD54 expression in THP-1 cells (human monocytic leukemia cell line). In our previous Japanese inter-laboratory study, we reported that the transferability and reproducibility of the h-CLAT is basically good. The aim of this study was to define the criteria for selecting appropriate THP-1 cells in the h-CLAT. In this study, new THP-1 cell lots were obtained from three cell banks: one in America Europe and Japan. Using these three lots plus the cell lot we had previously used and obtained from ATCC we investigated the CD86/CD54 expression following exposure to two allergens (DNCB and Ni) and one non-allergen (SLS). Compared with the previous ATCC lot, two new lots showed similar results. Meanwhile, the third new lot showed distinctly different results in cell viability and CD86/CD54 augmentation induced by Ni compared to the other three lots. These results showed that the variability of cellular responses in the THP-1 cells depended on the cell source. In conducting the h-CLAT, it would be important to select appropriate THP-1 cells to predict correctly the skin sensitization potential.

Key words: *h-CLAT, skin sensitization, alternatives, THP-1, cell selection*

Introduction

Allergic contact dermatitis (ACD) resulting from skin sensitization is a common occupational and environmental health issue. In developing new cosmetics and toiletry products that come in contact with the skin, it is necessary to evaluate the skin sensitization potential of the ingredients/products. Traditionally, skin sensitization tests have been conducted in guinea pigs. Because of an increasing social concern for animal welfare, several cell based in vitro skin sensitization tests have been developed (Ryan *et al.*, 2001; Basketter *et al.*, 2005). As candidate cells, peripheral blood-derived dendritic cells (Aiba *et al.*, 1997; Coutant *et al.*, 1999; Tuschl *et al.*, 2001; Hulette *et al.*, 2002; Staquet *et al.*, 2004) and $CD34^+$ cord blood hematopoietic progenitor cells (De Smedt *et al.*, 2002; Boisleve *et al.*, 2004) were used. Although using these cells for identifying allergy potency in vitro has indicated promising data, there are still some

technical problems with the routine use of these cells for in vitro skin sensitization tests. Problems included difficulty in obtaining a sufficient number of cells and variability among human donors (Aiba *et al.*, 1997; Rougier *et al.*, 2000). In order to clarify these problems, several groups have proposed using other human cell lines; THP-1 cells, U937 cells (human histiocytic lymphoma cell line) and MUTZ-3 (human monocytic cell line) (Ashikaga *et al.*, 2002; Yoshida *et al.*, 2003; Ade *et al.*, 2006, Azam *et al.*, 2006). Previously, we have reported the usefulness of the h-CLAT using THP-1 cells (Ashikaga *et al.*, 2006; Sakaguchi *et al.*, 2006). Moreover, our Japanese inter-laboratory study found that the transferability and reproducibility of the h-CLAT was basically good (Ashikaga *et al.*, 2007).

In all of our previous studies, we used THP-1 cell lots obtained from American Type Culture Collection (ATCC). THP-1 cells are commonly available in other cell banks. Even though THP-1 cells are established as a cell line, the cells have not always had exactly the same property. Therefore, in this study, we obtained new THP-1 cell lots from three cell banks in order to confirm that those cell lots show a similar response as the previous lot. Furthermore, our final goal is to define the criteria for selecting appropriate THP-1 cells in the h-CLAT.

Materials and Methods

Collaborating laboratories

Laboratory A: Kao Safety Science Research Laboratory. Laboratory B: Kanebo Cosmetics Products Science Research Laboratory.

Cells and medium

A total of four THP-1 cell lots were evaluated. One lot had been purchased from ATCC (Manassas, VA, USA) and used in our previous Japanese inter-laboratory study. This lot served as the reference lot in this study. Three new THP-1 cell lots were obtained from three sources: American, European, and Japanese cell banks. Cells were cultured in RPMI 1640 (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; MP Biomedicals, Irvine, CA, USA, lot#. 2688H), 0.05mM 2-mercaptoethanol and 1% of antibiotic-antimycotic (Invitrogen Corp.).

Chemicals and applying doses

Dinitrochrolobenzene (DNCB) and nickel sulfate (Ni) known as allergens and sodium lauryl sulfate (SLS) known as a non-allergen were evaluated in the h-CLAT. All chemicals were purchased from SIGMA-ALDRICH (St. Louis, MO, USA). Ni and SLS were first dissolved in saline, and DNCB was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in culture media was less than 0.2%. In order to determine the test doses, each laboratory conducted a cytotoxicity test using propidium iodide (PI). From the cytotoxicity test, the dose for each chemical was set at the concentration giving 75% cell viability (CV75). In addition, as a positive control in the h-CLAT, 5 μg/mL of DNCB was evaluated.

Cytotoxicity test

THP-1 cells from each lot were cultured in 24-well plates (1.0×10^6 cells/1 mL/well) with various concentrations of chemicals for 24 h. Cells were washed twice, and then stained with 0.625μg/mL

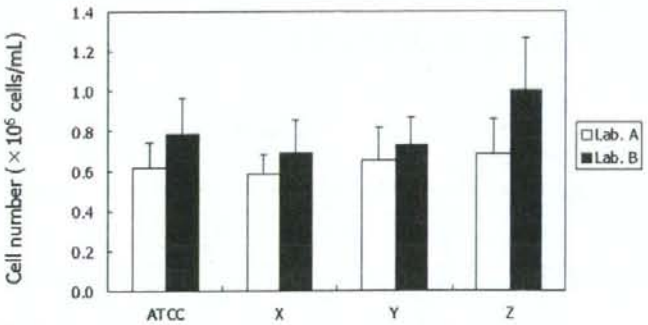

Figure1
Cell number of each THP-1 cell lot after 72h culture
Each laboratory evaluated the proliferation of each THP-1 cell lot once a week during two months (at week 3-9 cultures). Data are expressed as mean ± SD (n=7).

PI. Cell viability was measured by using flow cytometry. Total events for living cell counting were 10,000.

h-CLAT procedure

THP-1 cells from each lot were plated at 1.0 x 10^6 cells/mL and treated for 24 h with media or each chemical. After treatment, Fc receptor blocking procedure was conducted: 0.01% of Globlins Cohn fraction II, III (SIGMA-ALDRICH) was added to THP-1 cells for 10 min on 4°C. Next, cell staining was performed using the following FITC-conjugated monoclonal antibodies (mAbs): anti-human CD54 (clone; 6.5B5) from DAKO (Glostrup, Denmark), anti-human CD86 (clone; Fun-1) from BD-PharMingen (San Diego, CA, USA) and FITC labeled-mouse IgG1 (clone; DAK-G01) from DAKO. Using the manufacturer's recommended dilutions, cells were incubated with the above mAbs at 6μL/3x10^5 cells/50μL for the anti-human CD86 mAb and 3μL/3x10^5 cells/50 μL for the anti-human CD54 mAb. Also, FITC labeled-mouse IgG1 was used as an isotype control at a dilution of 3μL/3x10^5 cells/50 μL. Cells were incubated with these mAbs for 30 min at 4°C. After washing and resuspending with PBS (-) supplemented with 0.1% bovine serum albumin (BSA), the fluorescence intensities of the THP-1 cell surface markers were analyzed by flow cytometry (FACS Calibur Cell Quest, Becton Dickinson, San Jose, CA, USA). A solution of 0.625μg/mL PI was used to gate out dead cells. A total of 10,000 living cells were analyzed.

Data analysis

The relative fluorescence intensity (RFI) was used as an indicator of CD86/ CD54 expression and was calculated by the following formula:

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

MFI = (Geometric) Mean fluorescence intensity

RFI values above 150 and 200 for CD86 and CD54 expression, respectively, were considered positive response following exposure to a chemical. When the cell viability was less than 50% in even one experiment, the data at that concentration was excluded from the analysis of the data. The reason is that the diffuse labeling cytoplasmic structures that occur due to cell membrane destruction will interfere with fluorescent measurements (Becker *et al.*, 1994). Also, data were not included in the analysis when the cell viability with DNCB at CV75 was not within a range of 60-90%.

Statistical analysis

The Student's *t*-test was used to analyze data for significant differences. The value was regarded as significant at $p<0.01$.

Results

Proliferation of THP-1 cells

A total of four THP-1 cell lots were cultured during two months by both labs. Each lab evaluated the proliferation of each THP-1 cell lot once a week during the two months (at week 3 through week 9 cultures). After culturing for 72h, the cell number of each THP-1 cell lot in each lab is shown in Figure 1. *P* values were calculated by Student's *t*-test in order to evaluate a statistical significance between the "ATCC" lot and the each new lot. There were no significant differences in cell number after 72h culture in both laboratories (Table 1).

Cell viability of THP-1 cells treated for 24h with culture media

The cell viability of each THP-1 cell lot treated for 24h with culture media is shown in Table 2. The

Table 1
Statistical analysis of cell number of each THP-1 cell lot after 72h culture

THP-1 cell lot	*P* value	
	Lab. A	Lab. B
ATCC	-	-
X	0.63	0.31
Y	0.68	0.51
Z	0.42	0.10

P values were calculated by Student's t-test in order to evaluate a statistical significance between the "ATCC" lot, served as the reference lot in this study, and the each new lot.

data represent the average ± standard deviation (SD) of seven independent experiments for each laboratory. The two laboratories had almost similar results. Compared to the older "ATCC" lot, which we used in the previous ring study, the new lots, "X" and "Y", showed similar cell viability (approximately 96%) and a low SD value. In contrast, lot "Z" had significantly lower cell viability, which was approximately 85% in both laboratories.

CD86 and CD54 expression induced by chemical treatment

The CV75 (estimated concentration giving 75% cell viability) of each chemical calculated in week 3 for each laboratory is shown in Table 3. Using the THP-1 cells at 3rd week, we examined CD86 and CD54 expression induced by chemical treatment. The calculated RFI values for CD86 and CD54 expression following DNCB, Ni, and SLS exposure for 24h are shown in Figure 2. The two laboratories had almost similar results in CD86/CD54 expression.

For lot "ATCC", DNCB at 5.0 μg/mL and Ni at CV75 enhanced both CD86 and CD54 expression over the positive criterion (RFI value of 150 and 200, respectively). SLS did not induce the augmentation of either CD86 or CD54 expression. As previously observed in the inter-laboratory study, lot "ATCC" could correctly evaluate the skin sensitization potential of DNCB, Ni, and SLS. For DNCB at CV75, the cell viability was 4% for laboratory A and 56% for laboratory B (below 60% in both laboratories), so the data could not be calculated. The cell viability was re-evaluated using

Table 2
Cell viability of each THP-1 cell lot treated for 24h with culture media

THP-1 cell lot	Cell viability (%)	
	Lab. A	Lab. B
ATCC	97.8 ± 0.5	96.4 ± 0.9
X	97.6 ± 0.5	95.7 ± 1.4
Y	97.6 ± 0.7	95.3 ± 0.9
Z	85.1 ± 3.2	85.4 ± 3.5

Data are expressed as mean ± SD (n=7).
Statistical significance compared to the reference lot "ATCC" was calculated by Student's t-test (*p<0.01).

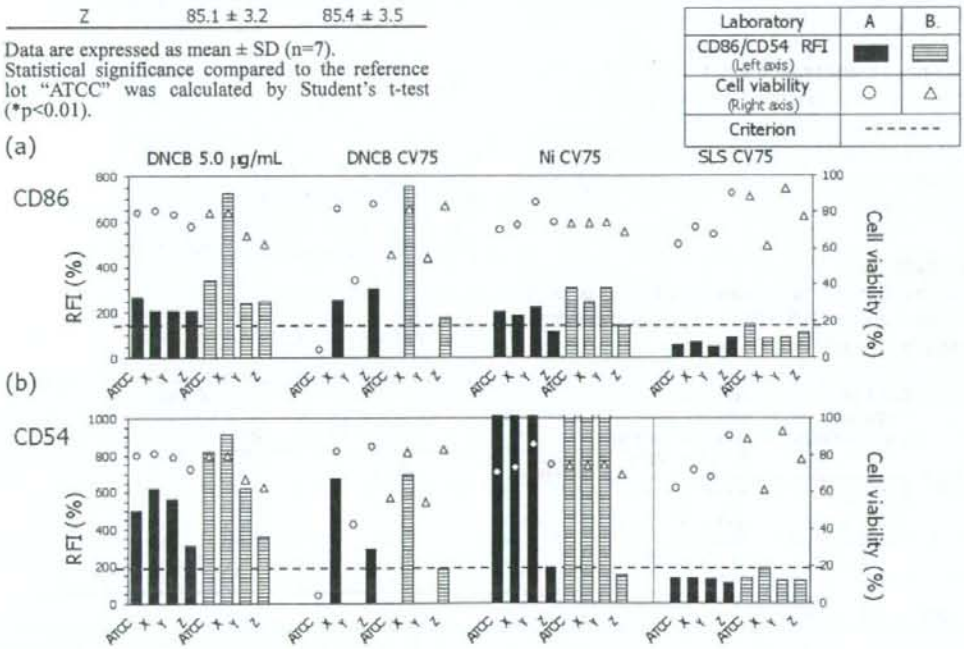

Figure 2
CD86/CD54 expression induced by DNCB, Ni, and SLS
After 3 weeks culture, each THP-1 cell lot was treated with DNCB 5.0 μg/mL, DNCB at CV75, Ni at CV75 and SLS at CV75. The CV75 values of each test chemical in each laboratory are shown in Table 3. The augmentation of surface marker expression for CD86 (a) and CD54 (b) are shown. For DNCB at CV75, cell viability was below 60% for lot "ATCC" and "Y", which resulted in not being able to calculate the augmentation for CD86/CD54 expression.

4-week-cultures. Results of the re-evaluation can be found below.

For lot "X" and "Y", the RFI values were almost similar to lot "ATCC" values. Both lot "X" and "Y" could correctly evaluate the skin sensitization potential of all three chemicals. In contrast, for lot "Z", Ni did not induce either CD86 or CD54 augmentation over the positive criterion in both laboratories. Even though cell viability was similar between the other lots and the Ni-treated lot "Z", Ni gave a negative finding in this test. Particulary for CD54 expression, the RFI values were very high (over 1000) in the other three lots whereas the RFI values were 194 (laboratory A) and 149 (laboratory B) for lot "Z".

Re-evaluation of CD86 and CD54 expression induced by DNCB at CV75

As mentioned earlier, the cell viability for lot "Y" as well as lot "ATCC" with DNCB at CV75 was below 60% in both laboratories. Seeing this discrepancy, we conducted the cytotoxicity test again using THP-1 cells at 4th week. For these two lots, the re-evaluated CV75 of DNCB is shown in Table 4. For lot "Y" in laboratory A, the re-evaluated

CV75 was higher than that calculated in week 3 cells. A value of 6.90 μg/mL was calculated as the CV75 in week 3 cells and this concentration had a cell viability less than 60%. So the higher concentration (7.83μg/mL) obtained in the re-evaluation was estimated to be more cytotoxic.

Table 3
CV75 values (μg/mL) for DNCB, Ni, and SLS
CV75 values (μg/mL) for each THP-1 cell lot after 3 weeks culture was determined for each chemical using the propidium iodine (PI) cytotoxicity test. CV75 values are presented for each chemical and cell lot for both laboratories. In addition, the common CV75 value used in previous h-CLAT studies is shown for comparison.

THP-1 cell lot	DNCB CV75 (μg/mL)			Ni CV75 (μg/mL)			SLS CV75 (μg/mL)		
	Common CV75 used in previous study	Lab. A	Lab. B	Common CV75 used in previous study	Lab. A	Lab. B	Common CV75 used in previous study	Lab. A	Lab. B
ATCC	5.0	8.08	5.66	150	272	145	60.0	67.5	51.8
X	Not done	4.81	4.22	Not done	207	121	Not done	64.4	54.0
Y	Not done	6.90	7.53	Not done	170	113	Not done	69.7	47.3
Z	Not done	2.89	2.68	Not done	118	84.8	Not done	40.5	40.5

Table 4
Re-evaluation of CV75 values (μg/mL) for DNCB
Using the THP-1 cell lot "ATCC" and "Y" after 4 weeks culture, we re-examined CV75 values for both laboratories. The calculated CV75 values in both week 3 and week 4 are shown for each laboratory.

(a)

THP-1 cell lot	Lab. A			
	CV75 (μg/mL)	CD86 RFI (%)	CD54 RFI (%)	Cell viability (%)
ATCC	6.72	257	425	75.4
Y	6.90	125	217	61.7

(b)

THP-1 cell lot	Lab. B			
	CV75 (μg/mL)	CD86 RFI (%)	CD54 RFI (%)	Cell viability (%)
ATCC	4.20	209	426	62.8
Y	4.56	243	379	71.1

Table 5
Re-evaluation of CD86/CD54 expression induced by DNCB at CV75
The THP-1 cell lot "ATCC" and lot "Y" after 4 weeks culture were treated with CV75 value for DNCB determined in week 4, except for the lot "Y" in laboratory A. For laboratory A, lot "Y" was treated with DNCB at CV75 determined in week 3. The RFI values indicative of CD86 and CD54 expression and the cell viability obtained by each lab are shown.

THP-1 cell lot	Lab. A		Lab. B	
	Week 3	Week 4	Week 3	Week 4
ATCC	8.08	6.72	5.66	4.20
Y	6.90	7.83	7.53	4.56

Therefore, for lot "Y", the previous CV75 (6.90μg/mL) determined in week 3 in laboratory A was used for re-testing while the CV75 calculated in week 4 was used for lot "Y" by laboratory B. Except for lot "Y" in laboratory A, the CV75 calculated in week 4 cells was used for re-evaluation. The re-evaluated RFI values for CD86 and CD54 expression following DNCB at CV75 exposure are shown in Table 5. DNCB at CV75 augmented both CD86 and CD54 expression over the positive criterion except for lot "Y" in laboratory A. For lot "Y", DNCB at CV75 enhances only CD54 expression over the positive criterion.

Discussion

THP-1 cells were established by Tsuchiya *et al* (1980) in Japan. After establishment as a cell line, many researchers used THP-1 cells for several purposes in the world. Currently we can obtain THP-1 cells from several cell banks not only in Japan but also America (including ATCC) and Europe. We already reported a useful in vitro skin sensitization test using THP-1 cells named the h-CLAT (Ashikaga *et al*., 2006 and Sakaguchi *et al*., 2006). Up to this point, we have used THP-1 cells obtained only from ATCC and we have gotten good results. If the h-CLAT using THP-1 cells was to be adopted across the world, researchers may obtain these cells not only from ATCC but also other cell banks. Therefore, in order to develop a robust in vitro skin sensitization test using THP-1 cells, we need to clarify the criteria for selection of THP-1 cells. To clarify this purpose, we evaluated three new obtained THP-1 cell lots from American, European, and Japanese cell banks, against our reference THP-1 cells from ATCC (lot #: 33664116).

In this study, we evaluated the CD86/CD54 expression following exposure to typical sensitizers (DNCB and Ni) and a non-sensitizer (SLS) by using new THP-1 cell lots obtained from three different cell banks. Two of the three THP-1 cell lots (lot "X" and "Z") showed similar results compared with the previous ATCC lot. Meanwhile, the one THP-1 cell lot (lot "Y") showed distinctly different results in the cell viability and CD86/CD54 augmentation induced by Ni.

The previously used THP-1 cell lot (ATCC) and two lots (X and Y) of THP-1 cells from two cell banks showed more than 95% cell viability when cells were treated for 24h with culture media. On the other hand, the cell viability was lower (below 86%) for the remaining one lot (Z). The cell viability value for lot "Z" is close to the range (85% - 90%) reported by Python *et al* (2007) for their negative control in their test using U937 cells. As cells to be used in a cell based in vitro skin sensitization test, the cell viability of lot "Z" was not too low. However, Miyazawa *et al* (2008a) showed that the cell viability of non-treated THP-1 cells (media only) was over 95%. We have even evaluated more than 10 lots of THP-1 cells from ATCC and all lots showed more than 95% cell viability (data not shown). Based on these data, cell viability of lot "Z" was evidently low. If non-treated cells showed a low cell viability, we considered the possibility of getting unexpected skin sensitization results if used in the h-CLAT.

In fact, lot "Z" with a low cell viability had a different response in the h-CLAT following treatment with Ni. Lot "Z" was the only lot that did not augment CD86/CD54 expression over the set criteria for the h-CLAT compared to the other lots tested. When we compared the four THP-1 cell lots including the reference lot (ATCC) following treatment with Ni at each CV75 dose, cell viability was similar among all four lots with a viability range of 65% to 85%. The result is in line with Sakaguchi *et al* (2006). Sakaguchi *et al* (2006) showed that THP-1 cells treated with Ni in the h-CLAT augmented CD86 and CD54 expression in the cell viability range of 53% to 97% for CD86 expression and 53% to 90% for CD54.

Judging from the cell viability caused by Ni treatment in this study, the exposure procedure for all lots, including lot "Z", was considered to be properly conducted for CD86/CD54 augmentation. Therefore, the unresponsiveness to Ni of CD86/CD54 on THP-1 cells from lot "Z" might be due to differences specific to lot "Z". Even though cells can be established as a cell line, some unexpected circumstances (e.g., contamination of other cells, mix-up of cells) have occurred (Reid *et al*., 1995 and Lacroix *et al*., 2008) that have caused problems for the investigators. From the unexpected troubles, one possibility is that the cell line has undergone a mutation. In fact, the cell bank disclosed to us that the newly obtained THP-1 cell lot "Z", which had distinctly different results in the h-CLAT, is different from the ATCC lot in terms of DNA pattern sequence (personal communication). Although the THP-1 cell lot "Z" did not induce CD86/CD54 augmentation following Ni treatment, DNCB at 5 μg/mL induced both CD86 and CD54 augmentation. Aiba *et al* (2003) showed that DNCB and $NiCl_2$ stimulate different signal trans-

duction pathways in monocyte-derived dendritic cells. Miyazawa *et al* (2008b) also showed that different signaling pathways regulated the THP-1 cell activation induced by DNCB and $NiSO_4$. Therefore, both DNCB and Ni can be used to establish whether THP-1 cells are functional: both DNCB and Ni result in stimulation of at least two signal transduction pathways and lead to the CD86/CD54 augmentation.

From these results, the variability in cellular responses appeared to be dependent on the cell source (i.e., the cell banks). Such variability could have grievous consequences if the unresponsive cells were to be used in the h-CLAT. Therefore, guidance on selecting appropriate THP-1 cells is needed. We propose the following criteria for selecting an appropriate THP-1 cell lot: 1) the cell viability of THP-1 cells for 24h with culture media needs to be above 90%, 2) the THP-1 cells can correctly evaluate the skin sensitization potential of DNCB, Ni and SLS. This means DNCB and Ni are positive with both CD86 and CD54 expression over the positive criterion. In contrast, SLS is negative.

In order to select appropriate THP-1 cells according the above criteria, appropriate test doses of DNCB, Ni, and SLS are needed. Sakaguchi *et al* (in press) reported that most allergens, including DNCB and Ni, had a positive result at CV75. Considering these data, the CV75 doses for each chemical were used in our study. But for DNCB, the calculated CV75 had a strong cytotoxicity (cell viability below 60%). Thus, the RFI values could not be calculated in two of the four THP-1 cell lots (reference "ATCC" and new ATCC lot "Y"). For these two THP-1 cell lots, except for the lot "Y" in laboratory A, a new CV75 calculated in week 4 was lower than the one calculated in week 3. When the new CV75 was re-tested, DNCB had a positive response. The changes in CV75 at two different weeks may be due to the cell variability.

Though THP-1 cells are a cell line, cell conditions seemed to differ day by day. In fact, the cell number after 72h culture in each week was a little bit different. Therefore, the CV75 of DNCB at week 3 and 4 were different. On the other hand, DNCB 5.0 μg/mL enhanced both CD86 and CD54 expression over the criterion in all of THP-1 cell lots. Sakaguchi *et al* (in press) showed the positive range for DNCB was not wide at the doses from 1.9 to 5.6 μg/mL. These results with observer with DNCB suggest that perhaps two or more concentrations (CV75 plus one other) need to be used in order to better select appropriate THP-1 cells. Therefore, further investigations will be necessary in order to define the more detailed criteria, which including the reference doses for DNCB, Ni, and SLS, for selecting an appropriate THP-1 cells.

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