平成21年度　厚生労働科学研究費補助金（化学物質リスク研究事業）
分担研究報告書

研究課題名：国際協調により公的な試験法を確立するための手順に関する研究

分担研究課題名：遺伝毒性試験（トランスジェニックアッセイ）のバリデーションに関する基盤的研究

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研究要旨

トランスジェニック(TG)遺伝毒性試験は、遺伝子変異を多臓器において定量的に解析することができるため、発がんの標的臓器で遺伝毒性を評価できる特徴を持つ。点突然変異と欠失突然変異を検出することができるレポーターであるλEG10 DNAを発がん試験に汎用されるF344ラットに導入した*gpt* deltaラットを樹立し、技術普及を進めた。TG 遺伝毒性試験のバリデーションに関する基盤的研究として、平成 21 年にスイスのバーゼルで開催された IWGT (International Workshop on Genotoxicity Testing)の"Strategy for genotoxicity testing"のグループに発言討論者として参加し、F344 *gpt* deltaラットの開発状況を報告した。国内約10機関が参加した共同研究を開始し、発がん物質(2,4-ジアミノトルエン，アリストロキア酸、亜硫化ニッケル)および構造の類似した非発がん物質(2,6-ジアミノトルエン)の *in vivo* 遺伝毒性を検索する。

キーワード:*in vivo* 遺伝毒性試験、*gpt* deltaトランスジェニックラット、発がん標的臓器

A. 研究目的

遺伝毒性試験は、(1)Ames 試験 (2)*in vitro* 染色体異常試験 (CA) あるいはマウスリンフォーマ遺伝子突然変異試験 (MLA) (3)マウス小核試験 (*in vivo* MN) の3試験が、多くのガイドラインで推奨されてきた。だが(2) *in vitro* CA, MLAで陽性となった物質の中には、実験動物に発がん性を示さない物質(false positive)が多数含まれることが明らかとなり、ICH(日米EU医薬品規制調和国際会議)では、従来の試験バッテリー以外に、(1) Ames 試験 (2) *in vivo* MN (3) 第二の *in vivo* 試験 からなる別な選択肢が提唱され、議論されている。

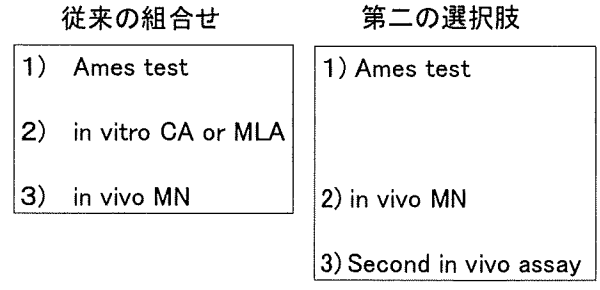

図1　遺伝毒性試験バッテリーの再構築

第二の *in vivo* 遺伝毒性試験の候補の一つと考えられているのが、変異検出用のレポーター遺伝子を導入したトランスジェニック(TG)動物を用いる遺伝毒性試験である。この試験では、TGマウスあるいはラットを化学物質に曝露し、任意の臓器・組織からレポーター遺伝子を *in vitro* パッケージング法によりλファージ粒子として回収して大腸菌に感染させ、動物個体で起きた変異を、大腸菌を用いて検出する。検出された変異体は、DNA シークエンス解析により分子解析することができる。TG 遺伝毒性試験では、遺伝子変異を多臓器において定量的に解析することができるため、発がんの標的臓器で遺伝毒性を評価できるという特徴がある。

我々は、従来のTG遺伝毒性試験法では検出しにくかった欠失変異を効率良く検出することを目的に、新規なλファージλEG10 を開発し、これをC57BL6/Jマウスの受精卵に導入することで点突然変異と欠失変異を検出できる *gpt* delta マウスを樹立した。またλEG10DNAを Sprague Dawley (SD)ラットに導入して *gpt* delta ラットを作成した。さらに、発がん試験において汎用されている F344 系統にバッククロスを行い、F344 *gpt* deltaラットを樹立した。ラットは、一般毒性試験、発がん試験に汎用されており、TG ラットを用いて *in vivo* 遺伝毒性試験と一般毒性試験(あるいは短期発がん試験)を統合することは、動物愛護の点から望ましい方向と考えられる。

本研究では、F344 *gpt* delta ラットを用いた共同研究を通じて、TG 遺伝毒性試験の技術普及とバリデーションに関する基盤的知見を得ることを目的とする。共同研究には、国内約10機関が参加し、3種類の発がん物質[2,4-ジアミノトルエン(2,4-DAT), アリストロキア酸、亜硫化ニッケル]および構造の類似した非発がん物質[2,6-ジアミノトルエン(2,6-DAT)]の *in vivo* 遺伝毒性を検索する。

B. 研究方法

1) F344 *gpt* delta ラットの樹立

SD *gpt* delta ラットの雄を F344 ラットの雌と15世代戻し交配を行い、F344 *gpt* delta ラットを樹立した。

2) *gpt* delta ラット共同研究の概要

共同研究には国内約10施設が参加した。まず、参加施設に共通の陽性・陰性対照としてF344 *gpt* delta ラット凍結組織を配布し、TGアッセイの技術的検討を行った。陽性対照としてはジエチルニトロサミン (DEN) 20 mg/kg を毎週1回、13週間腹腔内投与したラット肝臓を用いた。陰性対照は溶媒対照群のラット肝臓を用いた。組織からのゲノムDNA抽出、*in vitro* パッケージングによるレポーター遺伝子の回収効率、および *gpt* アッセイによる突然変異頻度の測定を行った。

本試験では、発がん物質(2,4-DAT, アリストロキア酸、亜硫化ニッケル)および非発がん物質(2,6-DAT)の *in vivo* 遺伝毒性を検索した。IWGT (International Workshop on Genotoxicity Testing)が推奨する、28日間の連続投与、最終投与後3日目に組織を採取するという試験プロトコルを基本に動物実験をデザインした。参加施設を3グループに分け、各化合物の試験を行うこととした。各グループの1施設が動物実験を担当し、また、TGアッセイに熟練した施設が各グループに少なくとも1施設含まれるようにグループ分けを行った。本試験では、共通の陽性対照群として、7週齢雄のF344 *gpt* deltaラットにエチルニトロソ尿素(ENU) 50 mg/kg を5日間腹腔内投与し、試験31日目(最終投与後26日目)に採取した肝臓組織を全参加施設に配布して用いた。

	グループ1	グループ2	グループ3
発がん物質	2,4-ジアミノトルエン (2,4-DAT)	アリストロキア酸	亜硫化ニッケル
(標的臓器)	肝臓	腎臓	肺
(非標的臓器)	腎臓	肝臓	
(投与方法)	28日間反復経口投与	28日間反復経口投与	週1回気管内投与を4週間
非発がん物質	2,6-ジアミノトルエン (2,6-DAT)		

※ 国内の約10参加機関を3グループに分け、各化合物の試験を担当する。

図2　*gpt* delta ラット共同研究の概要

3) 試験物質の投与

3-1) 2,4-DAT および 2,6-DAT

2,4-DAT は、げっ歯類に肝癌を誘発するが、異性体の 2,6-DAT は肝癌を誘発しない。両化合物は Ames 試験の S9mix 存在下でともに陽性結果を示す変異原物質である。今回、両化合物の *in vivo* 遺伝毒性評価を行った。2,4-DAT の投与量は 10 および 30 mg/kg/day、2,6-DAT の投与量は 60 mg/kg/day とした。雄の *gpt* delta ラットに 1 日 1 回、28 日間反復経口投与し、最終投与の 3 日後に臓器を採取した。肝臓より抽出した DNA を用いて *gpt* アッセイを実施した。

3-2) アリストロキア酸

アリストロキア酸はハーブや生薬に含まれ、腎障害、遺伝毒性および発がん性が報告されている。アリストロキア酸(0.3 mg/kg, 1 mg/kg)を *gpt* delta rat に 28 日間反復経口投与し、最終投与日から 3 日後に剖検し、肝臓および腎臓を用いて *gpt* アッセイを行った。

3-3)亜硫化ニッケル

亜硫化ニッケル(Ni_3S_2)は不溶性の金属化合物であり、ラットに吸入又は気管内投与すると肺がんを誘発することが報告されている。亜硫化ニッケルをパラフルオロカーボンに懸濁し、0、0.5、1.0 mg/匹の用量で、週 1 回の気管内投与を 4 回行った。初回投与後 28 日目及び 90 日目に肺を採取し、*gpt*アッセイを行った。さらに、欠失変異の誘発を検索するために Spi$^-$ アッセイを行った。

4) 突然変異体頻度の測定(*gpt* アッセイ)

F344 *gpt* delta ラットの臓器(肝臓、腎臓、肺)から RecoverEase DNA isolation kit (Stratagene)を用いてゲノム DNA を採取した。その後、Transpack packaging extract (Stratagene)を用いて *in vitro* パッケージング反応を行い、ゲノム DNA からλEG10 をファージ粒子として回収した。ファージは大腸菌 YG6020 に感染させ、6-チオグアニンとクロラムフェニコールを含む培地上に播種し耐性となったコロニー(*gpt* 変異体候補コロニー)を検出した。検出したコロニーは、再度、6-チオグアニンとクロラムフェニコールを含む培地上にストリークして、*gpt* 変異体を確認した。回収したファージの一部は適宜希釈した後 YG6020 に感染させ、クロラムフェニコールのみを含む培地上に播種し、耐性コロニー数を計測して回収したレポーター遺伝子の総数を求めた。*gpt* 変異体数を回収したレポーター遺伝子数で除して *gpt* 突然変異体頻度を算出した。

(倫理面への配慮)

本研究は、実験動物を用いたものであり、ヒトに関する倫理上の問題はない。また、全ての実験は、各参加施設における遺伝子組換え実験および動物実験に関する規定に準拠して行った。

C. 研究結果

1) TG 遺伝毒性試験のバリデーションに関する基盤的研究として、平成 21 年 8 月 18 日と 19 日にスイスのバーゼルで開催された IWGT の"Strategy for genotoxicity testing"のグループに発言討論者として参加し、F344 *gpt* delta ラットの開発状況を報告した。本グループの討

論内容については、国際学術雑誌に投稿の予定である。*gpt* delta ラットと *gpt* delta マウスは、現在、市販されており、受託研究機関、製薬企業等においても、購入して試験に用いることが可能になった。

2) F344 *gpt* delta ラットを用いた共同研究を開始した。参加施設に共通の陽性・陰性対照として F344 *gpt* delta ラット凍結組織を配布し、TG アッセイの技術的検討を行った。その結果、肝臓 DNA からのλEG10 ファージ回収効率は、パッケージング1反応あたり10～40万 pfu であった。陰性対照サンプル(溶媒対照群ラット肝臓)の *gpt* 変異体頻度の値は1～8.6 x 10^{-6} であり、平均値は 4.5±1.9(SD) x 10^{-6} であった。一方、陽性対照サンプル(DEN 投与ラット肝臓)における *gpt* 変異体頻度は 180～1200 x 10^{-6} であり、平均値は464±327(SD) x 10^{-6} であった。

本試験では、参加施設を3グループに分け、2,4-DAT および 2,6-DAT、アリストロキア酸、亜硫化ニッケルの *in vivo* 遺伝毒性を検索した。投与群は2用量を設定し、試験物質を28日間連続投与し最終投与後3日目に組織を採取する試験プロトコルを用いた。ただし、亜硫化ニッケルについては気管内投与の負担を考慮し、週1回の気管内投与を4回行うプロトコルを用いた。実験には各群5匹の雄ラットを用いた。なお、亜硫化ニッケルについては他グループと異なり解析対象が肺となるため、ENU 投与(50 mg/kg, 5日間連続腹腔内投与)ラット肺サンプルをグループ施設に配布し、肺からの DNA 抽出、レポーター遺伝子の回収および *gpt* アッセイの技術的検討を事前に行い、技術的に問題がないことを確認した。

現在、各試験物質の動物実験が終了し、変異解析を進めている。

D. 考　察

TG 遺伝毒性試験は、多臓器において遺伝子変異を定量的に解析することができるため、発がんの標的臓器で遺伝毒性を評価できる *in vivo* 試験として有望である。*In vivo* 遺伝毒性試験においては、その位置づけの重要性から sensitivity とともに高い specificity が求められており、TG 試験の有用性を評価するための基盤的研究が重要である。IWGT での議論を基に作成された OECD の Detailed Review Paper on Transgenic Rodent Mutation Assays (2009)には、既存の TG 試験データおよび推奨プロトコルがまとめられている。この中で、28日間の連続投与、最終投与後3日目に組織を採取するという試験プロトコルが提案されているが、実際に同プロトコルで行われた試験データは不足している。また、既存の試験データが遺伝毒性発がん物質に偏っている点も指摘されている。

今回樹立した F344 *gpt* delta ラットは、発がん試験に汎用される F344 ラットを遺伝的背景にしており、発がんと遺伝毒性の関連を調べる上でも有用な試験法と考えられる。また、一般毒性試験(あるいは短期発がん試験)との統合によって使用動物数の削減が可能と考えられる。このことから、今回の共同研究では、発がん物質と非発がん物質、遺伝毒性が弱いかまたは認められない発がん物質を対象として、IWGT 推奨プロトコルを基本とした TG 試験を行い、ラット発がん標的臓器における遺伝毒性を検索することとした。参加施設には TG 試験の経験のない施設が含まれていたため、本試験前に共通の陽性・陰性対照サンプルを用いた技術的検討を行った。その結果、DNA 抽出およびトランスジーン回収効率は各施設ともに試験が可能な水準であった。*gpt* アッセイにおいても、多くの施設で適切な陰性対照値と有意に高い陽性対照値が得られた。個別の問題点については試験見学等のフォローアップによって改善されるものと考えられた。本試験では、参加施設を3グループに

分け、2,4-DAT、2,6-DAT、アリストロキア酸、亜硫化ニッケルをそれぞれ投与し、変異解析を進める。

E. 結　論

TG動物を用いる *in vivo* 遺伝毒性試験の評価および技術普及のため、F344 *gpt* delta ラットを用いた共同研究を行った。陽性・陰性対照サンプルを用いて技術的検討を行った後、既知の発がんおよび非発がん物質を用いて遺伝毒性の検索を行った。

F. 健康危機情報

特になし

G. 研究発表

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H. 知的所有権の取得状況

特になし

平成21年度　厚生労働科学研究費補助金（化学物質リスク研究事業）
分担研究報告書

研究課題名：国際協調により公的な試験法を確立するための手順に関する研究

分担研究課題名：in vitro 皮膚感作性試験代替法のバリデーション研究

研究分担者：　大野　泰雄　　国立医薬品食品衛生研究所　副所長

研究協力者：　足利　太可雄　　株式会社資生堂
　　坂口　斉　　花王株式会社

研究要旨

我々は、今までの厚生労働科学研究を通じて、ヒト単球由来の細胞株であるTHP-1 細胞の CD86 および CD54 の発現亢進を指標にした皮膚感作性試験代替法(human Cell Line Activation Test: h-CLAT)を開発し、その試験法の詳細について検討を行い、試験法のプロトコールを確立した。そこで、これを OECD の試験法とするため、ECVAM (欧州動物実験代替法検証センター)に h-CLAT を推薦し、共同で国際バリデーションすることにした。

本年度は、欧州で開催された３回のバリデーション実行委員会に参加し、研究計画の構築に協力した。これを経て、2010 年 3 月よりプレバリデーションが開始された。

A. 研究目的

我々は、皮膚感作性試験代替法として、ヒト単球細胞株であるTHP-1細胞を用い、CD86 および CD54 の発現亢進を指標とした試験法(human Cell Line Activation Test: h-CLAT)を開発した[1, 2, 3]。

本試験法を国際的標準法として磨きあげるため、厚生労働科学研究（医薬安全総合研究事業）において、国内7施設による共同研究を実施し、本試験法は技術移転が容易であり、基本的に施設間再現性が良好であることを明らかにした[4]。さらに、本試験法の汎用性を向上させることを目的として、細胞と血清のロット差および前培養条件に関する検討[5, 6, 7]や、予測モデル、陽性対照物質(2,4-Dinitrochlorobennzene: DNCB)の推奨適用濃度、および測定指標である CD86/CD54 発現亢進最小濃度の算出方法を見直した。また、化粧品原料に関する h-CLAT の有用性を目的として防腐剤、染毛剤および香料 30 品を評価し、h-CLAT の有用性を示した[8, 9, 10]。

本試験法の信頼性を、さらに向上させることを目的として、課題と考えられる３つのテーマについて背景データを取得した。具体的には、①細胞選択時の対照物質である Ni および SLS の推奨濃度の決定、②細胞継代方法の違いが結果に与える影響、③測定に用いるフローサイトメーターの精度管理に関する基礎的研究を行った。

このような経緯を経て、試験法のプロトコールが確立できたことから、本研究班では OECD ガイドライン化のための最短経路として、ECVAM（欧州動物実験代替法バリデーションセンター）と共同で h-CLAT バリデーションを行うこととし、ECVAM で行う３つの皮膚感作性試験代替法のバリデーションに組み込んだ。

B. 研究方法

h-CLAT の開発者である 2 施設（花王株式会社および株式会社資生堂）をリード施設として、本バリデーションへの参加を委託し、リード施設および日本の代表として、ECVAM が開催した３回のバリデーション実行委員会への参加を促した。本年度はこれらの会議を経て、研究計画の構築を実施した。

バリデーション実行委員会の開催日
第一回　2009 年 9 月 30 日～10 月 1 日
第二回　2009 年 12 月 2 日～3 日
第三回　2010 年 1 月 12 日～13 日

いずれも開催地は、イタリア、イスプラ市の ECVAM であった。

（倫理面への配慮）

本研究は動物実験における 3R の原則を推進するため、皮膚感作性試験代替法を開発するものである。実験動物は行わず in vitro の実験のみを行い、ヒトや動物の権利や福祉に抵触するところはない。

C. 研究結果

3回のバリデーション実行委員会にて、実験スケジュール、被験物質数、被験物質の種類、繰り返しの回数等が決定した。添付資料1および2に、第2および3回の会議議事録を示す。

本バリデーションは、プレバリデーションの位置付けとして、PhaseⅢプレバリデーションと名付けられた。バリデーションの目的は、技術移転および再現性の確認である。本バリデーションの概要を以下に示す。

1）h－CLAT協力施設

資生堂、花王（以上、リード施設）、IVM（In-house, validation and Training Laboratory, ECVAM）、Bioassay GmbH（ドイツ）の4施設

2）被験物質数 24（皮膚感作性陽性物質16、陰性物質8）

これ以上の情報はバリデーションが終了するまで公開されない。

3）バリデーションの段階

・技術移転

Phase A Stage Ⅰ：リード施設による技術指導

Phase A Stage Ⅱ：各施設での予備試験（コード化なし）

・再現性の確認

Phase B Stage Ⅰ：コード化された9物質を用いて1回／施設

Phase B Stage Ⅱ：コード化された15物質を用いて3回／施設

4）日程

以下の表に日程を示す。リード施設による指導としては、3月1～5日に食品薬品安全センター 秦野研究所をお借りして実施した。すなわち、3月1日より、本プレバリデーションが開始された。そのプログラムおよび技術移転実施報告書を添付資料3および4に示した。当日試験法の説明に用いた資料を別添5および6に示した。

5）統計学的検定 ECVAMの統計学者担当

これらの詳細は、実験デザイン（添付資料7）および計画（添付資料8）に記載されている。

表．今後の計画案

段階	終了期限
Phase A Stage Ⅰ	2010年3月31日
Phase A Stage Ⅱ	2010年6月30日
Phase B Stage Ⅰ	2010年9月30日
Phase B Stage Ⅱ	2011年7月31日
データ解析	2011年9月30日
(ECVAM 統計学者)	
最終報告書案 (ECVAM および VMG)	2012年2月29日
最終報告書 (ECVAM および VMG)	2012年4月30日

D. 考察

3回のバリデーション実行委員会には、リード施設である資生堂および花王の代表者のうち、少なくとも1名が参加した。日本からは日程および予算の関係で、他の参加者はおらず、その影響で実行委員会のメンバーから日本人が外れてしまった。本件を共同研究と唱える日本の主張を考慮しないものとECVAMに抗議し、調整を進めている。

これまで、日本として本バリデーションの計画に対して、被験物質の選定および統計学的な処理に対する意見を送ってきた。しかし、ECVAMから満足な回答は得られていない。そこで、国内の専門家、統計学者に協力を願い、h-CLATワーキンググループを立ち上げ、より的確な意見を、日本からの正式なコメントとして送るシステムを構築し、ECVAMとの意思疎通を図ることにした。今後、本グループの代表も実行委員会に参画できるようECVAMに要望していく。

特に、統計学的な処理に関する意思疎通が重要である。次回の会議が予定されている6月末までには日本の正式な意見を伝え、データ解析の再考に反映させていきたい。

E. 結論

h-CLATの国際バリデーションが開始された。本バリデーション実行委員会には、日本の代表を送り、ECVAMとの共同バリデーションがスムースに進行するよう支えていきたい。

F. 健康危険情報

なし

G. 知的財産権の出願・登録状況

なし

H. 参考文献

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J. 添付資料

1) Direct Peptide Reactivity Assay, human Cell Line Activation Test, Myeloid U937 Skin Sensitisation Test, Phase III Prevalidation. 2nd Validation Management Team Meeting minutes
2) Direct Peptide Reactivity Assay, human Cell Line Activation Test, Myeloid U937 Skin Sensitisation Test, Phase III Prevalidation. 3rd Validation Management Team Meeting minutes
3) 技術移転スケジュール (Time schedule)
4) h-CLAT トレーニング報告書
5) human Cell Line Activation Test : h-CLAT (in Vitro Skin Sensitization Test)
6) How to do "h-CLAT"
7) Phase III Pre-validation Study draft experimental design
8) Draft Project Plan

Direct Peptide Reactivity Assay, human Cell Line Activation Test, Myeloid U937 Skin Sensitisation Test
Phase III Prevalidation

2nd Validation Management Team Meeting minutes

December 2nd and 3rd 2009, ECVAM, Ispra, Italy

Meeting participants:

David Basketter (DB; chair), Silvia Casati (SC; co-chair), Alexandre Angers (AA; ECVAM representative), Pierre Aeby (PA; external expert), Sebastian Hoffmann (SH, external expert), Thomas Cole (TC; chair of the Chemicals Selection Group (CSG)), Jon Richmond (JR; external expert), Andre Kleesang (AK; ECVAM biostatistician), Anna Compagnoni (AC, ECVAM biostatistician), Luca Tosti (LT; CSG member), Takao Ashikaga (TA; h-CLAT lead laboratory representative), Hitoshi Sakaguchi (HS; h-CLAT lead laboratory representative), Jean-Marc Ovigne (JMO; MUSST lead laboratory representative).

Maurice Whelan (MW; Head of Unit of the System Toxicology Unit, IHCP) attended the first day of the meeting.

Frank Gerberick (FG; DPRA lead laboratory representative), Leslie Foertsch (LF; DPRA lead laboratory expert), William Stokes (WS, NICEATM), Dave Allen (DA; NICEATM) and Eleni Salicru (ES; NICEATM) participated at the meeting via teleconference during the afternoon sessions.

Introduction

The meeting started with the Agenda presentation and approval by the meeting participants. The nature and structure of the printed documents were explained.

Session 1: Review of Minutes and Actions

The minutes of the 1st VMT meeting were endorsed by all the participants, as only minor typos were noticed. Going through the list of Actions showed that all of them have been addressed by the responsible parties, without going into further detail at this time.

Session 2: h-CLAT, review of progress done.

The first document presented by TA and HS, in response to Action 9 of the previous meeting, was a troubleshooting table where they describe how to deal when cells are not responding as expected. The section "No cytotoxicity is observed (>90% viability) at the maximum applying dose", was clarified to have consequence about the classification of the chemical, since negative results would be considered inconclusive, rather than being part of a troubleshooting guide. Also, the mention that this would not apply to proteins should be removed, since the method is proposed for the testing of chemical substances only. This should be made clear in the text of the SOP

Action (TA & HS): Clarify in the SOP that the method is proposed to test chemicals

Other details of the troubleshooting instructions should be corrected (eg. "more than 90%" instead of "less than 90%"). An additional general comment on this table was that this information should be presented in the relevant sections of the SOP rather than be gathered in a separate table

Action (TA & HS): Correct, and incorporate the troubleshooting instructions in the text of the SOP

SC added that ECVAM will go through the MUSST and h-CLAT SOPs as it was done for the DPRA and make suggestions in tracked changes for the parts that seem not to be completely clear or detailed enough, and send the document to the test submitters so that they can approve or not the suggested changes. Ultimately it will be the VMG responsibility to endorse the SOP before these are sent to the other laboratories participating in the study.

Action (ECVAM): revise the h-CLAT and MUSST SOP with tracked changes and send to the respective test submitters.

JMO asked whether it would be possible to organize a teleconference to discuss the proposed changes it was replied that this was done with the DPRA with very efficient results.

Regarding Action 13 on the patents implication, discussions started with what the patent office told SC. The current validation exercise doesn't fall within the patent because it is considered a research activity. The same feedback was received from l'Oréal. It was suggested that this could be an issue for the contract laboratories who will be paid for performing the study, but it was commented that the contract laboratories will not make profits from this study. MW suggested we should discuss internally the patent issue since this applies to other validation studies as well. Regarding the Japanese Shiseido's statement was read and accepted by the VMT.

Discussions then moved to the requirement for the third run in the h-CLAT if the two first run give identical results, since in this case the results of a third run would never affect the final conclusion for the chemical. TA agreed that for classifying the chemical the third run is not required, but expressed the concern that comparing the RFIs values would be complicated if the chemicals are not analysed in the same numbers of runs. AA explained that this scenario is the current situation for the MUSST, and that the ECVAM statisticians have expressed no concerns about analysing such situations. JR suggested that for the validation we keep the requirement for three runs but we need to consider that at the end of the study the proposed protocol might incorporate the need only of two runs. HS and JMO suggested that the evaluation of the EC150 or EC200 values will be more precise with 3 runs, which is important for the purpose of the GHS classifications.

Action (TA & HS): Include in the SOP a section on EC150 and EC200 (how to calculate them, how to report them...)

SH commented that with the primary goal of the study the information gained from the third run is almost zero. JR added that if there is a third test we might spend a lot of time discussing about the meaning of the third result. SH added that you would discard the third

run in any case if discordant during the statistical evaluation. But since there was no statistical justification for deciding two or three runs, the conclusion was that the PM should be applied as it was submitted.

Session 2: MUSST, review of progress done.

For the action related to limiting the number of testing, JMO repeated the occasional need for up to five valid runs for deriving conclusions. The occurrence of invalid runs is very small, and he would be amenable to the idea of establishing a limit of 6, with a maximum of one invalid run. In any case, after two consecutive invalid runs the testing should be halted and the problem investigated. JMO expressed a concern that having the runs spread over many months might increase the odds of having more than one invalid run per chemical.

JMO having supplied an updated SOP, a quick comment from ECVAM was the absence of an introductory paragraph at the beginning of the SOP where the scope and nature of the test would be described. Also, a clear list of reagents and instruments needed should be included.

Action (JMO) Include these sections at the beginning of the SOP

As a list of equipment has been compiled by ECVAM for the purpose of the tender, it was proposed that this could be shared as a starting point.

Action (ECVAM) Provide as a starter the list done for the call for tender to JMO

JR enquired whether it was necessary to specify the day in the culture and pre-culture table, since contract research organizations might organize their workforce differently and be available to do work in the week-end. JMO agreed to change Monday into day zero and the rest of the table accordingly, but he will need to introduce some additional guidelines to ensure that the cells are not overgrown before testing.

Action (JMO) Make the changes in the SOP

The analyses supplied in response to the Action about analysing the MUSST results in the light of the GHS classification system was deemed to be insufficient by ECVAM, as it only took into account the classified and non classified. The point was to test if the MUSST can give initial insights whether a chemical is a strong or weak sensitiser, i.e. distinguish between class 1A and 1B. Concerns were raised regarding evaluating these methods for their ability to provide information on potency when they where submitted only for the purpose of discriminating positive versus negative compounds. It was clarified that this would not be the primary aim of the study but would simply represent additional info and not a key decision criteria regarding the performance of the test. It was agreed that such type of analysis should be performed in the context of this evaluation rather than having it performed by somebody else afterwards.

Action (JMO): Try to see if the results from the MUSST can classify the chemicals according to GHS classes 1A and 1B.

A general comment was raised that it would be important for ECVAM to confirm that the three laboratories assigned consistent GSH classes to the chemicals they used when establishing the Cooper's statistics tables.

Action (ECVAM) Verify concordance in the *in vivo* classifications between the three tests

AA added that if IC150 and CV70 values are calculated, they should be mentioned in the reporting section. JMO replied that this was already part of the Excel reporting template that he has supplied in addition he clarified that in-house they base the calculation of the IC150 only on two positive runs. If a third run is performed and this is negative, this is not considered since it might impact the calculation of the EC150. It was mentioned that within the validation study the IC150s will be calculated in the same way but since all the data are captured they could be recalculated in future analyses.

The updated prediction model was then clarified as now requiring only two increasingly positive results for a run to be classified as positive.

Action 18, on the differences between the final SOP and the one used in the ring trial, was thought by ECVAM not to have been performed, but JMO clarified that this was included in the "Response to comments" word file that he supplied in response to Action 19

Action (ECVAM): Check the results of Action 18 and comment before next meeting.

Session 3: Study Plan

TA and HS agree on the wording of the financing section between JaCVAM and the lead laboratories.

JMO mentioned that the automated plate sampler for the flow cytometry is not required by the MUSST, but that they will be using one in their part of the study. L'Oreal expressed concerns regarding the fact that the MUSST is not performed in the IVM laboratory. JR mentioned that in this situation, there are two decisions, one that ECVAM will perform only one test, and the second about which test is chosen, and for the second one there is a transparency issue. ECVAM clarified that there was no "choice" being made of one method over the other, but that this decision was based on the fact that the MUSST SOP has been interpreted as being written for acquisition in a 96-well plate, and that the equipment could not be acquired in time for the beginning of the experimental phase.

MW would like to have general internal discussions about the choice of methods for in-house validation, including the idea to discuss the criteria of selecting external laboratories with the test submitter. SC mentioned that this was impossible to do because the tender had to be submitted before the first VMT meeting in order to complete the procedure in time.

SC mentioned that the current plan is that the third laboratory work will be contracted, and this should be done within the next two months. As part of the discussions, JMO mentioned that automated acquisition in 96-well plates should take into consideration, as an important factor, the time of acquisition between the first and the last well.

Action (JMO): insert time considerations for acquisition in the MUSST protocol.

Action (ECVAM): have internal discussions about a transparent explanation about the choice of MUSST instead of h-CLAT as the method validated in-house.

HS asked whether the 3rd laboratories will be the same for all methods, and SC updated the VMT about the tender situation, and concluded that the evaluation was finished but that the chosen laboratories could not be revealed at this time until the contract has been signed. In any case, there was no requirement that the same laboratory would participate to all three methods.

HS asked if within ECVAM different persons would be performing the different assays to make sure the person can not "break the code" of the chemicals by comparing the chemical's physical characteristics and using this to influence the analyses. This was confirmed to be the case, although these considerations were not thought to be of great importance.

Action (S&K): communicate the names of study directors, quality assurance and safety officers

AK expressed the view that it is absolutely required that the QA officer and the study director are a different person.

Action (L'Oréal): find a study director different from the quality assurance officer.

JMO expressed the fact that this is an issue for him because of the lack of QA officers in the current organization of his research facility. It was mentioned that Cecile Piroird's role is more compatible with the role of the study director.

JR noticed that the wording of the spirit of GLP minimum requirements should be changed to make sure that these were instructions, not suggestions.

Action (ECVAM): replace the words "should" with the words "shall" in the spirit of GLP minimum requirements

PA suggested that we should add that the data generated should be recorded "in a dedicated notebook". DB mentioned we don't need to be that precise in our instructions and leave it to the laboratories how to comply with the requirements. It was agreed to leave it open the possibility to the labs to chose their own system

On page eleven phase B first paragraph the goals should be harmonized.

JMO suggests that the sealed envelope that comes with the coded chemical should be one envelope per chemical to make sure the whole experiment is not affected if there is an accident with one chemical. TM mentioned that in any case, chemicals with dangerous properties would not be selected in the study. JR added that we should make sure the warning that comes with the chemicals should be adapted to all the local rules where the study will be performed.

Session 4: DPRA, review of progress done.

For this session and the next one, FG, LF, WS, DA and ES joined by teleconference.

FG reviewed the work done related to the Actions of last meeting and mentioned that they are in agreement with 99% of the modifications suggested by ECVAM on the DPRA SOP which was sent the preceding week with tracked changes in the document. He added that the SOP is currently being worked on by LF and that they hope to send us their revised version by next Monday (the 7th of December 2009).

Session 5: Experimental Design

The updated proposed Experimental Design, including the document that summarizes the results of Actions 20 to 22 of the previous meeting, was presented. In this new version, the calculations for the determination of sample size are now performed in two ways: first, using the concordance of the predictions and second, using the raw data. Both agree on the number of 21 chemicals. The main difference with the previous version is that the statistical power has been reduced to a level which is still acceptable for reproducibility. The lower number of chemical has the disadvantage of decreasing the strength of the preliminary evaluation of the predictive capacity of the methods, but since this is a secondary goal of the study, and as the amount of work to be done is a concern, this compromise was deemed necessary. The same number was calculated for the between and within laboratory reproducibility.

At this point DB asked the Liaisons for reactions. DA and WS's main comment was whether this number was sufficient to correctly evaluate the reproducibility of chemicals with different physico-chemical properties, and that a sample size based on simple power calculations, might not cover these considerations. Other comments included certain concerns about the amount of work required for a pre-validation, and that it would be important to consider for the chemical selection how much additional work would be needed for the subsequent evaluation of the relevance of the test. Furthermore, they considered the reliability of the tests to be more informative with sensitiser of low and moderate potency, rather than non-sensitisers or extreme sensitisers, and questioned the decision to split the selection with equal number of sensitisers and non-sensitisers.

Discussions moved to the requirement to have the WLR evaluated in each of the laboratories, or if it could be evaluated in a single laboratory. The biostatistician's response to this related to the fact that the WLR is very much dependent on the experience of the laboratory. If it was possible to know in advance which laboratory would have the worse reproducibility, then it would be sufficient to have it performed there, but this information is not available beforehand.

HS mentioned a suggestion from a Japanese statistician who indicated that the WLR could be assessed with a subset of the chemicals (e.g. 7). DA expressed concerns about the impact of selecting a subset of chemicals, since differences in physico-chemical properties, and potency classes could have a great impact on the reproducibility.

The Japanese document "Comments on the statistical aspect of "Phase III Pre-validation Study draft experimental design" for Direct Peptide Reactivity Assay (DPRA), human-Cell Line Activation Test (h-CLAT), and Myeloid U937 Skin Sensitisation Test (MUSST)", by Isao Yoshimura (Prof., Tokyo University of Science), Takashi Omori (Prof., Kyoto University), Takashi Sozu (Prof., Osaka University) was introduced and discussed.

Because of the "concordance" has been wrongly defined by the authors as the concordance between the prediction model result and the in vivo classification of the chemicals, and because there is no mention of the requirements of evaluating the WLR, it is ECVAM's opinion that the conclusions of this document do no challenge the proposed Experimental Design.

The table provided in this document can still be used to confirm the number of chemicals required to evaluate the BLR, i.e. 36 divided by the square root of 3, which is 21. SH's suggestion was to see if this table could be used to justify a lower number chemicals used for the WLR, by accepting the assumption that the WLR will be lower than the BLR, which has been historically shown to be the case. He sent by e-mail a request to the authors of the document to ask the value of N with an expected concordance of 0.95, which is not shown in the original version of the document. This number should be available for the discussions of the next day.

WS also proposed to share a document explaining the design of a validation study for methods evaluating endocrine disrupters, which he will present on the next day's session "Feedback from Liaison Representatives".

2nd Day

Since it was in the morning, FG, LF, WS, DA and ES were not attending this part of the session.

DB repeated the importance of coming to an agreement on the study design in order to start defining the study timelines. The main discussions revolved around the strategies to decrease the work involved in the evaluation of the WLR in each laboratory.

An initial suggestion to separate the chemicals in three groups so that each laboratory evaluates a third or the chemicals was rejected on the basis that it adds an additional unknown in the analyses, i.e. the fact that the chemicals are different and could have different intrinsic reproducibility. Furthermore, it is important to evaluate WLR in a statistically significant way within each laboratory, and pooling results from different laboratories assuming they came from a single one was not considered appropriate by the biostatisticians. Strategies involving overlaps of subsets of chemicals were proposed, but the reduction of work they presented compared to the increase in complexity was deemed to be disputable.

AC performed the calculations using the method proposed in the Experimental Design, but using parameters resulting from assuming a higher concordance of results for the WLR. Her calculations showed that, with this assumption, the required number of chemicals was found to be 13-14 chemicals. Additionally, the Japanese biostatistician informed SH that the N for the concordance of 0.95, using their own method of calculation, was 19, which divided by square root of three, is 11.

It was then decided to use this fresh input to justify a lower number of chemicals for the evaluation of the WLR, which allowed reaching a consensus. Since these numbers represented the minimal number of chemical required, DB proposed an experimental design that would include addition chemicals for safety:

- For evaluation of the BLR, 24 chemicals tested once in every laboratory
- For evaluation of the WLR, 15 chemicals tested two further times in each laboratory, the same subset being used at every site.

No objections were raised against this design. Furthermore, HS mentioned that he consulted his Japanese biostatisticians with this proposal, and that they were in agreement.

It was clarified that, in this case, the subset of chemicals to be selected would be chosen randomly from the chemicals selected for the evaluation of the BLR, as this was the only method that was statistically defendable.

Session 6: Study Timelines

Chemical selection:

TC said that since the number of required chemical had been decided, the list should be ready for VMG approval for the next meeting in January 2010.

Study plan:

SC commented that the study plan should be sent to the laboratories involved in the study, as they will need to read it and sign it. The Experimental Design will be ultimately incorporated in the study plan since the laboratories need to know the work involved. The tentative date for the final draft of the study plan is also the next meeting, in January 2010.

Chemical coding and distribution:

This phase could be overlapping with the training phase of the laboratories, the most critical factor being obtaining the chemical from their commercial sources.

Training:

For the MUSST, the training could be done in a week, although JMO requested to know in advance so that they can be prepared. Also, he requested that all laboratories be trained at the same time. The test submitters for the h-CLAT will need to choose which of the two laboratories will be involved in the training, since the suggestion of having the two naïve laboratories trained one at each site was refused by ECVAM. Also, since the travel expenses during the training phase is paid by the participating laboratory, the training period can not be too long. Tentative final date to end the training: end of March 2010.

It was also discussed that the pace of the three methods can be independent; however, the laboratories involved in the evaluation of the same test will need to meet at the three milestones, at the end of Phase A Stage I, Phase A Stage II and Phase B Stage I, before proceeding.

Transferability:

It is the responsibility of the lead laboratories to define a transfer protocol, i.e. which chemicals should be used for the transfer, as well as the criteria that will be set to decide if the

transfer was successful. It is not necessary that the details are harmonized between the methods.

Action (lead laboratories): Write a transfer protocol

Action (ECVAM): find a sample transfer protocol from previous studies to distribute as a guide.

It should also be discussed whether the lead laboratories will be responsible for supplying the chemicals used in this phase. JMO mentioned that L'Oréal does not normally send chemicals for legal reasons and suggests a commercial source to the laboratories. It was also decided that, despite ICCVAM's comment on the study plan, the chemicals will not be coded at this phase.

For the timeline, it was suggested that this phase should last between one and two months, putting the tentative deadline at mid-June 2010.

The discussion then moved to ICCVAM's comment about establishing criteria for the reproducibility to be acceptable.

AC started by saying that when you establish the parameters for the calculations of the sample size, you make the assumptions about what the results should be, so the minimum standard is built in the calculations. AK shared a discussion in the Eye Irritation validation study where lead laboratories were not amenable to the idea of setting the criteria into stone, so the criteria would be established as "guidelines" on what they would like to obtain, adding flexibility clauses that would allow, for example, excluding chemicals based on the applicability domain.

JR mentioned that in many reviewing processes, the pass/fail criteria are not set and it is the reviewer himself who makes the judgement. Expectations should be set rather than pass/fail criteria, i.e. "in the order of". SH suggested the wording "we expect around 80% concordance for the chemicals for which we did not foresee any complications." JMO's main concern is that someone might challenge a study where the criteria were not established from the beginning on the basis that those criteria could have been determined post-hoc to ensure that the methods would pass them.

Action (ECVAM): include in the agenda of next meeting "discussions about the criteria" and "transferability planning".

The session on the study plan comments ended with a clarification that the "X Weekly report", which is important for the VMG to know which stages the participating laboratories have reached and know of any problem as they arise, should be set up as "monthly"

Session 7: Comments from Liaison Representatives

For this session, FG, LF, WS, DA and ES joined by teleconference.

WS (ICCVAM) went through the presentation of the Lumicell validation and explained the experimental design for this study.