新規試験法提案書

皮膚感作性試験代替法（LLNA-DA 法）

平成25年 1 月

国立医薬品食品衛生研究所

新規試験法提案書

平成 25 年 1 月 20 日
No. 2012-05

皮膚感作性試験代替法 Local Lymph Node Assay (LLNA):DA の判定基準の変更に関する提案

平成 24 年 10 月 1 日に東京、国立医薬品食品衛生研究所にて開催された新規試験法評価会議（通称：JaCVAM 評価会議）において以下の提案がなされた。

提案内容：皮膚外用剤として用いる医薬品、医療機器、化粧品、皮膚適用の医薬部外品、農薬等に含まれる物質又はそれらの製品の皮膚感作性を予測する皮膚感作性試験代替法 Local Lymph Node Assay (LLNA): DA は、RI を使用せずとも従来試験法と同等の結果が得られることから、行政上利用することは可能である。

この提案書は、米国Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) LLNA: DA Evaluation Report (2010)、LLNA: DAのJaCVAM評価報告 (2011) およびOECD Test Guideline (TG) 442Aをもとに、皮膚感作性試験代替法評価委員会によりまとめられた文書を用いてJaCVAM評価会議が評価および検討した結果、その有用性が確認されたことから作成された。

以上の理由により、行政当局の安全性評価方法として「皮膚感作性試験代替法LLNA:DAの判定基準の変更」に関する提案をするものである。

吉田武美

JaCVAM 評価会議　議長

西川秋佳

JaCVAM 運営委員会　委員長

JaCVAM 評価会議

吉田武美（日本毒性学会）：座長
浅野哲秀（日本環境変異原学会）
五十嵐良明（国立医薬品食品衛生研究所　生活衛生化学部）
大島健幸（日本化学工業協会）
小笠原弘道（独立行政法人　医薬品医療機器総合機構）
小野寺博志（独立行政法人　医薬品医療機器総合機構）
黒澤　努（日本動物実験代替法学会）
杉山真理子（日本化粧品工業連合会）
西川秋佳（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）
長谷川隆一（独立行政法人　製品評価技術基盤機構）
牧　栄二（日本免疫毒性学会）
増田光輝（座長推薦）
横関博雄（日本皮膚アレルギー・接触皮膚炎学会）
吉田　緑　（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　病理部）
吉村　功（座長推薦）
渡部一人（日本製薬工業協会）

任期：平成 24 年 4 月 1 日～平成 26 年 3 月 31 日

JaCVAM運営委員会

西川秋佳（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）：委員長
大野泰雄（国立医薬品食品衛生研究所）
小川久美子（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　病理部）
菅野　純（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　毒性部）
斎藤和幸（独立行政法人　医薬品医療機器総合機構）
佐々木正広（厚生労働省　医薬食品局　化学物質安全対策室）
関野祐子（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）
高木篤也（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　毒性部　動物管理室）
長谷部和久（厚生労働省　医薬食品局　化学物質安全対策室）
広瀬明彦　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　総合評価研究室）
本間正充（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　変異遺伝部）
光岡俊成（厚生労働省　医薬食品局　審査管理課）
小島　肇（（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　新規試験表評価室）：事務局

JaCVAM statement
on the Local Lymph Node Assay (LLNA): DA
for skin sensitization assay

At the meeting concerning the above method, held on 1 October 2012 at the National Institute of Health Sciences (NIHS), Tokyo, Japan, the members of the Japanese Center for the Validation of Alternative Methods (JaCVAM) Regulatory Acceptance Board unanimously endorsed the following statement:

The LLNA: DA can be used to identify substances as potential skin sensitizers or nonsensitizers as well as LLNA for regulatory use, without Radio-isotope.

Following the review of the results of the ICCVAM(Interagency Coordinating Committee on theValidation of Alternative Methods, USA) Evaluation Report, JaCVAM peer review panel reports, and OECD (Organisation for Economic Co-operation and Development) Test Guideline revised No. 442A, it is concluded that the LLNA: DA for skin sensitization assay is clearly beneficial.

The JaCVAM Regulatory Acceptance Board has been regularly kept informed of the progress of the study, and this endorsement is based on an assessment of various documents, including, in particular, the evaluation report prepared by the JaCVAM ad hoc peer review panel for skin sensitization assay.

Takemi Yoshida
Chairperson
JaCVAM Regulatory Acceptance Board

Akiyoshi Nishikawa
Chairperson
JaCVAM Steering Committee

20 January, 2013

The JaCVAM Regulatory Acceptance Board was established by the JaCVAM Steering Committee, and is composed of nominees from the industry and academia.

Mr. Takemi Yoshida (Japanese Society of Toxicology): Chairperson
Mr. Norihide Asano (Japanese Environmental Mutagen Society)
Mr. Yoshiaki Ikarashi (National Institute of Health Sciences: NIHS)
Mr. Takeyuki Oshima (Japan Chemical Industry Association)
Mr. Hiromichi Ogasawara (Pharmaceuticals and Medical Devices Agency)
Mr. Hiroshi Onodera (Pharmaceuticals and Medical Devices Agency)
Mr. Tsutomu Miki Kurosawa (Japanese Society for Animal Experimentation)
Ms. Mariko Sugiyama (Japan Cosmetic Industry Association)
Mr. Akiyoshi Nishikawa (Biological Safety Research Center: BSRC, NIHS)
Mr. Ryuichi Hasegawa (National Institute of Technology and Evaluation)
Mr. Eiji Maki (Japanese Society of Immunotoxicology)
Mr. Mitsuteru Masuda(nominee by Chairperson)
Mr. Hiroo Yokozeki (Japanese Society for Dermatoallergology and Contact Dermatitis)
Ms. Midori Yoshida (BSRC, NIHS)
Mr. Isao Yoshimura (nominee by Chairperson)
Mr. Kazuto Watanabe (Japan Pharmaceutical Manufacturers Association)

This statement was endorsed by the following members of the JaCVAM steering Committee after receiving the report from JaCVAM Regulatory Acceptance Board:

Mr. Akiyoshi Nishikawa (BSRC, NIHS): Chairperson
Mr. Yasuo Ohno (NIHS)
Ms. Kumiko Ogawa (Division of Pathology, BSRC, NIHS)
Mr. Jun Kanno (Division of Cellular and Molecular Toxicology, BSRC, NIHS)
Mr. Kazuyuki Saito (Pharmaceutical & Medical Devices Agency)
Mr. Masahiro Sasaki (Ministry of Health, Labour and Welfare)
Ms. Yuko Sekino (Division of Pharmacology, BSRC, NIHS)
Mr. Atsuya Takagi (Animal Management Section of the Division of Cellular and Molecular Toxicology, BSRC, NIHS)
Mr. Kazuhisa Hasebe (Ministry of Health, Labour and Welfare)
Mr. Akihiko Hirose (Division of Risk Assessment, BSRC, NIHS)
Mr. Masamitsu Honma (Division of Genetics and Mutagenesis, BSRC, NIHS)
Mr. Toshinari Mitsuoka (Ministry of Health, Labour and Welfare)
Mr. Hajime Kojima (Section for the Evaluation of Novel Methods, Division of Pharmacology, BSRC, NIHS):Secretary

皮膚感作性試験代替法 Local Lymph Node Assay (LLNA): DA

目次

皮膚感作性試験代替法 Local Lymph Node Assay(LLNA)：DA の判定基準の変更に関する評価会議報告

JaCVAM 評価会議

平成 24 年（2012 年）10 月 1 日

JaCVAM 評価会議

吉田武美（日本毒性学会）：座長
浅野哲秀（日本環境変異原学会）
五十嵐良明（国立医薬品食品衛生研究所）
大島健幸（日本化学工業協会）
小笠原弘道（独立行政法人　医薬品医療機器総合機構）
小野寺博志（独立行政法人　医薬品医療機器総合機構）
黒澤　努（日本動物実験代替法学会）
杉山真理子（日本化粧品工業連合会）
西川秋佳（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）
長谷川隆一（独立行政法人　製品評価技術基盤機構）
牧　栄二（日本免疫毒性学会）
増田光輝（座長推薦）
横関博雄（日本皮膚アレルギー・接触皮膚炎学会）
吉田　緑　（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）
吉村　功（座長推薦）
渡部一人（日本製薬工業協会）

任期：平成 24 年 4 月 1 日～平成 26 年 3 月 31 日

以上

皮膚感作性試験代替法 LLNA:DA については、既に JaCVAM 評価会議でその妥当性が評価されている [1)]。今回 LLNA:DA の判定基準の変更に関する感作性試験評価委員会からの報告 [2)]を受け、以下の 10 項目について評価したので報告する。

＜審議内容＞

1. 当該試験法は、どのような従来試験法を代替するものか。または、どのような毒性を評価あるいは予測するものか。

当該 Local Lymph Node Assay (LLNA):DA は、化学物質等の皮膚感作性を評価するモルモット Maximization Test (GPMT)あるいは Buehler Test (BT)の代替試験法であるマウス LLNA の改良試験法である。従って、当該試験法の予測するところは、従来試験法の LLNA が予測する化学物質等の皮膚感作性である。

2. 当該試験法と従来試験法の間にどのような科学的なつながりがあるか。

従来試験法は、感作に基づく耳介リンパ節の細胞増殖反応を放射性物質の[^{3}H-methyl]-thymidine (^{3}H-TdR)の DNA への取込みを指標として検出する試験法である。当該試験法は、感作誘導によるリンパ節細胞の増殖を検出するという試験法の原理は従来試験法と同じであるが、放射性物質の ^{3}H-TdR の代わりに、細胞中のアデノシン三リン酸（ATP）量をルシフェリンールシフェラーゼ反応による化学発光で計測するものである。

3. 当該試験法とそのデータは、透明で独立な科学的評価を受けているか。

ICCVAM は、第三者評価委員会を組織し、JaCVAM が実施した当該試験法の検証試験 [3)]で得られた 14 物質の試験成績を含む 44 物質の試験成績をレトロスペクティブに解析し、併せて従来試験法による結果と比較した。その解析において、当該試験法における皮膚感作性の判定基準としてカットオフ値≧1.8 を使用することにより、従来試験法と同等の結果が得られることを示し、当該試験法の精度、感度および特異性を評価した [4)]。その組織および評価結果は、ICCVAM のホームページで公表されている。

また、我が国の LLNA:DA 感作性試験評価委員会は、JaCVAM で実施された当該試験法の検証報告と上記 ICCVAM の評価結果を対比して評価した。

よって、当該試験法の判定基準の変更は、透明で独立な評価を受けていると判断される。

4. 当該試験法は、従来試験法の代替法として、どのような物質又は製品を評価することを目的としているか。

当該試験法は、皮膚外用剤として用いる医薬品、医療機器、化粧品、皮膚適用の医薬部外品、農薬等に含まれる物質又はそれらの製品に求められる皮膚感作性を評価する従来試験法の代替法としての使用を目的とする。

5. 当該試験法は、ハザード評価あるいはリスク評価のどちらに有用であるか。

当該試験法は、上記の物質又は製品における皮膚感作性のハザード評価に有用である。

6. **当該試験法は、目的とする物質又は製品の毒性を評価できるか。その場合、当該試験法の適用条件が明確になっているか。**

当該試験法の妥当性を示すデータは、JaCVAM が検証試験に使用した 14 物質を含む 44 物質（化粧品、化成品、農薬、医薬品、殺菌消毒剤、合成中間体および原材料、食品添加物、香料、衛生材料および溶剤）の試験成績である。よって、当該試験法は、これらを包括する物質又は製品の皮膚感作性を評価することができるといえる。

当該試験法においては､従来試験法と同様に過度の局所刺激性や明らかな全身毒性を示さない用量を最高用量とする。偽陰性を排除するため、皮膚感作性の陽性判定基準を JaCVAM で検証したカットオフ値≧3.0 から≧1.8 に変更した。この変更された判定基準においては、偽陽性を示す物質も存在するため、皮膚感作性陽性の最終判定は、被験物質に関する付加的情報（例えば、用量反応情報、全身毒性若しくは過剰な局所皮膚刺激性の証拠、タンパク結合性、分子量、関連化学物質の成績等）を考慮して行う必要がある。

適用限界は、従来試験法の LLNA と同様である。

7. **当該試験法はプロトコルの微細な変更に対して頑健であるか。**

当該試験法は、従来試験法と原理的に同じであることから、当該試験法の精度並びに施設内および施設間再現性および頑健性は、従来試験法と同じであると考えられる。当該試験法は、リンパ節細胞中の ATP 含量を指標とするため､ATP 合成およびその測定に影響を及ぼす物質の検討には適さない。また、リンパ節摘出後、ATP 含量は経時的に低下することから、リンパ節摘出から発光測定までの操作は、個体毎の時間経過を揃えることが望ましい。更に、発光量や減衰性は、使用する市販の ATP 測定試薬によっても差がある。しかし、当該試験法は、ICCVAM が推奨する最新のプロトコルに従い実施するならば、微細な変更に対して頑健な方法である。

8. **当該試験法の技術習得は、適切な訓練と経験を経ている担当者にとって容易なものであるか。試験法の実施に特殊な設備が必要か。**

当該試験法は、適切な訓練を受け、経験を積んだ担当者にとってその技術習得は容易である。従来試験法に比べ、放射性取扱施設等の特殊な設備は必要ない。

9. **当該試験法は、従来試験法と比べて時間的経費的に優れているか。**

従来試験法は、リンパ球の増殖反応を測定する方法として放射性物質（RI）を使用するため、特殊な実験施設や設備を必要とし、放射能管理、廃棄物の処理問題等、試験を実施する上で種々の制約があった。一方、当該試験法は、通常の実験設備が使用でき、また、RI を使用しないことより、特殊設備や廃棄物処理の管理が不要であり、時間的経費的に優れている。

10. **当該試験法は、動物福祉の観点及び科学的見地から、目的とする物質又は製品の毒性を評価する代替法として、行政上利用することは可能か。**

当該試験法は、動物を用いない代替試験法ではない。しかし、GPMT 等他の皮膚感作性試験法と比較して、動物に与えるストレスは少なく、苦痛の軽減という点で優れている。皮膚外用剤として用いる医薬品、医療機器、化粧品、皮膚適用の医薬部外品、農薬等に含まれる物質又はそれらの製品の

皮膚感作性を予測する当該試験法は、判定基準のカットオフ値を下げることにより偽陰性の排除が可能になるとともに、RI を使用せずとも従来試験法と同等の結果が得られることから、行政上利用することは可能である。

参考文献

1) 皮膚感作性試験代替法（LLNA：DA 法）の評価会議報告書、JaCVAM 評価会議（平成 20 年 10 月、平成 23 年 4 月改定）
2) 皮膚感作性試験代替法 Local Lymph Node Assay (LLNA)-DA の第三者評価報告（平成 24 年 7 月）
3) Takashi Omori, et al. Interlaboratory validation of the modified murine local lymph node assay based on adenosine triphosphate measurement. Journal of Pharmacological and Toxicological Methods. 58: 11-26 (2008)
4) ICCVAM (2010). ICCVAM Test Method Evaluation Report on the Murine Local Lymph Node Assay: DA, a Nonradioactive Alternative Test Method to Assess the Allergic Contact Dermatitis Potential of Chemicals and Products. NIH Publication No. 10-7551. Research Triangle Park, NC: National Institute of Environmental Health Sciences

Local Lymph Node Assay: DA (LLNA: DA、局所リンパ節試験:DA)の概要およびその調査結果

平成 24 年 6 月 25 日

JaCVAM 皮膚感作性試験第三者評価委員会

委員長　金澤 由基子 [(独) 医薬品医療機器総合機構]
委員　　牧 栄二 [前 (財) 食品農医薬品安全性評価センター]

要旨

マウスにおける Local Lymph Node Assay (LLNA，局所リンパ節試験)は、感作に基づく耳介リンパ節の細胞増殖反応を放射性物質の[^{3}H-methyl]-thymidine（^{3}H-TdR）の取り込み量を測定することで定量的かつ客観的に判定する試験法である。LLNA-DA は、放射性物質の ^{3}H-TdR の取り込み量の代わりに、感作に基づく増殖耳介リンパ節細胞中のアデノシン三リン酸（ATP）量を測定することにより化学物質の皮膚感作性を判定するもので、本邦にて開発された LLNA の変法であるが、被験物質の皮膚感作性陽性を判断するカットオフ値［溶媒処置群（陰性対照群）に対する被験物質処置群の ATP 量の比（Stimulation index、SI 値)］に本邦と海外において違いが生じている。

本報告では、JaCVAM で実施された LLNA-DA の検証報告（2008）と ICCVAM の LLNA-DA Evaluation Report (2010) を対比し、両者のカットオフ値（SI 値）の違いについて調査を行った。その結果、ICCVAM が検証の対象とした皮膚感作性陽性 32 物質全てを陽性と判定するカットオフ値 1.8 を判定基準として採用することが妥当であると考えた。また、LLNA-DA に関する OECD ガイドライン（2010）においても、皮膚感作性陽性を示すカットオフ値として 1.8 が設定されている。このような状況を踏まえると、本邦においても 1.8 をカットオフ値として設定することが妥当であると考える。一方、SI 値が 1.8 から 3 の間には、皮膚感作性偽陽性を示す化合物も存在するため、化合物の皮膚感作性の最終判定においては、ICCVAM が勧告する付加的な情報（例えば、用量反応情報、全身毒性若しくは過剰な局所皮膚刺激の証拠、必要に応じて、処置群と溶媒対照群の統計的な比較、ペプチド反応性、分子量、関連物質の結果、他の試験データ）を考慮して決定する必要がある。

はじめに

マウスにおける Local Lymph Node Assay (LLNA，局所リンパ節試験)は、皮膚外用剤として用いる医薬品ならびに化粧品原料を含む化学物質等の皮膚接触感作性のリスクを動物で予測するモルモットにおける Maximization Test (GPMT)或いは Buehler Test (BT)の代替試験法であり、その予測率は、GPMT に劣らないとされ [1]、国際的に認知されている。LLNA は、感作に基づく耳介リンパ節の細胞増殖反応を放射性物質の[^{3}H-methyl]-thymidine（^{3}H-TdR）の取り込み量を測定することで定量的かつ客観的に判定する試験法である。LLNA-DA は、放射性物質の ^{3}H-TdR の取り込み量の代わりに、感作に基づく増殖耳介リンパ節細胞中のアデノシン三リン酸（ATP）量を測定することにより化学物質の皮膚感作性を判定するもので、本邦にて開発された LLNA の変法である。LLNA-DA の試験法の原理並びに簡便性は、海外においても認められるところであるが、被験物質の皮膚感作性陽性を示すカットオフ値［溶媒処置群（陰性対照群）に対する被験物質処置群の ATP 量の比 (Stimulation index、SI 値)］に本邦と海外において違いが生じている。

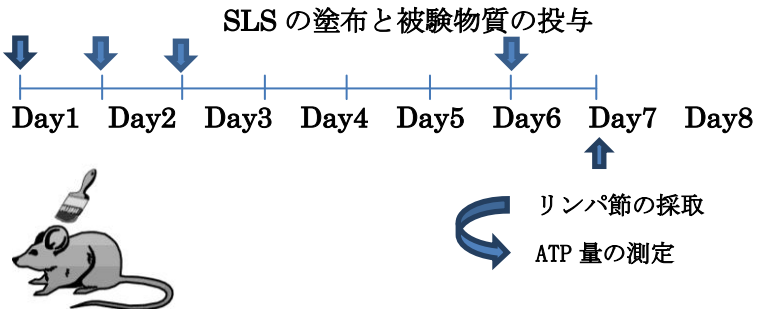
本報告では、JaCVAM で実施された LLNA-DA の検証報告（2008）[2]と ICCVAM の

LLNA-DA Evaluation Report (2010) [3] を対比し、両者のカットオフ値の違いについて調査を行ったので、その結果並びに JaCVAM 皮膚感作性試験第三者評価委員会（以下、委員会）としての提案を述べる。

１．試験法

LLNA-DA（図1）の試験法の原理は、概ね原法のLLNAと同じである。LLNA-DAでは1、2 および 3日目に加え、7日目にも被験物質を耳介に経皮投与し、更に、各回の経皮投与に先立って感作を増強させる目的で投与部位にsodium lauryl sulfate（SLS）の塗布を行う。8日目に採取した耳介リンパ節の感作に伴う細胞増殖を検出する。即ち、皮膚感作性を有する低分子化合物が経皮投与されると、皮膚組織中のタンパク質と結合し、感作抗原として皮膚の樹状細胞に認識される。その後、樹状細胞は活性化しながら皮膚から所属リンパ節へ遊走し、抗原提示を行い抗原特異的なTリンパ球細胞の増殖を誘導する。この一連の生体応答が感作誘導期である。LLNAでは、感作誘導期のリンパ節における抗原特異的なTリンパ球細胞の増殖を放射性物質の^{3}H-TdRのDNAへの取り込みを指標として検出するが、LLNA-DAでは、感作終末点の細胞中のATP含量をルシフェリン-ルシフェラーゼ反応による化学発光を計測することにより検出するものである。

図１　LLNA-DA の概略



２．JaCVAM の LLNA-DA 検証試験における SI 値

JaCVAM の行った検証試験では、17 施設が参加し、14 化合物（abietic acid、3-aminophenol、cobalt chloride、dimethyl isophthalate、2,4-dinitrochlorobenzen、formaldehyde、glutaraldehyde、hexyl cinnamic aldehyde、isoeugenol、isopropanol、lactic acid、methyl salicylate、nickel sulfate、および potassium dichromate)について盲検下にて試験された。

初回の検証試験では、3 化合物（2,4-dinitrochlorobenzene、hexyl cinnamic aldehyde および isopropanol）が 10 施設で検証され、9 化合物（abietic acid、3-aminophenol、cobalt chloride、dimethyl isophthalate、formaldehyde、glutaraldehyde、isoeugenol、methyl

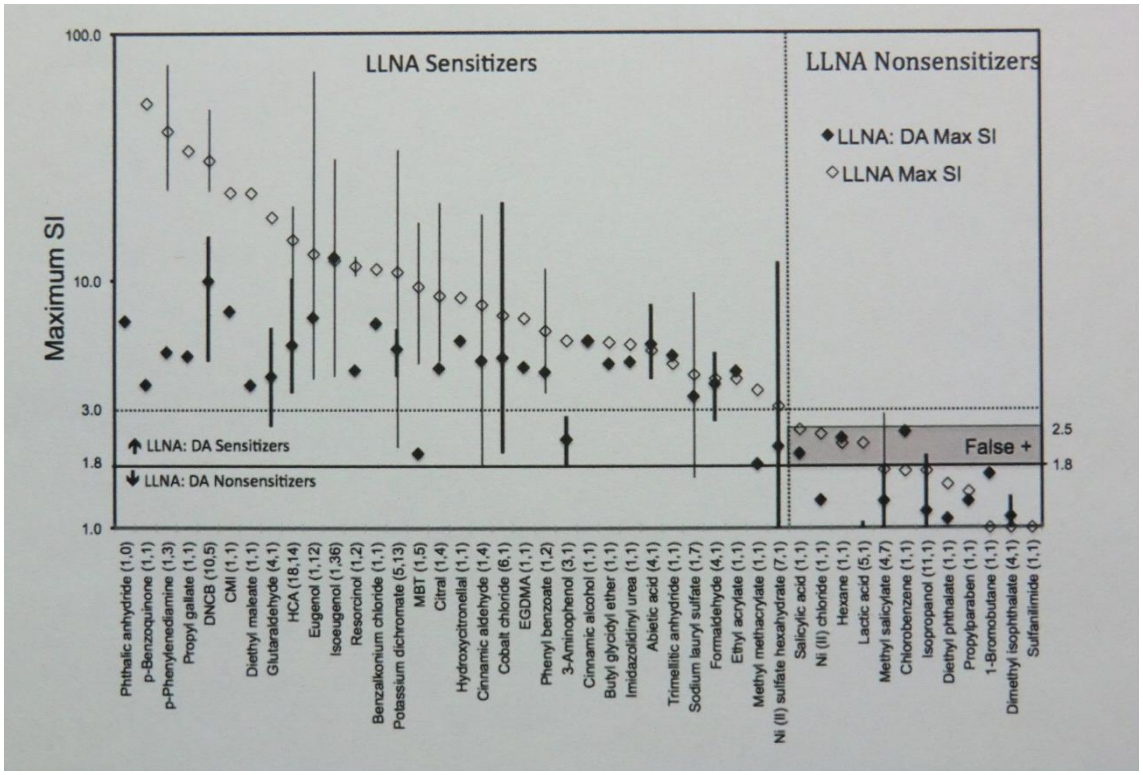
salicylate および nickel sulfate）が 3 施設で検証された。2 回目の検証試験では、1 化合物（hexyl cinnamic aldehyde）が 7 施設で、4 化合物（cobalt chloride、lactic acid、nickel sulfate および potassium dichromate）が 4 施設で検証された。試験結果は、各々の化合物処置群について ATP 含量をルシフェリンールシフェラーゼ法で測定し、化学発光量として示され、各々の化合物処置群の SI 値が同時に実施された溶媒対照群に対する ATP 含量の比として求められた。その結果、皮膚感作性陽性を示す SI 値は、≧3 と設定された。

初回の検証試験で 12 化合物について得られた結果は、それら化合物の SI 値において施設間バラツキは小さく、一貫性のあるものであった。GPMT/BT の結果のない glutaraldehyde を除く 11 化合物の SI 値≧ 3 における GPMT/BT に対する感度、特異性および精度は、各々7/8 (87.5%)、3/3 (100%)および 10/11 (90.0%) であった。2 回目の検証試験において、試験された全 5 化合物の施設間バラツキは、許容できる小さなものであった。

全 2 回の検証に使用された合計 14 化合物の内、皮膚感作性物質は 11 化合物であり、LLNA-DA ではその内の 2 化合物が偽陰性となった。偽陰性の 1 化合物の nickel sulfate は、原法の LLNA でも偽陰性と評価されている。

３． ICCVAM の LLNA-DA Evaluation Report における SI 値（図 2 参照）

図 2　LLNA-DA の SI 値と原法 LLNA の SI 値の比較



（化合物名の横の括弧内の数字は、LLNA-DAに続いて原法のLLNAの試験数を

示す。試験数は、類似の
　試験の最高用量のみが記載されているので、得られた試験の総数とは異なる
場合がある。）

ICCVAM は、次のように結論している。即ち、LLNA-DA の精度および信頼性は、化合物を、潜在的に皮膚感作性を有する物質或いは非感作性物質として検出するためには十分であり、皮膚感作性試験として LLNA-DA を支持するものである。JaCVAM の検証試験で得られた 14 化合物のデータを含む 44 化合物の検証データベースの retrospective 解析において、LLNA-DA は、LLNA で皮膚感作性物質として評価された 32 化合物全て（0%[0/32]の偽陰性）と LLNA で非感作性物質と評価された 12 化合物の内 9 化合物（25%[3/12]の偽陽性）を正確に検出した。ICCVAM は、潜在的に皮膚感作性を有する化合物を検出する判定基準としてカットオフ値 1.8 を使用することを勧告した。ICCVAM のこの勧告は、SI 値≧1.8 を使用する場合、原法の LLNA による最新の検証データベースに対して偽陰性が生じないことに基づくものである。

LLNA-DA の欠点として、1.8 から 2.5 の間の SI 値で陽性反応が得られる場合、偽陽性の結果を生じる可能性があることが挙げられている。更に、LLNA-DA は、ATP 量に影響を及ぼす化合物（例えば、ATP 阻害剤として作用される化合物）や細胞内 ATP の測定に影響を及ぼす化合物（例えば、ATP 分解酵素やリンパ節の細胞外における ATP の存在）の検討には適切ではないことが挙げられている。

LLNA-DA の精度は、原法の LLNA の精度に匹敵するものであった。最適な LLNA-DA の遂行は、皮膚感作性物質と非感作性物質を分類するためにカットオフ値 1.8 を使用することにより達成された。原法の LLNA と比較すると、精度は、偽陽性率 25% (3/12)、偽陰性率 0% (0/32)の 93%（41/44）であった。LLNA-DA において SI 値≧1.8 を使用すると、1.8 から 2.5 の間の SI 値を示す 3 偽陽性化合物（salicylic acid、hexane および chlorobenzene）が生じた。それ故、1.8 から 2.5 の境界域の SI 値を示した化合物が潜在的に皮膚感作性を有する化合物であるか否かを確認するために、他に利用できる情報、例えば、用量反応性、全身毒性あるいは過度な局所刺激性の証拠、必要に応じて SI 値と共に統計的有意性を考慮すべきである。また、その考察には、既知の皮膚感作性物質との構造類似性も含め、被験物質の種々の性質も加えるべきであるとしている。

４．ICCVAM の勧告

最終的に ICCVAM は、次のように勧告している。即ち、カットオフ値 1.8 という一つの判定基準を潜在的な皮膚感作性物質を分類するために使用すべきである、何故なら、この基準が使用されるとき、原法の LLNA による最新の検証データベースにおいて偽陰性は認められなかったからである。しかしながら、判定基準としてカットオフ値 1.8 を使用すると、原法の LLNA と比較して 25% (3/12)の偽陽性が生じる。LLNA-DA において 3 偽陽性物質

が 1.8 から 2.5 の間の SI 値を示したことから、この範囲内での成績については、真に陽性である否かを確認するために付加的な情報（例えば、用量反応情報、全身毒性若しくは過剰な局所皮膚刺激の証拠、必要に応じて、処置群と溶媒対照群の統計的な比較、ペプチド反応性、分子量、関連物質の結果、他の試験データ）を考慮すべきである。

５．委員会としての提案

表１　ICCVAM と JaCVAM の LLNA- DA の検証の比較

研究組織	LLNA-DA	
	検証に使用された化合物数	陽性判定の SI 値
ICCVAM	44	≧ 1.8
JaCVAM	14	≧ 3

JaCVAM と ICCVAM の判定基準のカットオフ値の違いは､検証に使用した化合物数の違いも一因と考える。両者には検証に使用した化合物数に差があり、化合物数を多くすれば試験の精度は高まるが、皮膚感作性の判定基準であるカットオフ値は低くなることが予想される（表 1 参照)。

ICCVAM の検証において、検証の対象とされた 32 皮膚感作性物質の内 SI 値＜3 の皮膚感作性物質は 4 化合物（2-mercaptobenzothiazole [MBT]、3-aminophenol、methyl methacrylate および nickel (II) sulfate hexahydrate）である。また、ICCVAM の基準である SI 値≧1.8 においては、12 非感作性物質の内偽陽性を示す 3 化合物（salicylic acid、hexane および chlorobenzene）が存在することも事実である。

以上の結果を総合的に判断し、委員会としては、ICCVAM が検証の対象とした皮膚感作性陽性物質全てを陽性と判定するカットオフ値 1.8 を判定基準として採用することが妥当であると考えた。また、LLNA-DA に関する OECD ガイドライン（2010）[4] においても、皮膚感作性陽性を示すカットオフ値として 1.8 が設定されている。このような状況を踏まえると､本邦においても 1.8 をカットオフ値として設定することが妥当であると考える。一方、SI 値≧1.8 においては、皮膚感作性偽陽性を示す化合物も存在するため、化合物の皮膚感作性の最終判定においては、ICCVAM が勧告する付加的な情報（例えば、用量反応情報、全身毒性若しくは過剰な局所皮膚刺激の証拠、必要に応じて処置群と溶媒対照群の統計的な比較、ペプチド反応性、分子量、関連物質の結果、他の試験データ）を考慮して決定する必要がある。

6. 文献

1) ICCVAM and NICEATM. The Murine Local Lymph Node Assay: A Test Method for

Assessing the Allergic Contact Dermatitis Potential of Chemicals/Compounds. The Results of an Independent Peer Review Evaluation Coordinated by ICCVAM and NICEATM. NIH Publication No. 99-4494. February (1999)

2) Takashi Omori, et al. Interlaboratory validation of the modified murine local lymph node assay based on adenosine triphosphate measurement. Journal of Pharmacological and Toxicological Methods. 58: 11-26 (2008)

3) ICCVAM (2010). ICCVAM Test Method Evaluation Report on the Murine Local Lymph Node Assay: DA, a Nonradioactive Alternative Test Method to Assess the Allergic Contact Dermatitis Potential of Chemicals and Products. NIH Publication No. 10-7551. Research Triangle Park, NC: National Institute of Environmental Health Sciences.

4) OECD Guideline for the Testing of Chemicals: Skin Sensitization: Local Lymph Node Assay: DA (TG 442A), 22 July, 2010

Adopted:
22 July 2010

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Skin Sensitization: Local Lymph Node Assay: DA

INTRODUCTION

1. OECD Guidelines for the Testing of Chemicals are periodically reviewed in light of scientific progress, changing regulatory needs, and animal welfare considerations. The first Test Guideline (TG) for the determination of skin sensitization in the mouse, the Local Lymph Node Assay (LLNA; TG 429) was adopted in 2002, and has since then been revised (1). The details of the validation of the LLNA and a review of the associated work have been published (2) (3) (4) (5) (6) (7) (8) (9). In the LLNA, radioisotopic thymidine or iodine is used to measure lymphocyte proliferation and therefore the assay has limited use in regions where the acquisition, use, or disposal of radioactivity is problematic. The LLNA: DA (developed by Daicel Chemical Industries, Ltd.) is a non-radioactive modification to the LLNA, which quantifies adenosine triphosphate (ATP) content via bio-luminescence as an indicator of lymphocyte proliferation. The LLNA: DA test method has been validated and reviewed and recommended by an international peer review panel as considered useful for identifying skin sensitizing and non-sensitizing substances, with certain limitations (10) (11) (12) (13). This Test Guideline is designed for assessing skin sensitization potential of chemicals in animals. TG 406 utilises guinea pig tests, notably the guinea pig maximisation test and the Buehler test (14). The LLNA (TG 429) and the two non-radioactive modifications, LLNA: DA (TG 442 A) and LLNA: BrdU-ELISA (TG 442 B), all provide an advantage over the guinea pig tests in TG 406 (14) in terms of reduction and refinement of animal use.

2. Similar to the LLNA, the LLNA: DA studies the induction phase of skin sensitization and provides quantitative data suitable for dose-response assessment. Furthermore, an ability to detect skin sensitizers without the necessity for using a radiolabel for DNA eliminates the potential for occupational exposure to radioactivity and waste disposal issues. This in turn may allow for the increased use of mice to detect skin sensitizers, which could further reduce the use of guinea pigs to test for skin sensitization potential (*i.e.* TG 406) (14).

DEFINITIONS

3. Definitions used are provided in Annex 1.

INITIAL CONSIDERATIONS AND LIMITATIONS

4. The LLNA: DA is a modified LLNA method for identifying potential skin sensitizing test substances, with specific limitations. This does not necessarily imply that in all instances the LLNA: DA should be used in place of the LLNA or guinea pig tests (*i.e.* TG 406) (14), but rather that the assay is of equal merit and may be employed as an alternative in which positive and negative results generally no longer require further confirmation (10) (11). The testing laboratory should consider all available information on the test substance prior to conducting the study. Such information will include the identity and chemical structure of the test substance; its physicochemical properties; the results of any other *in vitro* or *in vivo* toxicity tests on the test substance; and toxicological data on structurally related test substances. This information should be considered in order to determine whether the LLNA: DA is appropriate for the test substance (given the incompatibility of limited types of test substances with the LLNA: DA [see paragraph 5]) and to aid in dose selection.

5. The LLNA: DA is an *in vivo* method and, as a consequence, will not eliminate the use of animals in the assessment of allergic contact sensitizing activity. It has, however, the potential to reduce animal use for this purpose when compared to the guinea pig tests (TG 406) (14). Moreover, the LLNA: DA offers a substantial refinement (less pain and distress) of the way in which animals are used for allergic contact sensitization testing, since unlike the TG 406, the LLNA: DA does not require that challenge-induced dermal hypersensitivity reactions be elicited. Despite the advantages of the LLNA: DA over TG 406 (14), there are certain limitations that may necessitate the use of TG 406 (*e.g.* the testing of certain metals, false positive findings with certain skin irritants [such as some surfactant-type substances] (6) (1), solubility of the test substance). In addition, test substance classes or substances containing functional groups shown to act as potential confounders (16) may necessitate the use of guinea pig tests (*i.e.* TG 406 (14)). Limitations that have been identified for the LLNA (1) have been recommended to apply also to the LLNA: DA (10). Additionally, the use of the LLNA: DA might not be appropriate for testing test substances that affect ATP levels (*e.g.* test substances that function as ATP inhibitors) or those that affect the accurate measurement of intracellular ATP (*e.g.* presence of ATP degrading enzymes, presence of extracellular ATP in the lymph node). Other than such identified limitations, the LLNA: DA should be applicable for testing any test substances unless there are properties associated with these substances that may interfere with the accuracy of the LLNA: DA. In addition, consideration should be given to the possibility of borderline positive results when Stimulation Index (SI) values between 1.8 and 2.5 are obtained (see paragraphs 31-32). This is based on the validation database of 44 substances using an $SI \geq 1.8$ (see paragraph 6) for which the LLNA: DA correctly identified all 32 LLNA sensitizers, but incorrectly identified three of 12 LLNA non-sensitizers with SI values between 1.8 and 2.5 (*i.e.* borderline positive) (10). However, as the same dataset was used for setting the SI-values and calculating the predictive properties of the test, the stated results may be an over-estimation of the real predictive properties.

PRINCIPLE OF THE TEST

6. The basic principle underlying the LLNA: DA is that sensitizers induce proliferation of lymphocytes in the lymph nodes draining the site of test substance application. This proliferation is proportional to the dose and to the potency of the applied allergen and provides a simple means of obtaining a quantitative measurement of sensitization. Proliferation is measured by comparing the mean proliferation in each test group to the mean proliferation in the vehicle treated control (VC) group. The ratio of the mean proliferation in each treated group to that in the concurrent VC group, termed the SI, is determined, and should be ≥ 1.8 before further evaluation of the test substance as a potential skin sensitizer is warranted. The methods described here are based on the use of measuring ATP content by bioluminescence (known to correlate with living cell number) (17) to indicate an increased number of proliferating cells in the draining auricular lymph nodes (18) (19). The bioluminescent method utilises the luciferase enzyme to catalyse the formation of light from ATP and luciferin according to the following reaction:

$$ATP + Luciferin + O_2 \xrightarrow{Luciferase} Oxyluciferin + AMP + PP_i + CO_2 + Light$$

The emitted light intensity is linearly related to the ATP concentration and is measured using a luminometer. The luciferin-luciferase assay is a sensitive method for ATP quantitation used in a wide variety of applications (20).

DESCRIPTION OF THE ASSAY

Selection of animal species

7. The mouse is the species of choice for this test. Validation studies for the LLNA: DA were conducted exclusively with the CBA/J strain, which is therefore considered the preferred strain (12) (13).

Young adult female mice, which are nulliparous and non-pregnant, are used. At the start of the study, animals should be between 8-12 weeks old, and the weight variation of the animals should be minimal and not exceed 20% of the mean weight. Alternatively, other strains and males may be used when sufficient data are generated to demonstrate that significant strain and/or gender-specific differences in the LLNA: DA response do not exist.

Housing and feeding conditions

8. Mice should be group-housed (21), unless adequate scientific rationale for housing mice individually is provided. The temperature of the experimental animal room should be 22 ± 3ºC. Although the relative humidity should be at least 30% and preferably not exceed 70%, other than during room cleaning, the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water.

Preparation of animals

9. The animals are randomly selected, marked to permit individual identification (but not by any form of ear marking), and kept in their cages for at least five days prior to the start of dosing to allow for acclimatisation to the laboratory conditions. Prior to the start of treatment all animals are examined to ensure that they have no observable skin lesions.

Preparation of dosing solutions

10. Solid test substances should be dissolved or suspended in solvents/vehicles and diluted, if appropriate, prior to application to an ear of the mice. Liquid test substances may be applied neat or diluted prior to dosing. Insoluble test substances, such as those generally seen in medical devices, should be subjected to an exaggerated extraction in an appropriate solvent to reveal all extractable constituents for testing prior to application to an ear of the mice. Test substances should be prepared daily unless stability data demonstrate the acceptability of storage.

Reliability check

11. Positive controls (PC) are used to demonstrate appropriate performance of the assay by responding with adequate and reproducible sensitivity to a sensitizing test substance for which the magnitude of the response is well characterised. Inclusion of a concurrent PC is recommended because it demonstrates competency of the laboratory to successfully conduct each assay and allows for an assessment of intra-, and inter-laboratory reproducibility and comparability. Some regulatory authorities also require a PC for each study and therefore users are encouraged to consult the relevant authorities prior to conducting the LLNA: DA. Accordingly, the routine use of a concurrent PC is encouraged to avoid the need for additional animal testing to meet such requirements that might arise from the use of a periodic PC (see paragraph 12). The PC should produce a positive LLNA: DA response at an exposure level expected to give an increase in the SI $\geq$ 1.8 over the negative control (NC) group. The PC dose should be chosen such that it does not cause excessive skin irritation or systemic toxicity and the induction is reproducible but not excessive (*e.g.* SI > 10 would be considered excessive). Preferred PC test substances are 25% hexyl cinnamic aldehyde (Chemical Abstracts Service [CAS] No 101-86-0) and 25% eugenol (CAS No 97-53-0) in acetone: olive oil (4:1, v/v). There may be circumstances in which, given adequate justification, other PC test substances, meeting the above criteria, may be used.

12. While inclusion of a concurrent PC group is recommended, there may be situations in which periodic testing (*i.e.* at intervals ≤6 months) of the PC test substance may be adequate for laboratories that conduct the LLNA: DA regularly (*i.e.* conduct the LLNA: DA at a frequency of no less than once per

month) and have an established historical PC database that demonstrates the laboratory's ability to obtain reproducible and accurate results with PCs. Adequate proficiency with the LLNA: DA can be successfully demonstrated by generating consistent positive results with the PC in at least 10 independent tests conducted within a reasonable period of time (*i.e.* less than one year).

13. A concurrent PC group should always be included when there is a procedural change to the LLNA: DA (*e.g.* change in trained personnel, change in test method materials and/or reagents, change in test method equipment, change in source of test animals), and such changes should be documented in laboratory reports. Consideration should be given to the impact of these changes on the adequacy of the previously established historical database in determining the necessity for establishing a new historical database to document consistency in the PC results.

14. Investigators should be aware that the decision to conduct a PC study on a periodic basis instead of concurrently has ramifications on the adequacy and acceptability of negative study results generated without a concurrent PC during the interval between each periodic PC study. For example, if a false negative result is obtained in the periodic PC study, negative test substance results obtained in the interval between the last acceptable periodic PC study and the unacceptable periodic PC study may be questioned. Implications of these outcomes should be carefully considered when determining whether to include concurrent PCs or to only conduct periodic PCs. Consideration should also be given to using fewer animals in the concurrent PC group when this is scientifically justified and if the laboratory demonstrates, based on laboratory-specific historical data, that fewer mice can be used (22).

15. Although the PC test substance should be tested in the vehicle that is known to elicit a consistent response (*e.g.* acetone: olive oil; 4:1, v/v), there may be certain regulatory situations in which testing in a non-standard vehicle (clinically/chemically relevant formulation) will also be necessary (23). If the concurrent PC test substance is tested in a different vehicle than the test substance, then a separate VC for the concurrent PC should be included.

16. In instances where test substances of a specific chemical class or range of responses are being evaluated, benchmark test substances may also be useful to demonstrate that the test method is functioning properly for detecting the skin sensitization potential of these types of test substances. Appropriate benchmark substances should have the following properties:

- structural and functional similarity to the class of the test substance being tested;
- known physical/chemical characteristics;
- supporting data from the LLNA: DA;
- supporting data from other animal models and/or from humans.

TEST PROCEDURE

Number of animals and dose levels

17. A minimum of four animals is used per dose group, with a minimum of three concentrations of the test substance, plus a concurrent NC group treated only with the vehicle for the test substance, and a PC (concurrent or recent, based on laboratory policy in considering paragraphs 11-15). Testing multiple doses of the PC should be considered, especially when testing the PC on an intermittent basis. Except for absence of treatment with the test substance, animals in the control groups should be handled and treated in a manner identical to that of animals in the treatment groups.

18. Dose and vehicle selection should be based on the recommendations given in references (2) and (24). Consecutive doses are normally selected from an appropriate concentration series such as 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, etc. Adequate scientific rationale should accompany the selection of the concentration series used. All existing toxicological information (*e.g.* acute toxicity and dermal irritation) and structural and physicochemical information on the test substance of interest (and/or structurally related test substances) should be considered, where available, in selecting the three consecutive concentrations so that the highest concentration maximises exposure while avoiding systemic toxicity and/or excessive local skin irritation (24) (25). In the absence of such information, an initial pre-screen test may be necessary (see paragraphs 21-24).

19. The vehicle should not interfere with or bias the test result and should be selected on the basis of maximising the solubility in order to obtain the highest concentration achievable while producing a solution/suspension suitable for application of the test substance. Recommended vehicles are acetone: olive oil (4:1 v/v), *N*,*N*-dimethylformamide, methyl ethyl ketone, propylene glycol, and dimethyl sulphoxide (6) but others may be used if sufficient scientific rationale is provided. In certain situations it may be necessary to use a clinically relevant solvent or the commercial formulation in which the test substance is marketed as an additional control. Particular care should be taken to ensure that hydrophilic substances are incorporated into a vehicle system, which wets the skin and does not immediately run off, by incorporation of appropriate solubilisers (*e.g.* 1% Pluronic® L92). Thus, wholly aqueous vehicles are to be avoided.

20. The processing of lymph nodes from individual mice allows for the assessment of inter-animal variability and a statistical comparison of the difference between test substance and VC group measurements (see paragraph 33). In addition, evaluating the possibility of reducing the number of mice in the PC group is only feasible when individual animal data are collected (22). Further, some national regulatory authorities require the collection of individual animal data. Regular collection of individual animal data provides an animal welfare advantage by avoiding duplicate testing that would be necessary if the test substance results originally collected in one manner (*e.g.* via pooled animal data) were to be considered later by regulatory authorities with other requirements (*e.g.* individual animal data).

Pre-screen test

21. In the absence of information to determine the highest dose to be tested (see paragraph 18), a pre-screen test should be performed in order to define the appropriate dose level to test in the LLNA: DA. The purpose of the pre-screen test is to provide guidance for selecting the maximum dose level to use in the main LLNA: DA study, where information on the concentration that induces systemic toxicity (see paragraph 24) and/or excessive local skin irritation (see paragraph 23) is not available. The maximum dose level tested should be 100% of the test substance for liquids or the maximum possible concentration for solids or suspensions.

22. The pre-screen test is conducted under conditions identical to the main LLNA: DA study, except there is no assessment of lymph node proliferation and fewer animals per dose group can be used. One or two animals per dose group are suggested. All mice will be observed daily for any clinical signs of systemic toxicity or local irritation at the application site. Body weights are recorded pre-test and prior to termination (Day 8). Both ears of each mouse are observed for erythema and scored using Table 1 (25). Ear thickness measurements are taken using a thickness gauge (*e.g.* digital micrometer or Peacock Dial thickness gauge) on Day 1 (pre-dose), Day 3 (approximately 48 hours after the first dose), Day 7 (24 hours prior to termination) and Day 8. Additionally on Day 8, ear thickness could be determined by ear punch weight determinations, which should be performed after the animals are humanely killed. Excessive local irritation is indicated by an erythema score $\geq$3 and/or ear thickness of $\geq$25% on any day of measurement (26) (27). The highest dose selected for the main LLNA: DA study will be the next lower dose in the pre-

screen concentration series (see paragraph 18) that does not induce systemic toxicity and/or excessive local skin irritation.

Table 1. Erythema Scores

Observation	Score
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to eschar formation preventing grading of erythema	4

23. In addition to a 25% increase in ear thickness (26) (27), a statistically significant increase in ear thickness in the treated mice compared to control mice has also been used to identify irritants in the LLNA (28) (29) (30) (31) (32) (33) (34). However, while statistically significant increases can occur when ear thickness is less than 25% they have not been associated specifically with excessive irritation (30) (31) (32) (33) (34).

24. The following clinical observations may indicate systemic toxicity (35) when used as part of an integrated assessment and therefore may indicate the maximum dose level to use in the main LLNA: DA: changes in nervous system function (*e.g.* pilo-erection, ataxia, tremors, and convulsions); changes in behaviour (*e.g.* aggressiveness, change in grooming activity, marked change in activity level); changes in respiratory patterns (*i.e.* changes in frequency and intensity of breathing such as dyspnea, gasping, and rales), and changes in food and water consumption. In addition, signs of lethargy and/or unresponsiveness and any clinical signs of more than slight or momentary pain and distress, or a >5% reduction in body weight from Day 1 to Day 8 and mortality, should be considered in the evaluation. Moribund animals or animals showing signs of severe pain and distress should be humanely killed (36).

Main study experimental schedule

25. The experimental schedule of the assay is as follows:

- *Day 1:*

  Individually identify and record the weight of each animal and any clinical observation. Apply 1% sodium lauryl sulfate (SLS) aqueous solution to the dorsum of each ear by using a brush dipped in the SLS solution to cover the entire dorsum of each ear with four to five strokes. One hour after the SLS treatment, apply 25 µL of the appropriate dilution of the test substance, the vehicle alone, or the PC (concurrent or recent, based on laboratory policy in considering paragraphs 11-15), to the dorsum of each ear.

- *Days 2, 3 and 7:*

  Repeat the 1% SLS aqueous solution pre-treatment and test substance application procedure carried out on Day 1.

- *Days 4, 5, and 6:*

  No treatment.

- *Day 8:*

Record the weight of each animal and any clinical observation. Approximately 24 to 30 hours after the start of application on Day 7, humanely kill the animals. Excise the draining auricular lymph nodes from each mouse ear and process separately in phosphate buffered saline (PBS) for each animal. Details and diagrams of the lymph node identification and dissection can be found in reference (22). To further monitor the local skin response in the main study, additional parameters such as scoring of ear erythema or ear thickness measurements (obtained either by using a thickness gauge, or ear punch weight determinations at necropsy) may be included in the study protocol.

Preparation of cell suspensions

26. From each mouse, a single-cell suspension of lymph node cells (LNC) excised bilaterally is prepared by sandwiching the lymph nodes between two glass slides and applying light pressure to crush the nodes. After confirming that the tissue has spread out thinly pull the two slides apart. Suspend the tissue on both slides in PBS by holding each slide at an angle over the Petri dish and rinsing with PBS while concurrently scraping the tissue off of the slide with a cell scraper. Further, the lymph nodes in NC animals are small, so careful operation is important to avoid any artificial effects on SI values. A total volume of 1 mL PBS should be used for rinsing both slides. The LNC suspension in the Petri dish should be homogenised lightly with the cell scraper. A 20 μL aliquot of the LNC suspension is then collected with a micropipette, taking care not to take up the membrane that is visible to the eye, and subsequently mixed with 1.98 mL of PBS to yield a 2 mL sample. A second 2 mL sample is then prepared using the same procedure so that two samples are prepared for each animal.

Determination of cellular proliferation (measurement of ATP content of lymphocytes)

27. Increases in ATP content in the lymph nodes are measured by the luciferin/luciferase method using an ATP measurement kit, which measures bioluminescence in Relative Luminescence Units (RLU). The assay time from time of animal sacrifice to measurement of ATP content for each individual animal should be kept uniform, within approximately 30 minutes, because the ATP content is considered to gradually decrease with time after animal sacrifice (12). Thus, the series of procedures from excision of auricular lymph nodes to ATP measurement should be completed within 20 minutes by the pre-determined time schedule that is the same for each animal. ATP luminescence should be measured in each 2 mL sample so that a total of two ATP measurements are collected for each animal. The mean ATP luminescence is then determined and used in subsequent calculations (see paragraph 31).

OBSERVATIONS

Clinical observations

28. Each mouse should be carefully observed at least once daily for any clinical signs, either of local irritation at the application site or of systemic toxicity. All observations are systematically recorded with records being maintained for each mouse. Monitoring plans should include criteria to promptly identify those mice exhibiting systemic toxicity, excessive local skin irritation, or corrosion of skin for euthanasia (36).

Body weights

29. As stated in paragraph 25, individual animal body weights should be measured at the start of the test and at the scheduled humane kill.

CALCULATION OF RESULTS

30. Results for each treatment group are expressed as the mean SI. The SI is derived by dividing the mean RLU/mouse within each test substance group and the PC group by the mean RLU/mouse for the solvent/VC group. The average SI for the VCs is then one.

31. The decision process regards a result as positive when SI $\geq$ 1.8 (10). However, the strength of the dose-response relationship, the statistical significance and the consistency of the solvent/vehicle and PC responses may also be used when determining whether a borderline result (*i.e.* SI value between 1.8 and 2.5) is declared positive (2) (3) (37).

32. For a borderline positive response between an SI of 1.8 and 2.5, users may want to consider additional information such as dose-response relationship, evidence of systemic toxicity or excessive irritation, and where appropriate, statistical significance together with SI values to confirm that such results are positives (10). Consideration should also be given to various properties of the test substance, including whether it has a structural relationship to known skin sensitizers, whether it causes excessive skin irritation in the mouse, and the nature of the dose-response relationship observed. These and other considerations are discussed in detail elsewhere (4).

33. Collecting data at the level of the individual mouse will enable a statistical analysis for presence and degree of dose-response relationship in the data. Any statistical assessment could include an evaluation of the dose-response relationship as well as suitably adjusted comparisons of test groups (*e.g.* pair-wise dosed group versus concurrent solvent/vehicle control comparisons). Statistical analyses may include, *e.g.* linear regression or Williams's test to assess dose-response trends, and Dunnett's test for pair-wise comparisons. In choosing an appropriate method of statistical analysis, the investigator should maintain an awareness of possible inequalities of variances and other related problems that may necessitate a data transformation or a non-parametric statistical analysis. In any case, the investigator may need to carry out SI calculations and statistical analyses with and without certain data points (sometimes called "outliers").

DATA AND REPORTING

Data

34. Data should be summarised in tabular form showing the individual animal RLU values, the group mean RLU/animal, its associated error term (*e.g.* SD, SEM), and the mean SI for each dose group compared against the concurrent solvent/vehicle control group.

Test report

35. The test report should contain the following information:

Test substance and control test substances:

- identification data (*e.g.* CAS number, if available; source; purity; known impurities; lot number);
- physical nature and physicochemical properties (*e.g.* volatility, stability, solubility);
- if formulation, composition and relative percentages of components;

Solvent/vehicle:

- identification data (purity; concentration, where appropriate; volume used);

– justification for choice of vehicle;

Test animals:

– source of CBA mice;
– microbiological status of the animals, when known;
– number and age of animals;
– source of animals, housing conditions, diet, etc;

Test conditions:

– the source, lot number and manufacturer's quality assurance/quality control data for the ATP kit;
– details of test substance preparation and application;
– justification for dose selection (including results from pre-screen test, if conducted);
– vehicle and test substance concentrations used, and total amount of test substance applied;
– details of food and water quality (including diet type/source, water source);
– details of treatment and sampling schedules;
– methods for measurement of toxicity;
– criteria for considering studies as positive or negative;
– details of any protocol deviations and an explanation on how the deviation affects the study design and results;

Reliability check:

– a summary of results of latest reliability check, including information on test substance, concentration and vehicle used;
– concurrent and/or historical PC and concurrent negative (solvent/vehicle) control data for testing laboratory;
– if a concurrent PC was not included, the date and laboratory report for the most recent periodic PC and a report detailing the historical PC data for the laboratory justifying the basis for not conducting a concurrent PC;

Results:

– individual weights of mice at start of dosing and at scheduled kill; as well as mean and associated error term (*e.g.* SD, SEM) for each treatment group;
– time course of onset and signs of toxicity, including dermal irritation at site of administration, if any, for each animal;
– time of animal sacrifice and time of ATP measurement for each animal;
– a table of individual mouse RLU values and SI values for each dose treatment group;
– mean and associated error term (*e.g.* SD, SEM) for RLU/mouse for each treatment group and the results of outlier analysis for each treatment group;
– calculated SI and an appropriate measure of variability that takes into account the inter-animal variability in both the test substance and control groups;
– dose response relationship;
– statistical analyses, where appropriate;

Discussion of results:

– a brief commentary on the results, the dose-response analysis, and statistical analyses, where appropriate, with a conclusion as to whether the test substance should be considered a skin sensitizer.

LITERATURE

(1) OECD (2010), *Skin Sensitization: Local Lymph Node Assay*, Test Guideline No. 429, Guidelines for the Testing of Chemicals, OECD, Paris. Available at: [http://www.oecd.org/env/testguidelines]

(2) Chamberlain, M. and Basketter, D.A. (1996), The local lymph node assay: status of validation. *Food Chem, Toxicol*., 34, 999-1002.

(3) Basketter, D.A., Gerberick, G.F., Kimber, I. and Loveless, S.E. (1996), The local lymph node assay: A viable alternative to currently accepted skin sensitisation tests. *Food Chem, Toxicol*., 34, 985-997.

(4) Basketter, D.A., Gerberick, G.F. and Kimber, I. (1998), Strategies for identifying false positive responses in predictive sensitisation tests. *Food Chem. Toxicol.*, 36, 327-333.

(5) Van Och, F.M.M., Slob, W., De Jong, W.H., Vandebriel, R.J. and Van Loveren, H. (2000), A quantitative method for assessing the sensitizing potency of low molecular weight chemicals using a local lymph node assay: employment of a regression method that includes determination of uncertainty margins. *Toxicol*., 146, 49-59.

(6) ICCVAM (1999), The murine local lymph node Assay: A test method for assessing the allergic contact dermatitis potential of chemicals/compounds: The results of an independent peer review evaluation coordinated by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program Center for the Evaluation of Alternative Toxicological Methods (NICETAM). NIH Publication No: 99-4494. Research Triangle Park, N.C. Available at: http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf]

(7) Dean, J.H., Twerdok, L.E., Tice, R.R., Sailstad, D.M., Hattan, D.G., Stokes, W.S. (2001), ICCVAM evaluation of the murine local lymph node assay: II. Conclusions and recommendations of an independent scientific peer review panel. *Reg. Toxicol. Pharmacol.* 34, 258-273.

(8) Haneke, K.E., Tice, R.R., Carson, B.L., Margolin, B.H., Stokes, W.S. (2001), ICCVAM evaluation of the murine local lymph node assay: III. Data analyses completed by the national toxicology program interagency center for the evaluation of alternative toxicological methods. *Reg. Toxicol. Pharmacol. 34*, 274-286.

(9) Sailstad, D.M., Hattan, D., Hill, R.N., Stokes, W.S. (2001), ICCVAM evaluation of the murine local lymph node assay: I. The ICCVAM review process. *Reg. Toxicol. Pharmacol.* 34, 249-257.

(10) ICCVAM (2010), ICCVAM Test Method Evaluation Report. Nonradioactive local lymph node assay: modified by Daicel Chemical Industries, Ltd., based on ATP content test method protocol (LLNA: DA). NIH Publication No. 10-7551A/B. Research Triangle Park, NC: National Institute of Environmental Health Sciences. Available at: [http://iccvam.niehs.nih.gov/methods/immunotox/llna-DA/TMER.htm]

(11) ICCVAM (2009), Independent Scientific Peer Review Panel Report: Updated validation status of new versions and applications of the murine local lymph node assay: a test method for assessing the allergic contact dermatitis potential of chemicals and products. Research Triangle Park, NC: National Institute of Environmental Health Sciences. Available at: [http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2009.pdf].

(12) Idehara, K., Yamagishi, G., Yamashita, K. and Ito, M. (2008), Characterization and evaluation of a modified local lymph node assay using ATP content as a non-radio isotopic endpoint. *J. Pharmacol. Toxicol. Meth.*, 58, 1-10.

(13) Omori, T., Idehara, K., Kojima, H., Sozu, T., Arima, K., Goto, H., Hanada, T., Ikarashi, Y., Inoda, T., Kanazawa, Y., Kosaka, T., Maki, E., Morimoto, T., Shinoda, S., Shinoda, N., Takeyoshi, M., Tanaka, M., Uratani, M., Usami, M., Yamanaka, A., Yoneda, T., Yoshimura, I. and Yuasa, A. (2008), Interlaboratory validation of the modified murine local lymph node assay based on adenosine triphosphate measurement. *J. Pharmacol. Toxicol. Meth.*, 58, 11-26.

(14) OECD (1992), *Skin Sensitisation*, Test Guideline No. 406, Guidelines for Testing of Chemicals, OECD, Paris. Available at: [http://www.oecd.org/env/testguidelines]

(15) Kreiling, R., Hollnagel, H.M., Hareng, L., Eigler, L., Lee, M.S., Griem, P., Dreessen, B., Kleber, M., Albrecht, A., Garcia, C. and Wendel, A. (2008), Comparison of the skin sensitizing potential of unsaturated compounds as assessed by the murine local lymph node assay (LLNA) and the guinea pig maximization test (GPMT). *Food Chem. Toxicol*., 46, 1896-1904.

(16) Basketter, D., Ball, N., Cagen, S., Carrillo, J.C., Certa, H., Eigler, D., Garcia, C., Esch, H., Graham, C., Haux, C., Kreiling, R. and Mehling, A. (2009), Application of a weight of evidence approach to assessing discordant sensitisation datasets: implications for REACH. *Reg. Toxicol. Pharmacol*., 55, 90-96.

(17) Crouch, S.P., Kozlowski, R., Slater, K.J. and Fletcher J. (1993), The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. *J. Immunol. Meth*., 160, 81-88.

(18) Ishizaka, A., Tono-oka, T. and Matsumoto, S. (1984), Evaluation of the proliferative response of lymphocytes by measurement of intracellular ATP. *J. Immunol. Meth*., 72, 127-132.

(19) Dexter, S.J., Cámara, M., Davies, M. and Shakesheff, K.M. (2003), Development of a bioluminescent ATP assay to quantify mammalian and bacterial cell number from a mixed population. *Biomat.*, 24, 27-34.

(20) Lundin A. (2000), Use of firefly luciferase in ATP-related assays of biomass, enzymes, and metabolites. *Meth. Enzymol*., 305, 346-370.

(21) ILAR (1996), Institute of Laboratory Animal Research (ILAR) Guide for the Care and Use of Laboratory Animals. 7th ed. Washington, DC: National Academies Press.

(22) ICCVAM (2009), Recommended Performance Standards: Murine Local Lymph Node Assay, NIH Publication Number 09-7357, Research Triangle Park, NC: National Institute of Environmental Health Sciences. Available at: [http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna-ps/LLNAPerfStds.pdf]

(23) McGarry, H.F. (2007), The murine local lymph node assay: regulatory and potency considerations under REACH. *Toxicol*., 238, 71-89.

(24) Kimber, I., Dearman, R.J., Scholes E.W. and Basketter, D.A. (1994), The local lymph node assay: developments and applications. *Toxicol*., 93, 13-31.

(25) OECD (2002), *Acute Dermal Irritation/Corrosion*, Test Guideline No. 404, Guidelines for Testing of Chemicals, OECD, Paris. Available at: [http://www.oecd.org/env/testguidelines]

(26) Reeder, M.K., Broomhead, Y.L., DiDonato, L. and DeGeorge, G.L. (2007), Use of an enhanced local lymph node assay to correctly classify irritants and false positive substances. *Toxicologist*, 96, 235.

(27) ICCVAM (2009), Nonradioactive Murine Local Lymph Node Assay: Flow Cytometry Test Method Protocol (LLNA: BrdU-FC) Revised Draft Background Review Document. Research Triangle Park, NC: National Institute of Environmental Health Sciences. Available at: [http://iccvam.niehs.nih.gov/methods/immunotox/fcLLNA/BRDcomplete.pdf].

(28) Hayes, B.B., Gerber, P.C., Griffey, S.S. and Meade, B.J. (1998), Contact hypersensitivity to dicyclohexylcarbodiimide and diisopropylcarbodiimide in female B6C3F1 mice. *Drug. Chem. Toxicol*., 21, 195-206.

(29) Homey, B., von Schilling, C., Blumel, J., Schuppe, H.C., Ruzicka, T., Ahr, H.J., Lehmann, P. and Vohr, V.W. (1998), An integrated model for the differentiation of chemical-induced allergic and irritant skin reactions. *Toxicol. Appl. Pharmacol.*, 153, 83-94.

(30) Woolhiser, M.R., Hayes, B.B. and Meade, B.J. (1998), A combined murine local lymph node and irritancy assay to predict sensitization and irritancy potential of chemicals. *Toxicol. Meth.*, 8, 245-256.

(31) Hayes, B.B. and Meade, B.J. (1999), Contact sensitivity to selected acrylate compounds in B6C3F1 mice: relative potency, cross reactivity, and comparison of test methods. *Drug Chem. Toxicol*., 22, 491-506.

(32) Ehling, G., Hecht, M., Heusener, A., Huesler, J., Gamer, A.O., van Loveren, H., Maurer, T., Riecke, K., Ullmann, L., Ulrich, P., Vandebriel, R. and Vohr, H.W. (2005), A European inter-laboratory validation of alternative endpoints of the murine local lymph node assay: first round. *Toxicol*., 212, 60-68.

(33) Vohr, H.W. and Ahr, H.J. (2005), The local lymph node assay being too sensitive? *Arch. Toxicol*., 79, 721-728.

(34) Patterson, R.M., Noga, E. and Germolec, D. (2007), Lack of evidence for contact sensitization by Pfiesteria extract. *Environ. Health Perspect*., 115, 1023-1028.

(35) ICCVAM (2009), Report on the ICCVAM-NICEATM/ECVAM/JaCVAM Scientific Workshop on Acute Chemical Safety Testing: Advancing *In Vitro* Approaches and Humane Endpoints for Systemic Toxicity Evaluations. Research Triangle Park, NC: National Institute of Environmental Health Sciences. Available at: [http://iccvam.niehs.nih.gov/methods/acutetox/Tox_workshop.htm]

(36) OECD (2000), *Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation*, Environmental Health and Safety Monograph Series on Testing and Assessment No. 19, ENV/JM/MONO(2000)7, OECD, Paris. Available at: [http://www.oecd.org/env/testguidelines]

(37) Kimber, I., Hilton, J., Dearman, R.J., Gerberick, G.F., Ryan, C.A., Basketter, D.A., Lea, L., House, R.V., Ladies, G.S., Loveless, S.E. and Hastings, K.L. (1998), Assessment of the skin sensitization potential of topical medicaments using the local lymph node assay: An interlaboratory exercise. *J. Toxicol. Environ. Health*, 53 563-79.

(38) OECD (2005), *Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment*, Environment, Health and Safety Monograph Series on Testing and Assessment No. 34, ENV/JM/MONO(2005)14, OECD, Paris. Available at: [http://www.oecd.org/env/testguidelines]

ANNEX 1

DEFINITIONS

Accuracy: The closeness of agreement between test method results and accepted reference values. It is a measure of test method performance and one aspect of relevance. The term is often used interchangeably with "concordance" to mean the proportion of correct outcomes of a test method (38).

Benchmark substance: A sensitizing or non-sensitizing substance used as a standard for comparison to a test substance. A benchmark substance should have the following properties; (i) a consistent and reliable source(s); (ii) structural and functional similarity to the class of substances being tested; (iii) known physical/chemical characteristics; (iv) supporting data on known effects, and (v) known potency in the range of the desired response.

False negative: A substance incorrectly identified as negative or non-active by a test method, when in fact it is positive or active.

False positive: A substance incorrectly identified as positive or active by a test, when in fact it is negative or non-active.

Hazard: The potential for an adverse health or ecological effect. The adverse effect is manifested only if there is an exposure of sufficient level.

Inter-laboratory reproducibility: A measure of the extent to which different qualified laboratories, using the same protocol and testing the same test substances, can produce qualitatively and quantitatively similar results. Inter-laboratory reproducibility is determined during the pre-validation and validation processes, and indicates the extent to which a test can be successfully transferred between laboratories, also referred to as between-laboratory reproducibility (38).

Intra-laboratory reproducibility: A determination of the extent that qualified people within the same laboratory can successfully replicate results using a specific protocol at different times. Also referred to as within-laboratory reproducibility (38).

Outlier: An outlier is an observation that is markedly different from other values in a random sample from a population.

Quality assurance: A management process by which adherence to laboratory testing standards, requirements, and record keeping procedures, and the accuracy of data transfer, are assessed by individuals who are independent from those performing the testing.

Reliability: Measures of the extent that a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility (38).

Skin sensitization: An immunological process that results when a susceptible individual is exposed topically to an inducing chemical allergen, which provokes a cutaneous immune response that can lead to the development of contact sensitization.

Stimulation Index (SI): A value calculated to assess the skin sensitization potential of a test substance that is the ratio of the proliferation in treated groups to that in the concurrent vehicle control group.

Test substance: Any material tested using this TG, whether it is a single compound or consists of multiple components (e.g. final products, formulations). When testing formulations, consideration should be given to the fact that certain regulatory authorities only require testing of the final product formulation. However, there may also be testing requirements for the active ingredient(s) of a product formulation.

ICCVAM Test Method Evaluation Report on the Murine Local Lymph Node Assay: DA
A Nonradioactive Alternative Test Method to Assess the Allergic Contact Dermatitis Potential of Chemicals and Products

Interagency Coordinating Committee on the Validation of Alternative Methods

National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods

National Institute of Environmental Health Sciences
National Institutes of Health
U.S. Public Health Service
Department of Health and Human Services

March 2010

NIH Publication Number 10-7551

National Toxicology Program
P.O. Box 12233
Research Triangle Park, NC 27709

This document is available electronically at
http://iccvam.niehs.nih.gov/methods/immunotox/llna-DA/TMER.htm

When referencing this document, please cite as follows:
ICCVAM. 2010. ICCVAM Test Method Evaluation Report on the Murine Local Lymph Node Assay: DA, a Nonradioactive Alternative Test Method to Assess the Allergic Contact Dermatitis Potential of Chemicals and Products. NIH Publication No. 10-7551. Research Triangle Park, NC:National Institute of Environmental Health Sciences.

Table of Contents

List of Tables

List of Figures

This page intentionally left blank

List of Abbreviations and Acronyms

ACD	Allergic contact dermatitis
ACE	Acetone
AOO	Acetone: olive oil (4:1 by volume)
BRD	Background review document
BrdU	Bromodeoxyuridine
CASRN	Chemical Abstracts Service Registry Number
CI	Confidence interval
CMI	5-Chloro-2-methyl-4-isothiazolin-3-one
CPSC	U.S. Consumer Product Safety Commission
CV	Coefficient of variation
DMF	*N,N*-dimethylformamide
DMSO	Dimethyl sulfoxide
DNCB	2,4-Dinitrochlorobenzene
EC1.8	Estimated concentration needed to produce a stimulation index of 1.8
EC2.5	Estimated concentration needed to produce a stimulation index of 2.5
EC3	Estimated concentration needed to produce a stimulation index of 3.0
ECVAM	European Centre for the Validation of Alternative Methods
EGDMA	Ethylene glycol dimethacrylate
ELISA	Enzyme-linked immunosorbent assay
EPA	U.S. Environmental Protection Agency
ESAC	ECVAM Scientific Advisory Committee
FR	*Federal Register*
GP	Guinea pig
GPMT	Guinea pig maximization test
^{3}H	Tritiated
HCA	Hexyl cinnamic aldehyde
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ILS	Integrated Laboratory Systems
IWG	Immunotoxicity Working Group
JaCVAM	Japanese Center for the Validation of Alternative Methods
K_{ow}	Estimated log octanol-water partition coefficient
LLNA	Murine local lymph node assay
LLNA: DA	Murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content
LNC	Lymph node cells
Max.	Maximum
MBT	2-Mercaptobenzothiazole

MEK	Methyl ethyl ketone
NA	Not available
NC	Not calculated
Ni	Nickel
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NIEHS	National Institute of Environmental Health Sciences
No.	Number
OECD	Organisation for Economic Co-operation and Development
PBS	Phosphate buffered saline
rLLNA: DA	Reduced murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content
RLU	Relative luminescence units
SACATM	Scientific Advisory Committee on Alternative Toxicological Methods
SD	Standard deviation
SEM	Standard error of the mean
SI	Stimulation index
SLS	Sodium lauryl sulfate
TCA	Trichloroacetic acid
TG	Test Guideline
U.K.	United Kingdom
U.S.	United States
U.S.C.	United States Code

Interagency Coordinating Committee on the Validation of Alternative Methods: Agency Representatives

Agency for Toxic Substances and Disease Registry
* Moiz Mumtaz, Ph.D.
Bruce Fowler, Ph.D.
Edward Murray, Ph.D.
Eric Sampson, Ph.D.

Consumer Product Safety Commission
* Marilyn L. Wind, Ph.D. (Chair)
+ Kristina Hatlelid, Ph.D.
Joanna Matheson, Ph.D.

Department of Agriculture
* Jodie Kulpa-Eddy, D.V.M. (Vice-Chair)
+ Elizabeth Goldentyer, D.V.M.

Department of Defense
* Robert E. Foster, Ph.D.
+ Patty Decot
Harry Salem, Ph.D.
Peter J. Schultheiss, D.V.M., DACLAM

Department of Energy
* Michael Kuperberg, Ph.D.
+ Marvin Stodolsky, Ph.D.

Department of the Interior
* Barnett A. Rattner, Ph.D.
+ Sarah Gerould, Ph.D. (to Feb. 2009)

Department of Transportation
* George Cushmac, Ph.D.
+ Steve Hwang, Ph.D.

Environmental Protection Agency
Office of Pesticide Programs
* John R. "Jack" Fowle III, Ph.D., DABT
+ Vicki Dellarco, Ph.D.
+ Tina Levine, Ph.D.
Deborah McCall
Christine Augustyniak, Ph.D. (*U.S. Coordinator, OECD Test Guidelines Program)*
Office of Pollution Prevention and Toxics
Jerry Smrchek, Ph.D. (*U.S. Coordinator, OECD Test Guidelines Program,* to July 2009*)*
Office of Research and Development
Suzanne McMaster, Ph.D. (to Dec. 2008)
Julian Preston, Ph.D. (to July 2009)
Stephanie Padilla, Ph.D. (to July 2009)
Office of Science Coordination and Policy
Karen Hamernik, Ph.D. (to July 2009)

Food and Drug Administration
Office of the Commissioner
* Suzanne Fitzpatrick, Ph.D., DABT
Center for Biologics Evaluation and Research
Richard McFarland, Ph.D., M.D.
Ying Huang, Ph.D.
Center for Devices and Radiological Health
Melvin E. Stratmeyer, Ph.D.
Vasant G. Malshet, Ph.D., DABT
Center for Drug Evaluation and Research
+ Abigail C. Jacobs, Ph.D.
Paul C. Brown, Ph.D.
Center for Food Safety and Applied Nutrition
David G. Hattan, Ph.D.
Robert L. Bronaugh, Ph.D.
Center for Veterinary Medicine
Devaraya Jagannath, Ph.D.
M. Cecilia Aguila, D.V.M.
National Center for Toxicological Research
Paul Howard, Ph.D.
Donna Mendrick, Ph.D.
William T. Allaben, Ph.D. (to Jan. 2009)
Office of Regulatory Affairs
Lawrence D'Hoostelaere, Ph.D.

National Cancer Institute
* T. Kevin Howcroft, Ph.D.
Chand Khanna, D.V.M., Ph.D.
Alan Poland, M.D. (to Oct. 2008)

National Institute of Environmental Health Sciences
* William S. Stokes, D.V.M., DACLAM
+ Raymond R. Tice, Ph.D.
Rajendra S. Chhabra, Ph.D., DABT
Jerrold J. Heindel, Ph.D.

National Institute for Occupational Safety and Health
* Paul Nicolaysen, V.M.D.
+ K. Murali Rao, M.D., Ph.D.

National Institutes of Health
* Margaret D. Snyder, Ph.D.

National Library of Medicine
* Pertti (Bert) Hakkinen, Ph.D.
+ Jeanne Goshorn, M.S.

Occupational Safety and Health Administration
* Surender Ahir, Ph.D.

* Principal agency representative
\+ Alternate principal agency representative

Acknowledgements

Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Immunotoxicity Working Group (IWG)

U.S. Consumer Product Safety Commission
Joanna Matheson, Ph.D. (IWG Co-chair)
Marilyn L. Wind, Ph.D.

U.S. Environmental Protection Agency
Office of Pesticide Programs
Jonathan Chen, Ph.D.
Masih Hashim, D.V.M., Ph.D.
Marianne Lewis
Deborah McCall
Timothy McMahon, Ph.D.
John Redden, M.S.
Jenny Tao, Ph.D.
Office of Pollution Prevention and Toxics
Elizabeth Margosches, Ph.D.
Ronald Ward, Ph.D.
Office of Research and Development
Marsha Ward, Ph.D.
Office of Science Coordination and Policy
Karen Hamernik, Ph.D.

U.S. Food and Drug Administration
Center for Devices and Radiological Health
Vasant G. Malshet, Ph.D., DABT
Jeffrey Toy, Ph.D.
Center for Drug Evaluation and Research
Ruth Barratt, Ph.D., D.V.M.
Paul C. Brown, Ph.D.
Abigail C. Jacobs, Ph.D. (IWG Co-chair)
Jiaqin Yao, Ph.D.
Office of Science and Health Coordination
Suzanne Fitzpatrick, Ph.D., DABT

National Institute of Environmental Health Sciences
Dori Germolec, Ph.D.
William S. Stokes, D.V.M., DACLAM

National Institute for Occupational Safety and Health
B. Jean Meade, D.V.M., Ph.D.

National Library of Medicine
Pertti Hakkinen, Ph.D.

European Centre for the Validation of Alternative Methods – Liaison
Silvia Casati, Ph.D.
Alexandre Angers, Ph.D.

Japanese Center for the Validation of Alternative Methods – Liaison
Hajime Kojima, Ph.D.

Murine Local Lymph Node Assay
Independent Scientific Peer Review Panel
(March 4-6, 2008 and April 28-29, 2009)

Michael Luster, Ph.D. (Panel Chair)
Senior Consultant to the National Institute for Occupational Safety and Health
Health Effects Laboratory
Morgantown, WV

Nathalie Alépée, Ph.D.
Scientific Coordinator on Alternatives Methods in Life Science
L'Oréal Research and Development
Aulnay sous Bois, France

Anne Marie Api, Ph.D.
Vice President, Human Health Sciences
Research Institute for Fragrance Materials
Woodcliff Lake, NJ

Nancy Flournoy, M.S., Ph.D.
Professor and Chair
Department of Mathematics and Statistics
University of Missouri – Columbia
Columbia, MO

Thomas Gebel, Ph.D.[1]
Regulatory Toxicologist
Federal Institute for Occupational Safety & Health
Dortmund, Germany

Kim Headrick, B.Admin., B.Sc.[1]
International Harmonization and Senior Policy Advisor
Policy and Programme Service Office
Health Canada
Ottawa, Ontario, Canada

Dagmar Jírová, M.D., Ph.D.
Toxicologist, Research Manager
Head of Reference Center for Cosmetics and Reference Laboratory for Experimental Immunotoxicology
National Institute of Public Health
Prague, Czech Republic

David Lovell, Ph.D., FIBiol, CStat, CBiol
Reader in Medical Statistics
Postgraduate Medical School
University of Surrey
Guildford, Surrey, U.K.

Howard Maibach, M.D.
Professor, Department of Dermatology
University of California – San Francisco
San Francisco, CA

James McDougal, Ph.D.[1]
Professor and Director of Toxicology Research
Department of Pharmacology and Toxicology
Boonshoft School of Medicine
Wright State University
Dayton, OH

Michael Olson, Ph.D., A.T.S.
Director of Occupational Toxicology
Corporate Environment, Health and Safety
GlaxoSmithKline
Research Triangle Park, NC

Raymond Pieters, Ph.D.[2]
Associate Professor
Immunotoxicology Group Leader
Institute for Risk Assessment Sciences
Utrecht University
Utrecht, The Netherlands

Jean Regal, Ph.D.
Professor, Department of Pharmacology
University of Minnesota Medical School
Duluth, MN

Jonathan Richmond, MB ChB, FRCSEd[3]
Head, Animals Scientific Procedures Division
Home Office
London, U.K.

Peter Theran, V.M.D.
Consultant
Massachusetts Society for the Prevention of Cruelty to Animals
Novato, CA

Stephen Ullrich, Ph.D.
Professor of Immunology
Graduate School of Biomedical Sciences
University of Texas
M.D. Anderson Cancer Center – Houston
Houston, TX

Michael Woolhiser, Ph.D.
Science and Technology Leader
Toxicology and Environmental Research and Consulting
The Dow Chemical Company
Midland, MI

Takahiko Yoshida, M.D., Ph.D.
Professor, Department of Health Science
Asahikawa Medical College
Hokkaido, Japan

[1] Drs. Gebel and McDougal and Ms. Headrick were unable to attend the public meeting on April 28-29, 2009, and did not participate in the review.

[2] Dr. Pieters was unable to attend the public meeting on April 28-29, 2009. However, he was involved in the peer review of the documents and concurred with the conclusions and recommendations included in the *Independent Scientific Peer Review Panel Report – Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products.*

[3] Dr. Richmond was unable to attend the public meeting on March 4-6, 2008. However, he was involved in the peer review of the documents and concurred with the conclusions and recommendations included in the *Independent Scientific Peer Review Panel Report – Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products.*

National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

National Institute of Environmental Health Sciences

William Stokes, D.V.M., DACLAM
Director; Project Officer

Deborah McCarley
Special Assistant; Assistant Project Officer

NICEATM Support Contract Staff (Integrated Laboratory Systems [ILS], Inc.)

David Allen, Ph.D.
Thomas Burns, M.S.
Linda Litchfield
Steven Morefield, M.D.
Michael Paris
Eleni Salicru, Ph.D.
Catherine Sprankle
Frank Stack
Judy Strickland, Ph.D., DABT
Linda Wilson

Statistical Consultant for ILS, Inc.

Joseph Haseman, Ph.D.

Other Acknowledgements

ICCVAM and NICEATM gratefully acknowledge the following individuals and institutions that submitted data to NICEATM for the evaluation of the LLNA: DA test method.

Kenji Idehara, Ph.D.
Daicel Chemical Industries, Ltd.
Hyogo, Japan

Takashi Omori, Ph.D.
Kyoto University School of Public Health
Kyoto, Japan

Preface

Allergic contact dermatitis (ACD) is an adverse health effect that frequently develops in workers and consumers exposed to skin sensitizing chemicals and products. ACD results in lost workdays[1] and can significantly diminish quality of life (Hutchings et al. 2001; Skoet et al. 2003). To minimize the occurrence of ACD, regulatory authorities require testing to identify substances that may cause skin sensitization. Sensitizing substances must be labeled with a description of the potential hazard and the precautions necessary to avoid development of ACD.

Skin sensitization testing has typically required the use of guinea pigs (Buehler 1965; Magnusson and Kligman 1970). However, in 1998, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) evaluated and recommended an alternative test method known as the murine (mouse) local lymph node assay ("traditional LLNA").[2] The traditional LLNA provides several advantages compared to guinea pig test methods, including elimination of potential pain and distress, use of fewer animals, less time to perform, and availability of dose-response information. Based on the validation database and performance, ICCVAM recommended the LLNA as an alternative test method for assessing the skin sensitization potential of most types of substances (ICCVAM 1999). United States and international regulatory agencies subsequently accepted the traditional LLNA as a valid alternative test method for ACD testing.

In 2007, the U.S. Consumer Product Safety Commission (CPSC) requested that ICCVAM evaluate several modifications of the traditional LLNA, including a nonradioactive version of the LLNA developed by Dr. Kenji Idehara at Daicel Chemical Industries, Ltd. in Hyogo, Japan. This version (referred to as the "LLNA: DA") measures increases in ATP content instead of using a radioactive marker to measure lymphocyte proliferation. The validation studies were completed in coordination with the Japanese Center for the Validation of Alternative Methods (JaCVAM) at the National Institute of Health Sciences. ICCVAM assigned this activity a high priority after considering comments from the public and ICCVAM's Scientific Advisory Committee on Alternative Toxicological Methods (SACATM). As part of their ongoing collaboration with ICCVAM, scientists from the European Centre for Validation of Alternative Methods (ECVAM) and JaCVAM served as liaisons to the ICCVAM Immunotoxicity Working Group (IWG). A detailed timeline of the LLNA: DA evaluation is included with this report.

This Test Method Evaluation Report provides ICCVAM's recommendations regarding the LLNA: DA for assessing the ACD hazard potential of chemicals and products. Since the LLNA: DA does not require the use of a radioactive marker, it can be used by laboratories that currently cannot use the traditional LLNA because they do not have a license for using radioisotopes and in countries that severely limit or discourage the use of radioactive materials required by the traditional LLNA. The report also summarizes the validation status of the LLNA: DA and provides the ICCVAM-recommended LLNA: DA test method protocol.

Following independent scientific peer reviews in 2008 and 2009, ICCVAM submitted a proposed draft Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) for the LLNA: DA that was circulated in July 2009 to the 30 OECD member countries for review and comment. The U.S. CPSC and NICEATM-ICCVAM hosted an OECD Expert Consultation meeting on October 20-22, 2009, to evaluate the comments. A revised TG was distributed to the 30 OECD member countries in December 2009 for comment and then the final draft was forwarded to the

[1] Hhttp://www.blf.gov/IIF

[2] The "traditional LLNA" refers to the ICCVAM-recommended LLNA test method protocol, which measures lymphocyte proliferation based on incorporation of ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine into the cells of the draining auricular lymph nodes (ICCVAM 1999; Dean et al. 2001).

OECD Working Group of National Co-ordinators of the Test Guidelines Programme, which was approved as TG 442A at their March 23-25, 2010 meeting.

ICCVAM solicited and considered public comments and stakeholder involvement throughout the LLNA: DA evaluation process. ICCVAM considered the SACATM comments, the conclusions of the Panel and the OECD Expert Consultation, and all public comments before finalizing the ICCVAM test method recommendations for the LLNA: DA. The recommendations and the Background Review Document, which is provided as an appendix to this report, are incorporated in this ICCVAM Test Method Evaluation Report. As required by the ICCVAM Authorization Act (2000; Public Law 106-545, 42 United States Code 285*l*-3), ICCVAM will forward its recommendations to U.S. Federal agencies for consideration. Federal agencies must respond to ICCVAM within 180 days after receiving the ICCVAM test method recommendations. ICCVAM recommendations are available to the public on the NICEATM-ICCVAM website[3] and agency responses will also be made available on the website as they are received.

We gratefully acknowledge the many individuals who contributed to the preparation, review, and revision of this report. We especially recognize the Panel members for their thoughtful evaluations and generous contributions of time and effort. Special thanks are extended to Dr. Michael Luster for serving as the Panel Chair and to Dr. Michael Woolhiser, Dr. Michael Olson, Dr. Stephen Ullrich, and Kim Headrick for their service as Evaluation Group Chairs. We thank the IWG for assuring a meaningful and comprehensive review. We especially thank Dr. Joanna Matheson (CPSC) and Dr. Abigail Jacobs (U.S. Food and Drug Administration Center for Drug Evaluation and Research) for serving as Co-chairs of the IWG. We also acknowledge Integrated Laboratory Systems, Inc., the NICEATM support contractor, for providing excellent scientific and operational support, including Dr. David Allen, Thomas Burns, Michael Paris, Dr. Eleni Salicru, Frank Stack, and Dr. Judy Strickland. Finally, we thank Dr. Silvia Casati and Dr. Hajime Kojima, the IWG liaisons from ECVAM and JaCVAM, respectively, for their participation and contributions.

This comprehensive ICCVAM evaluation of the LLNA: DA should facilitate regulatory agency decisions on the acceptability of the method. Use of the method by industry can be expected to significantly reduce and refine animal use required for ACD testing while continuing to support the protection of human health.

Marilyn Wind, Ph.D.
Deputy Associate Executive Director
Directorate for Health Sciences
U.S. Consumer Product Safety Commission
Chair, ICCVAM

RADM William S. Stokes, D.V.M., DACLAM
Rear Admiral/Assistant Surgeon General, U.S. Public Health Service
Director, NICEATM
Executive Director, ICCVAM

[3] http://iccvam.niehs.nih.gov/methods/immunotox/llna-DA/TMER.htm

Executive Summary

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recently evaluated the validation status of a nonradioactive version of the murine local lymph node assay (LLNA) called the LLNA modified by Daicel Chemical Industries, Ltd., based on ATP content (LLNA: DA). The LLNA is used to identify chemicals and products that may cause allergic contact dermatitis (ACD), an allergic skin reaction characterized by redness, swelling, and itching. The LLNA: DA measures increases in ATP content by luciferin-luciferase assay as an indicator of increases in lymphocyte cell number while the traditional LLNA uses ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine uptake to measure lymphocyte proliferation.[4] This Test Method Evaluation Report provides ICCVAM's recommendations regarding the usefulness and limitations of the LLNA: DA as a variation of the traditional LLNA. The report includes the ICCVAM-recommended LLNA: DA test method protocol, the final LLNA: DA background review document (BRD) describing the validation status of the test method, and recommendations for future studies and performance standards.

Following nomination of the LLNA: DA by the U.S. Consumer Product Safety Commission (CPSC), the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), ICCVAM, and the ICCVAM Immunotoxicity Working Group prepared an initial draft BRD and draft test method recommendations. The drafts were provided to an independent international scientific peer review panel (Panel) and the public for comment. The Panel met twice in public session to review the initial and revised draft BRDs and draft ICCVAM recommendations. The initial draft BRD evaluated data for 29 substances. The Panel initially met in public session on March 4-6, 2008, to discuss its peer review of the ICCVAM draft BRD and to provide conclusions and recommendations regarding the validation status of the LLNA: DA test method. The Panel also reviewed how well the information in the draft BRD supported ICCVAM's draft test method recommendations. The Panel concluded that definitive test method recommendations could not be made until a detailed protocol and individual animal data were obtained and an evaluation of interlaboratory reproducibility was conducted.

NICEATM revised the draft BRD with additional information and data. The revised draft BRD evaluated data for 44 substances. The Panel reconvened in public session on April 28-29, 2009, to review the ICCVAM revised draft BRD and to finalize its conclusions and recommendations on the current validation status of the LLNA: DA test method.

Based on the revised draft ICCVAM recommendations and Panel reports, NICEATM submitted a proposed draft Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) for the LLNA: DA. The draft TG was circulated in July 2009 to the 30 OECD member countries for review and comment. The U.S. CPSC and NICEATM-ICCVAM hosted an OECD Expert Consultation meeting on October 20-22, 2009, to evaluate the comments. The expert group reviewed the draft OECD TG for the LLNA: DA and proposed responses to comments from member countries. A revised TG was distributed to the 30 OECD member countries in December 2009 for comment and then the final draft was forwarded to the OECD Working Group of National Co-ordinators of the Test Guidelines Programme, which approved the LLNA: DA as TG 442A at their March 23-25, 2010 meeting.

In finalizing this Test Method Evaluation Report and the BRD, which is included as an appendix, ICCVAM considered (1) the conclusions and recommendations of the Panel and the OECD Expert Consultation, (2) comments from ICCVAM's Scientific Advisory Committee on Alternative Toxicological Methods (SACATM), and (3) public comments.

---

[4] *Traditional LLNA* refers to the ICCVAM-recommended LLNA protocol, which measures lymphocyte proliferation based on incorporation of ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine into the cells of the draining auricular lymph nodes (ICCVAM 1999; Dean et al. 2001).

ICCVAM Recommendations: Test Method Usefulness and Limitations

ICCVAM concludes that the accuracy and reliability of the LLNA: DA support use of the test method to identify substances as potential skin sensitizers and nonsensitizers. For the validation database of 44 substances, the LLNA: DA correctly identified all 32 LLNA sensitizers (0% [0/32] false negatives), and nine of the 12 LLNA nonsensitizers (25% [3/12] false positives).[5] ICCVAM recommends that a stimulation index (SI) ≥ 1.8 be used as the decision criterion to identify substances as potential sensitizers. ICCVAM bases this recommendation on the fact that no false negatives, relative to the traditional LLNA, result with the current validation database when an SI ≥ 1.8 is used.

A limitation of the LLNA: DA is the potential for false positive results when borderline positive responses between an SI of 1.8 and 2.5 are obtained. Further, the use of the LLNA: DA might not be appropriate for testing substances that affect ATP levels (e.g., substances that function as ATP inhibitors) or those that affect the accurate measurement of intracellular ATP (e.g., presence of ATP degrading enzymes, presence of extracellular ATP in the lymph node).

ICCVAM Recommendations: Test Method Protocol

The ICCVAM-recommended LLNA: DA test method protocol, which is based on the protocol developed by Yamashita et al. (2005) and Idehara et al. (2008), incorporates all aspects of the ICCVAM-recommended traditional LLNA test method protocol except for those procedures unique to the conduct of the LLNA: DA. In testing situations that do not require dose-response information, or negative results are anticipated, the LLNA: DA should be considered for use as a reduced test method protocol. The reduced LLNA: DA tests only the high dose, thus further reducing animal use.

ICCVAM Recommendations: Future Studies

To further characterize the LLNA: DA test method, ICCVAM recommends that efforts be made to identify additional human data and human experience for test substances. These data may be used to further assess the usefulness and limitations of this and other versions of the LLNA for identifying human sensitizing substances. Such efforts might include postmarketing surveillance of consumers for allergic reactions and occupational surveillance of potentially exposed workers. Additional nonsensitizing skin irritants should be tested to determine the impact of such substances on the false positive rate of the LLNA: DA.

ICCVAM also recommends that efforts be made to further characterize the sensitization potential of borderline positive substances that produce SI values between 1.8 and 2.5 to determine if such results might be false positives. This could include (1) evaluations of peptide reactivity; (2) determination of molecular weight; (3) identification of results from related chemicals; (4) human studies where ethically and scientifically justified; and (5) review of occupational exposures, postmarketing experience or monitoring, and/or *in vitro* testing data. All decision criteria should be reassessed as additional discriminators and data become available.

ICCVAM Recommendations: Performance Standards

The ICCVAM-recommended performance standards for the traditional LLNA (ICCVAM 2009a) apply to the LLNA: DA because the test method is functionally and mechanistically similar to the traditional LLNA. Therefore, ICCVAM recommends that the ICCVAM-recommended performance standards for the traditional LLNA be used to evaluate any future modifications of the LLNA: DA.

Validation Status of the LLNA: DA

The mechanistic basis of the LLNA: DA is identical to that of the traditional LLNA. The traditional LLNA measures the lymphocyte proliferation in the draining lymph nodes for the skin area where the test article is applied. In the traditional LLNA, lymphocyte proliferation three-fold or more higher

[5] These results used the most prevalent outcome for substances that were tested multiple times.

than the vehicle control is considered a positive response indicative of a skin sensitizing substance. The LLNA: DA assesses cell proliferation by measuring increases in ATP content in the draining auricular lymph nodes as an indicator of cell number. The LLNA: DA also differs from the traditional LLNA in the test substance treatment and sampling schedule. In addition, the LLNA: DA includes pretreatment of the application site with an aqueous solution of 1% sodium lauryl sulfate (SLS).

The accuracy of the LLNA: DA was compared to that of the traditional LLNA. Optimal LLNA: DA performance was achieved using SI ≥ 1.8 to classify sensitizers versus nonsensitizers. Compared to the traditional LLNA, accuracy was 93% (41/44), with a false positive rate of 25% (3/12) and a false negative rate of 0% (0/32). The three false positive substances using SI ≥ 1.8 produced SI values between 1.8 and 2.5 in the LLNA: DA. Therefore, other available information, such as dose-response, evidence of systemic toxicity or excessive local irritation, and where appropriate, statistical significance together with SI values should be considered to confirm that such borderline positive results are potential skin sensitizers. Consideration should also be given to various properties of the test substance, including whether it is structurally similar to known skin sensitizers.

An evaluation to determine the robustness of the optimum SI ≥ 1.8 decision criterion indicated that the SI was quite stable. Taking different samples of the data as training and validation sets had relatively little impact on the cutoff SI criterion or on the resulting number of false or false negative results.

ICCVAM concludes that the reproducibility of the LLNA: DA supports the use of the method to identify substances as potential skin sensitizers and nonsensitizers. The validation database supported an assessment of both intra- and interlaboratory reproducibility. A two-phased study was conducted to assess interlaboratory reproducibility.

Intralaboratory reproducibility was assessed using a coefficient of variation (CV) analysis of EC3 (estimated concentration needed to produce an SI of 3.0) and EC1.8 values (estimated concentration needed to produce an SI of 1.8) for isoeugenol and eugenol. (Each substance was tested in three different experiments.) The mean EC3 value for isoeugenol was 2.74% ± 0.58%, with a corresponding CV of 21%. Eugenol had an EC3 of 5.06% ± 0.55% and a CV of 11%. The mean EC1.8 value and corresponding CV for isoeugenol and eugenol were 0.87% ± 0.31% (36% CV) and 3.38% ± 0.79% (23% CV), respectively.

Both phases of an interlaboratory validation study included qualitative analyses of LLNA: DA reproducibility. An SI ≥ 1.8 was used as the threshold to distinguish sensitizers from nonsensitizers. In the first phase, 12 substances (nine sensitizers and three nonsensitizers based on traditional LLNA test results) were tested in either three or 10 laboratories. There was 100% agreement among the laboratories for 10 substances (seven sensitizers and three nonsensitizers based on traditional LLNA results). There was 67% (2/3) agreement among the tests for the remaining two traditional LLNA sensitizers. Interlaboratory CV values for the EC1.8 values of the nine sensitizers ranged from 15% to 140%.

The second phase included five substances (four sensitizers and one nonsensitizer based on traditional LLNA test results) tested in either four or seven laboratories. There was 100% agreement among the laboratories for four substances (three sensitizers and one nonsensitizer based on traditional LLNA results). There was 75% (3/4) agreement among the tests for the remaining traditional LLNA sensitizer. Interlaboratory CV values for the EC1.8 values of the four traditional LLNA sensitizers ranged from 14% to 93%.

Reproducibility of results for the 14 substances (10 traditional LLNA sensitizers and four traditional LLNA nonsensitizers) that had three to 18 test results, regardless of whether the tests were performed in one laboratory or multiple laboratories, was assessed with respect to SI category. When the SI ≥ 1.8 decision criterion was used to classify sensitizers versus nonsensitizers the SI results for 80%

(8/10) of the sensitizers (based on traditional LLNA results) were 100% concordant (i.e., all tests for that substance yielded maximum SI ≥ 1.8) in the LLNA: DA for three to 18 tests. The SI results for 75% (3/4) of the nonsensitizers (based on traditional LLNA results) were 100% concordant in the LLNA: DA (i.e., all tests for that substance yielded SI < 1.8) for four to 11 tests. The other nonsensitizer had 91% concordance (10/11). This test for the nonsensitizer yielded SI values between 1.8 and 2.5, the narrow region in which false positive results occurred.

ICCVAM Consideration of Independent Peer Review Panel Report and Other Comments

The ICCVAM evaluation process incorporates a high level of scientific peer review and transparency. The evaluation process for the LLNA: DA included two public review meetings by an independent scientific peer review panel, multiple opportunities for public comments, consideration of reports from an OECD Consultation, and comments from the SACATM. ICCVAM and the Immunotoxicity Working Group considered the Panel report, conclusions of the OECD Expert Consultation, the SACATM comments, and all public comments before finalizing the ICCVAM Test Method Evaluation Report and final BRD for the LLNA: DA.

1.0 Introduction

The murine local lymph node assay (traditional LLNA)[1] is an alternative skin-sensitization test method that requires fewer animals and less time than currently accepted guinea pig tests (e.g., the guinea pig maximization test [GPMT] and the Buehler test). It also avoids animal discomfort that can occur in the guinea pig tests when substances cause allergic contact dermatitis (ACD). The LLNA measures cell proliferation in the draining auricular lymph nodes of the mouse by analyzing incorporation of a radioactive marker into newly synthesized DNA. The LLNA was the first alternative test method evaluated and recommended by the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). International regulatory authorities have now recognized the traditional LLNA as an acceptable alternative to guinea pig tests for most testing situations.

The LLNA modified by Daicel Chemical Industries, Ltd., based on ATP content (referred to hereafter as the "LLNA: DA") was one of several modified versions of the LLNA nominated by the U.S. Consumer Product Safety Commission (CPSC) for evaluation by ICCVAM and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM).[2] It is a nonradioactive version of the LLNA that assesses cell proliferation by detecting increases in ATP content as an indicator of cell number at the end of cell proliferation rather than by quantifying the incorporation of ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine. The increase in ATP content in lymph nodes from test animals compared to vehicle control animals is then quantified using a luciferin-luciferase assay. The LLNA: DA can reduce the use of animals for skin sensitization testing when it is used in place of guinea pig tests in countries that severely limit or discourage the use of radioactive materials that are required by the traditional LLNA.

In accordance with the ICCVAM Authorization Act of 2000 (Public Law 106-545, 42 United States Code 285*l*-3), ICCVAM coordinates the technical evaluations of new, revised, and alternative test methods with regulatory applicability. After considering comments from the public and ICCVAM's advisory committee, the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM), ICCVAM members unanimously agreed that the LLNA: DA should have a high priority for evaluation. A detailed timeline of the LLNA: DA evaluation is provided in **Appendix A**. The ICCVAM-recommended LLNA: DA test method protocol and the final LLNA: DA background review document (BRD) are provided in **Appendices B** and **C**, respectively.

The ICCVAM Immunotoxicity Working Group (IWG) was established to work with NICEATM to evaluate the LLNA: DA and other test methods and applications. The European Centre for the Validation of Alternative Methods (ECVAM) and the Japanese Center for the Validation of Alternative Methods (JaCVAM) designated liaison members to the IWG.

To facilitate peer review of the LLNA: DA test method, the IWG and NICEATM prepared a comprehensive draft BRD that provided information and data from validation studies and the scientific literature. A May 17, 2007, *Federal Register* (FR) notice (72 FR 27815)[3] requested data and information on these test methods and nominations of individuals to serve on an international independent scientific peer review panel (Panel). The request was also disseminated via the ICCVAM electronic mailing list and through direct requests to over 100 stakeholders. In response to this request, one individual submitted LLNA: DA data and three individuals or organizations nominated members to the Panel (see **Section 4.0**).

---

[1] The "traditional LLNA" refers to the ICCVAM-recommended LLNA test method protocol, which measures lymphocyte proliferation based on incorporation of ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine into the cells of the draining auricular lymph nodes (ICCVAM 1999; Dean et al. 2001).

[2] Available at http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf

[3] Available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf

In the initial draft BRD, ICCVAM examined data for 29 substances with adequate traditional LLNA data (19 sensitizers and 10 nonsensitizers, as classified by the traditional LLNA) that were tested in a single laboratory (Idehara et al. 2008). On January 8, 2008, ICCVAM announced the availability of the draft BRD to the public and a public Panel meeting to review the validation status of the LLNA: DA (and other LLNA-related activities) (73 FR 1360).[4] All of the information provided to the Panel, including the ICCVAM draft BRD, draft test method recommendations, and all public comments received prior to the Panel meeting, were made publicly available via the NICEATM-ICCVAM website.[5]

The first Panel meeting was a public session held on March 4-6, 2008, to review the validation status of the LLNA: DA and the completeness of the ICCVAM draft BRD (see **Appendix D**). The Panel evaluated (1) the extent to which the draft BRD addressed established validation and acceptance criteria and (2) the extent to which the draft BRD supported ICCVAM's draft proposed test method uses, recommended test method protocol, draft test method performance standards, and proposed future studies. Interested stakeholders from the public were provided opportunities to comment at the Panel meeting. The Panel considered these comments as well as those submitted prior to the meeting before concluding their deliberations. The Panel agreed with the draft ICCVAM recommendations that the LLNA: DA may be useful for identifying substances as potential skin sensitizers and nonsensitizers, but that more information and data were needed before definitive conclusions on the usefulness and limitations of the LLNA: DA could be made. The Panel noted that the following information was needed before definitive recommendations could be made: (1) a detailed test method protocol; (2) individual animal data for the validation database; and (3) an evaluation of interlaboratory reproducibility. On May 20, 2008, ICCVAM posted a report of the Panel's recommendations[6] (see **Appendix D**) on the NICEATM-ICCVAM website for public review and comment (announced in 73 FR 29136).[7]

ICCVAM provided SACATM with the draft BRD and draft test method recommendations, the Panel report, and all public comments for discussion at their meeting on June 18-19, 2008, where public stakeholders were given another opportunity to comment.

NICEATM subsequently obtained a detailed test method protocol and additional data and revised the draft BRD to include this new information. The revised draft BRD included an accuracy evaluation for the expanded database of individual animal results for 44 substances with adequate traditional LLNA data (32 sensitizers and 12 nonsensitizers, as classified by the traditional LLNA) as well as an evaluation of interlaboratory reproducibility. Based on the analyses included in the revised draft BRD, ICCVAM prepared revised draft test method recommendations for proposed test method uses and limitations, recommended test method protocol, test method performance standards, and future studies for the LLNA: DA.

On November 4, 2008, JaCVAM released a statement that at a meeting concerning the LLNA: DA at the National Institute of Health Sciences, Tokyo, Japan, on August 28, 2008, the noncommissioned members of the JaCVAM Regulatory Acceptance Board unanimously endorsed the following statement (see **Appendix E**): "Following the review of the results of the Ministry of Health, Labour and Welfare-funded validation study of the LLNA: DA coordinated by the Japanese Society for Alternative to Animal Experimentation, it is concluded that the LLNA: DA can be used for distinguishing between sensitizer and nonsensitizer chemicals within the context of the Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) 429 on skin sensitization: LLNA."

---

[4] Available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_25553.pdf
[5] Available at http://iccvam.niehs.nih.gov
[6] Available at http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf
[7] Available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR-E8-11195.pdf

ICCVAM released the revised draft documents to the public for comment on February 27, 2009, and announced a second meeting of the Panel (74 FR 8974).[8] The Panel reconvened on April 27-28, 2009, to reassess the validation status of the LLNA: DA (see **Appendix D**). The Panel also reviewed the completeness of the revised draft ICCVAM BRD and the extent to which the information therein supported the revised draft ICCVAM test method recommendations. On June 1, 2009, ICCVAM posted the second report of the Panel's recommendations[9] (see **Appendix D**) on the NICEATM-ICCVAM website for public review and comment (announced in 74 FR 26242).[10]

ICCVAM provided SACATM with the revised draft BRD, the second Panel report, and all public comments for discussion at their meeting on June 25-26, 2009, where public stakeholders were given another opportunity to comment.

Based on the revised draft ICCVAM recommendations, NICEATM submitted a proposed draft OECD TG for the LLNA: DA that was circulated in July 2009 to the 30 OECD member countries for review and comment via their National Co-ordinators, who distributed the draft TG to interested stakeholders. An OECD Expert Consultation Meeting was held on October 20-22, 2009, to evaluate the comments. Scientists from the National Institute of Environmental Health Sciences (NIEHS), the Environmental Protection Agency, the Food and Drug Administration, and the CPSC, as well as U.S. and international experts from industry and other stakeholder organizations participated in the meeting, which was co-hosted by CPSC and NICEATM-ICCVAM. The expert group reviewed the draft OECD TG for the LLNA: DA and proposed responses to comments from member countries. The OECD Expert Consultation convened a subsequent teleconference on December 1, 2009, to discuss outstanding issues identified at the October meeting. A revised TG was again distributed in December 2009 for review and comment to national experts and interested stakeholders of the 30 OECD member countries. A final teleconference of the OECD Expert Consultation was convened on January 29, 2010, to discuss the member country comments received during the last round of review, and a final draft TG was developed based on these discussions. This final draft was forwarded to the OECD Working Group of National Co-ordinators of the Test Guidelines Programme to consider for adoption at their March 23-25, 2010, meeting.

ICCVAM and the IWG considered the SACATM comments, the Panel report, conclusions of the OECD Expert Consultation, and all public comments before finalizing ICCVAM test method recommendations for the LLNA: DA. The recommendations (**Section 2.0**) and the final BRD (**Appendix C**) are incorporated in this ICCVAM Test Method Evaluation Report. As required by the ICCVAM Authorization Act of 2000 (Public Law 106-545, 42 United States Code 285l-3), ICCVAM will forward its recommendations to U.S. Federal agencies for consideration. Federal agencies must respond to ICCVAM within 180 days after receiving ICCVAM test method recommendations. ICCVAM recommendations are available to the public on the NICEATM-ICCVAM website, and agency responses will also be made available on the website as they are received.

[8] Available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR-E9-4280.pdf
[9] Available at http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2009.pdf
[10] Announced in 74 FR 26242 http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR-E9-12360.pdf

2.0 ICCVAM Recommendations for the Nonradioactive LLNA: DA Test Method

ICCVAM evaluated the validation status of the LLNA: DA as a nonradioactive modification of the traditional LLNA (ICCVAM 1999; Dean et al. 2001; Haneke et al. 2001; Sailstad et al. 2001) to identify substances that may cause ACD for regulatory hazard classification and labeling purposes. While the traditional LLNA assesses cell proliferation by measuring the incorporation of ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine into the DNA of dividing cells in the draining auricular lymph nodes, the LLNA: DA assesses cell proliferation by measuring increases in ATP content in the draining auricular lymph nodes as an indicator of the cell number at the end of cell proliferation. The LLNA: DA also differs from the traditional LLNA in the test substance treatment and sampling schedule, as well as pretreatment at the application site with an aqueous solution of 1% sodium lauryl sulfate (SLS) (see **Appendix B**). NICEATM and ICCVAM prepared a comprehensive report on the data and information supporting the validity of this test method, including its accuracy and reliability compared to the traditional LLNA (see **Section 3.0** and **Appendix C)**.

2.1 ICCVAM Recommendations: Test Method Usefulness and Limitations

ICCVAM concludes that the accuracy and reliability of the LLNA: DA support use of the test method to identify substances as potential skin sensitizers and nonsensitizers. For the validation database of 44 substances,[11] the LLNA: DA correctly identified all 32 LLNA sensitizers (0% [0/32] false negatives), and nine of the 12 LLNA nonsensitizers (25% [3/12] false positives). ICCVAM recommends that a stimulation index (SI) $\geq$ 1.8 be used as the decision criterion to identify substances as potential sensitizers. ICCVAM bases this recommendation on the fact that no false negatives, relative to the traditional LLNA, result with the current validation database when an SI $\geq$ 1.8 is used.

A limitation of the LLNA: DA is the potential for false positive results when borderline positive responses between an SI of 1.8 and 2.5 are obtained (see **Section 3.4**). ICCVAM considers the applicability domain for the LLNA: DA to be the same as the traditional LLNA unless there are properties associated with a class of materials that may interfere with the accuracy of the LLNA: DA. For instance, the use of the LLNA: DA might not be appropriate for testing substances that affect ATP levels (e.g., substances that function as ATP inhibitors) or those that affect the accurate measurement of intracellular ATP (e.g., presence of ATP degrading enzymes, presence of extracellular ATP in the lymph node). In contrast, the LLNA: DA can be used for testing metal compounds, with the exception of nickel. Inconsistent results for nickel sulfate in the interlaboratory validation study suggest that the LLNA: DA may not be suitable for testing substances containing nickel and therefore further testing using a different test system is recommended when negative results are obtained for such substances.

2.2 ICCVAM Recommendations: Test Method Protocol

ICCVAM recommends a LLNA: DA test method protocol (**Appendix B**) that is based on the test method protocol developed by Yamashita et al. (2005) and Idehara et al. (2008). The ICCVAM-recommended LLNA: DA test method protocol incorporates all aspects of the ICCVAM-recommended LLNA test method protocol (Appendix A of ICCVAM 2009a) except for those procedures unique to the conduct of the LLNA: DA (**Appendix B**). Key aspects from the ICCVAM-recommended LLNA test method protocol (Appendix A of ICCVAM 2009a) included in the ICCVAM-recommended LLNA: DA test method protocol (**Appendix B**) are the following:

[11] For the accuracy analyses, results for substances tested multiple times were combined so that each substance was represented by one result. In this case, the single result used for each substance represented the most prevalent outcome. Multiple tests were available for 14 substances tested with the LLNA: DA.

- The high dose should be the maximum possible concentration (for liquids, solids, or suspensions) that does not produce systemic toxicity and/or excessive local skin irritation. The measurement of ear thickness is a potentially valuable adjunct for identifying local skin irritation.
- A minimum of four animals per dose group is recommended.
- Collection of individual animal data is recommended.
- Inclusion of a concurrent vehicle control and concurrent positive control in each study is recommended.

Additionally, ICCVAM recommends that there should be a measure of variability of the positive control response over time. Laboratories should maintain a historical database of positive control SI values such that results can be compared to the mean historical SI. There could be cause for concern when a negative test substance result is accompanied by a concurrent positive control SI value significantly lower than the mean historical SI.

In testing situations where dose-response information is not required, or negative results are anticipated, ICCVAM recommends that the reduced LLNA: DA should be considered and used where determined appropriate. The reduced LLNA: DA test method protocol uses only the high dose (Kimber et al. 2006; ESAC 2007; ICCVAM 2009b), thus further reducing animal use by up to 40%.

2.3 ICCVAM Recommendations: Future Studies

ICCVAM recommends the following future studies to further characterize the usefulness and limitations of the LLNA: DA test method:

- Efforts should be made to identify additional human data and human experience for test substances. These data may be used to further assess the usefulness and limitations of this and other versions of the LLNA for identifying human-sensitizing substances. Such efforts might include postmarketing surveillance of consumers for allergic reactions and occupational surveillance of potentially exposed workers.
- Additional substances that are nonsensitizing skin irritants should be tested to determine the impact of such substances on the false positive rate of the LLNA: DA.
- Inconsistent results for nickel sulfate suggest that the LLNA: DA may not be suitable for testing nickel compounds. Therefore, the accrual of additional data from LLNA: DA studies on such compounds with comparative human and/or guinea pig data is needed in order to more comprehensively evaluate the suitability of the LLNA: DA for testing nickel compounds.
- Efforts should be made to further characterize the sensitization potential of borderline positive substances (i.e., those that produce SI values between 1.8 and 2.5) in the LLNA: DA to determine if such results might be false positives. This could include evaluations of peptide reactivity, determination of molecular weight, identification of results from related chemicals, human studies where ethically and scientifically justified, review of occupational exposures and postmarketing experience or monitoring, or *in vitro* testing data. All decision criteria should be reassessed as additional discriminators and data become available.

2.4 ICCVAM Recommendations: Performance Standards

ICCVAM concludes that the ICCVAM-recommended performance standards (ICCVAM 2009a) for the traditional LLNA can be used to evaluate any future modifications of the LLNA: DA. The ICCVAM-recommended performance standards for the traditional LLNA apply to the LLNA: DA because the test method is functionally and mechanistically similar to the traditional LLNA. ICCVAM, in conjunction with ECVAM and JaCVAM, developed the internationally harmonized test

method performance standards for the traditional LLNA (ICCVAM 2009a) to evaluate the performance of LLNA test methods that incorporate specific protocol modifications (e.g., procedures to measure lymphocyte proliferation) compared to the traditional LLNA. Thus, unique performance standards for the LLNA: DA are not proposed at this time.

3.0 Validation Status of the LLNA: DA Test Method

The ICCVAM BRD for the LLNA: DA test method (**Appendix C**) provides a comprehensive review of the current validation status of the LLNA: DA test method, including its accuracy and reliability, the substances tested, the rationale for the standardized test method protocol used for the validation studies, and all available data supporting its validity. This section provides a brief description and summary of the validation status of the LLNA: DA test method.

3.1 Test Method Description

Originally developed by Yamashita et al. (2005) and Idehara et al. (2008), the purpose of the LLNA: DA test method is to identify potential skin sensitizers by quantifying lymphocyte proliferation. Like the traditional LLNA, the magnitude of lymphocyte proliferation measured in the LLNA: DA correlates with the extent to which sensitization develops after a topical induction exposure to a potential skin sensitizing substance.

3.1.1 General Test Method Procedures

The test substance is administered topically on days one, two, three, and seven to the dorsum of the ears of mice at a concentration that provides maximum solubility of the test substance without producing systemic toxicity and/or excessive local skin irritation. One hour prior to each test substance application, an aqueous solution of 1% SLS is applied to the dorsum of the mouse ears to increase absorption of the test substance across the skin (van Och et al. 2000). Approximately 24 hours after the last test substance administration, the draining auricular lymph nodes are excised, and a single-cell suspension from the lymph nodes of each animal is prepared for quantifying the increase in ATP content, which serves as an indicator of cell number at the end of cell proliferation.

The increase in ATP content for each mouse is measured by luciferin-luciferase assay and is expressed in relative luminescence units (RLU). The SI is calculated as the ratio of the mean RLU/mouse for each treatment group against the mean RLU/mouse for the vehicle control group. Substances producing an SI greater than a specified threshold are considered to be potential skin sensitizers. Based on the accuracy evaluation described in **Section 3.4**, the optimum accuracy was at SI $\geq$ 1.8.

3.1.2 Similarities and Differences Between the Test Method Protocols for the Traditional LLNA and the LLNA: DA

While the traditional LLNA assesses cell proliferation by measuring the incorporation of radioactive thymidine or iodine into the DNA of dividing cells in the draining auricular lymph nodes (ICCVAM 1999; Dean et al. 2001), the LLNA: DA assesses cell proliferation by measuring increases in ATP content in the draining auricular lymph nodes as an indicator of cell number at the end of cell proliferation. The LLNA: DA also differs from the traditional LLNA in the test substance treatment and sampling schedule, as well as pretreatment at the application site with an aqueous solution of 1% SLS (see **Appendix B**).

In the traditional LLNA, the test substance is topically applied on three consecutive days. Two days after the last treatment, a radioactive marker such as ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine (in phosphate-buffered saline; 250 µL/mouse) is administered via the tail vein. Then, five hours later, the draining auricular lymph nodes are excised and prepared for quantifying the incorporation of radioactivity. By comparison, in the LLNA: DA, the test substance is administered topically on days one, two, three, and seven, with each treatment preceded by application of an aqueous solution of 1% SLS. The draining auricular lymph nodes are excised 24 hrs after the last test substance application

and prepared for quantifying the increase in ATP content, which does not require injection of a marker chemical.

3.2 Validation Database

The current validation database for the LLNA: DA includes results from studies for 46 substances that had previously been tested in the traditional LLNA. The LLNA: DA results were obtained from either the intralaboratory (Idehara et al. 2008; unpublished data) and/or the two-phased interlaboratory (Omori et al. 2008) validation study. These data were available and reviewed by the Panel in April 2009.

The reference test data for the 46 substances were obtained from traditional LLNA tests. Of the 46 substances, 33 were classified by the traditional LLNA as skin sensitizers, 12 were classified as nonsensitizers, and one (benzocaine) was classified as equivocal due to highly variable results (Basketter et al. 1995; ICCVAM 1999) and was not included in the performance analyses. Similar to benzocaine, traditional LLNA data for toluene 2,4-diisocyanate (van Och et al. 2000) were not suitable for comparison (i.e., a modified version of the traditional LLNA test method protocol was used that was not in accordance with OECD TG 429 [OECD 2002] or ICCVAM 1999 and Dean et al. 2001) and results for this test substance were not included in the performance analysis. Thus, the validation database is comprised of 44 substances tested in the LLNA: DA that have adequate traditional LLNA reference data for use in the performance analyses. Results from guinea pig skin sensitization testing and human skin sensitization testing and/or published clinical case report information are also provided where they were available (see **Appendix C, Annex III**). Of the 46 substances, 42 had guinea pig skin sensitization testing data and 43 had human skin sensitization testing data and/or published clinical case report information. Similar to LLNA: DA comparisons with the traditional LLNA, benzocaine and toluene 2,4-diisocyanate were not included in comparisons between the LLNA: DA and guinea pig or human outcomes.

Table 3-1 lists the chemical classifications, traditional LLNA EC3 values with maximum SI values, and LLNA: DA EC1.8 values with maximum SI values for the 44 substances with adequate comparative LLNA data that were evaluated in the LLNA: DA performance analyses. Twenty chemical classes were represented by the 44 substances evaluated in the LLNA: DA performance analyses; 13 substances were classified in more than one chemical class. The classes with the highest number of substances were carboxylic acids (16 substances) and phenols (5 substances). Further, of the 22 chemical classes represented in the NICEATM LLNA database by at least five substances (thereby providing a sufficiently large representation for further analyses), 20 classes had at least 60% of the traditional LLNA results identified as positive. For this database of more than 600 substances, these classes were identified as those most likely to be associated with skin sensitization. Seventeen of these classes were also represented in the LLNA: DA database (only amides, ketones, and macromolecular substances were not included). Among the chemical classes that have been previously identified as common skin allergens (e.g., aldehydes, ketones, quinones, and acrylates, [Gerberick et al. 2004]), only ketones were not included in the LLNA: DA database. Nevertheless, the Panel considered the database of substances tested in the LLNA: DA to be representative of a sufficient range of chemicals typically tested for skin sensitization potential. The traditional LLNA EC3 values (i.e., estimated concentration needed to produce an SI = 3) for the 32 sensitizers ranged from 0.009% to 90%.

Table 3-1 Product Use and Chemical Classification, Traditional LLNA EC3 Values, LLNA: DA EC1.8 Values, and Maximum SI Values for 44 Substances Evaluated in the LLNA: DA Performance Analyses

Substance Name	Product Use[1]	Chemical Class[2]	Trad. LLNA EC3 (%) (Max. SI)[3]	LLNA: DA EC1.8 (%) (Max. SI)[3]
5-Chloro-2-methyl-4-isothiazolin-3-one[4]	Cosmetics; Manufacturing; Pesticides	Sulfur Compounds; Heterocyclic Compounds	0.009 (27.7)	0.009 (7.5)
p-Benzoquinone[4]	Manufacturing; Pesticides; Pharmaceuticals	Quinones	0.010 (52.3)	0.003 (3.8)
2,4-Dinitrochlorobenzene[5, 6]	Manufacturing; Pesticides	Hydrocarbons, Cyclic; Hydrocarbons, Halogenated; Nitro Compounds	0.049 (43.9)	0.032 (15.1)
Benzalkonium chloride[5]	Cosmetics; Disinfectant; Manufacturing; Personal care products; Pesticides	Amines; Onium Compounds	0.070[7] (11.1)	0.402 (6.7)
Glutaraldehyde[5, 6]	Cosmetics; Disinfectant; Manufacturing; Pesticides	Aldehydes	0.083 (18.0)	0.118 (6.5)
p-Phenylenediamine[5]	Intermediate in chemical synthesis; Manufacturing	Amines	0.110 (26.4)	0.036 (5.1)
Potassium dichromate[5, 8]	Manufacturing; Pharmaceuticals	Inorganic Chemical, Chromium Compounds; Inorganic Chemical, Potassium Compounds	0.170 (33.6)	0.062 (6.4)
Propyl gallate[4]	Cosmetics; Food additive	Carboxylic Acids	0.320 (33.6)	0.225 (5.0)
Phthalic anhydride[5]	Intermediate in chemical synthesis; Manufacturing; Pharmaceuticals	Anhydrides; Carboxylic Acids	0.360 (26.0)	0.030 (6.9)
Formaldehyde[5, 6]	Disinfectant; Manufacturing	Aldehydes	0.495 (4.0)	0.699 (5.1)
Cobalt chloride[5, 6, 8]	Manufacturing; Pesticides	Inorganic Chemical, Elements; Inorganic Chemical, Metals	0.600 (7.2)	0.859 (20.6)
Isoeugenol[5, 6]	Food additive; Fragrance agent	Carboxylic Acids	1.540 (31.0)	1.477 (12.4)

continued

Table 3-1 Product Use and Chemical Classification, Traditional LLNA EC3 Values, LLNA: DA EC1.8 Values, and Maximum SI Values for 44 Substances Evaluated in the LLNA: DA Performance Analyses (continued)

Substance Name	Product Use[1]	Chemical Class[2]	Trad. LLNA EC3 (%) (Max. SI)[3]	LLNA: DA EC1.8 (%) (Max. SI)[3]
2-Mercaptobenzothiazole[5]	Manufacturing; Pesticides	Heterocyclic Compounds	1.700 (8.6)	7.992 (2.0)
Cinnamic aldehyde[5]	Cosmetics; Food additive; Fragrance agent; Intermediate in chemical synthesis; Personal care products; Pesticides	Aldehydes	1.910 (18.4)	0.635 (4.7)
3-Aminophenol[6]	Cosmetics; Pharmaceuticals	Amines; Phenols	3.200 (5.7)	1.841 (2.8)
Diethyl maleate[4]	Food additive; Intermediate in chemical synthesis	Carboxylic Acids	3.600 (22.6)	0.442 (3.8)
Trimellitic anhydride[5]	Manufacturing	Anhydride; Carboxylic Acids	4.710 (4.6)	0.058 (5.0)
Nickel (II) sulfate hexahydrate[5, 6, 8]	Manufacturing	Inorganic Chemical, Elements; Inorganic Chemical, Metals	4.800 (3.1)	2.606 (11.8)
Resorcinol[5]	Cosmetics; Manufacturing; Personal care products; Pesticides; Pharmaceuticals	Phenols	6.330 (10.4)	3.902 (4.3)
Sodium lauryl sulfate[5]	Cosmetics; Food additive; Manufacturing; Personal care products; Pesticides; Pharmaceuticals	Alcohols; Sulfur Compounds; Lipids	8.080 (8.9)	1.640 (3.4)
Citral[5]	Fragrance agent	Hydrocarbons, Other	9.170 (20.5)	2.053 (4.4)
Hexyl cinnamic aldehyde[5, 6, 8]	Food additive; Fragrance agent	Aldehydes	9.740 (20.0)	6.275 (10.2)

continued

Table 3-1 Product Use and Chemical Classification, Traditional LLNA EC3 Values, LLNA: DA EC1.8 Values, and Maximum SI Values for 44 Substances Evaluated in the LLNA: DA Performance Analyses (continued)

Substance Name	Product Use[1]	Chemical Class[2]	Trad. LLNA EC3 (%) (Max. SI)[3]	LLNA: DA EC1.8 (%) (Max. SI)[3]
Eugenol[5]	Cosmetics; Food additive; Intermediate in chemical synthesis; Manufacturing; Personal care products; Pharmaceuticals	Carboxylic Acids	10.090 (17.0)	2.629 (7.1)
Abietic acid[5, 6]	Manufacturing	Hydrocarbons, Cyclic; Polycyclic Compounds	11.920 (5.2)	4.530 (8.0)
Phenyl benzoate[4]	Manufacturing; Pesticides	Carboxylic Acids	13.600 (11.1)	0.653 (4.2)
Cinnamic alcohol[4]	Cosmetics; Food additive; Fragrance agent; Intermediate in chemical synthesis; Personal care products	Alcohols	21.000 (5.7)	5.218 (5.7)
Hydroxycitronellal[5]	Food additive; Fragrance agent; Personal care products	Hydrocarbons, Other	23.750 (8.5)	8.674 (5.7)
Imidazolidinyl urea[5]	Cosmetics; Personal care products; Pesticides	Urea	24.000 (5.5)	6.275 (4.7)
Ethylene glycol dimethacrylate[4]	Manufacturing	Carboxylic Acids	28.000 (7.0)	19.236 (4.5)
Butyl glycidyl ether[4]	Intermediate in chemical synthesis; Manufacturing	Ethers	30.900 (5.6)	17.507 (4.6)
Ethyl acrylate[4]	Manufacturing	Carboxylic Acids	32.800 (4.0)	6.790 (4.3)
Methyl methacrylate[4]	Manufacturing	Carboxylic Acids	90.000 (3.6)	99.347 (1.8)
1-Bromobutane[5]	Intermediate in chemical synthesis; Pharmaceuticals; Solvent	Hydrocarbons, Halogenated	NA (1.2)	NA (1.7)

continued

Table 3-1 Product Use and Chemical Classification, Traditional LLNA EC3 Values, LLNA: DA EC1.8 Values, and Maximum SI Values for 44 Substances Evaluated in the LLNA: DA Performance Analyses (continued)

Substance Name	Product Use[1]	Chemical Class[2]	Trad. LLNA EC3 (%) (Max. SI)[3]	LLNA: DA EC1.8 (%) (Max. SI)[3]
Chlorobenzene[5]	Manufacturing; Solvent	Hydrocarbons, Cyclic; Hydrocarbons, Halogenated	NA (1.7)	17.877 (2.4)
Diethyl phthalate[5]	Cosmetics; Manufacturing; Personal care products; Pesticides; Pharmaceuticals	Carboxylic Acids	NA (1.5)	NA (1.1)
Dimethyl isophthalate[4, 6]	Manufacturing; Fragrance agent	Carboxylic Acids	NA (1.0)	NA (1.3)
Hexane[5]	Manufacturing; Solvent	Hydrocarbons, Acyclic	NA (2.2)	82.232 (2.3)
Isopropanol[5, 6]	Cosmetics; Disinfectant; Food additive; Intermediate in chemical synthesis; Manufacturing; Personal care products; Pharmaceuticals; Solvent	Alcohols	NA (1.7)	NA (2.0)
Lactic acid[5, 8]	Food additive; Manufacturing; Pharmaceuticals	Carboxylic Acids	NA (2.2)	NA (1.1)
Methyl salicylate[5, 6]	Cosmetics; Food additive; Fragrance agent; Personal care products; Pharmaceuticals; Solvent	Carboxylic Acids; Phenols	NA (2.9)	NA (1.8)
Propylparaben[5]	Food additive; Pesticides; Pharmaceuticals	Carboxylic Acids; Phenols	NA (1.4)	NA (1.3)
Nickel (II) chloride[4]	Manufacturing; Pesticides	Inorganic Chemical, Elements; Inorganic Chemical, Metals	NA (2.4)	NA (1.3)
Salicylic acid[4]	Food additive; Manufacturing; Pharmaceuticals	Phenols; Carboxylic Acids	NA (2.5)	17.768 (2.0)

continued

Table 3-1 Product Use and Chemical Classification, Traditional LLNA EC3 Values, LLNA: DA EC1.8 Values, and Maximum SI Values for 44 Substances Evaluated in the LLNA: DA Performance Analyses (continued)

Substance Name	Product Use[1]	Chemical Class[2]	Trad. LLNA EC3 (%) (Max. SI)[3]	LLNA: DA EC1.8 (%) (Max. SI)[3]
Sulfanilamide[4]	Pharmaceuticals	Hydrocarbons, Cyclic; Sulfur Compounds	NA (1.0)	NA (0.9)

Abbreviations: EC3 = estimated concentration needed to produce a stimulation index of three; EC1.8 = estimated concentration needed to produce a stimulation index of 1.8; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; Max. = maximum; NA = not available; SI = stimulation index.

[1] Information for product use was gathered from the following databases:
Hazardous Substances Database - National Library of Medicine – TOXNET: http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB.
Haz-Map: National Library of Medicine-Toxicology and Environmental Health Information Program: http://hazmap.nlm.nih.gov/.
Household Products Database - National Library of Medicine: http://hpd.nlm.nih.gov/index.htm.
International Programme on Chemical Safety INCHEM database in partnership with Canadian Centre for Occupational Health and Safety: http://www.inchem.org/.
National Toxicology Program: http://ntp.niehs.nih.gov:8080/index.html?col=010stat

[2] Chemical classifications based on the Medical Subject Headings classification for chemicals and drugs, as developed by the National Library of Medicine: http://www.nlm.nih.gov/mesh/meshhome.html.

[3] The traditional LLNA EC3 or LLNA: DA EC1.8 values listed for each substance is averaged from respective studies. The substance was tested in the same vehicle in both the traditional LLNA and the LLNA: DA, except where noted. Numbers in parentheses indicate the maximum SI.

[4] Substance tested in the intralaboratory validation study (Idehara unpublished).

[5] Substance tested in the intralaboratory validation study (Idehara et al. 2008).

[6] Substance tested in phase one of the two-phased interlaboratory validation study (Omori et al. 2008).

[7] Benzalkonium chloride was tested in the LLNA: DA using acetone: olive oil (4:1) as the vehicle but the traditional LLNA EC3 value reported is based on results using acetone as the vehicle.

[8] Substance tested in phase two of a two-phased interlaboratory validation study (Omori et al. 2008).

Annex II of the BRD (**Appendix C**) lists various physicochemical properties for the substances tested in the LLNA: DA. For the 44 substances that were evaluated in the LLNA: DA performance analyses, the molecular weights ranged from 30 to 388 g/mol. Twenty-two of the 44 substances were solids, 21 were liquids, and one substance (benzalkonium chloride) exists as either a solid or a liquid. The estimated log octanol-water partition coefficients (K_{ow}) were available for 38 substances and ranged from -8.28 to 6.46. Peptide reactivity, which was available for 28 substances, ranged from high to minimal (Gerberick et al. 2004, 2007).

3.3 Reference Test Method Data

The traditional LLNA reference data used for the accuracy analyses were from ICCVAM (1999) for 34 of the 44 substances that were evaluated. The traditional LLNA reference data for the remaining 10 substances were obtained from the scientific literature (Gerberick et al. 1992; Hilton et al. 1998; Ryan et al. 2002; Basketter et al. 2005; Gerberick et al. 2005; Betts et al. 2006; Basketter et al. 2007). The reference data for the guinea pig tests (GPMT or Buehler test) and human tests (human maximization test, human patch test allergen, or other human data) were also obtained from the scientific literature. The LLNA, guinea pig, and human reference data and their sources for each of the 44 substances evaluated are provided in Annex III of the BRD (**Appendix C**).

3.4 Test Method Accuracy

The ICCVAM evaluation of the LLNA: DA included an assessment of multiple decision criteria (see **Table 3-2**) including SI ≥ 3.0, the threshold for distinguishing sensitizers and nonsensitizers that is recommended in the LLNA: DA developer's test method protocol. When the optimal decision criterion of SI ≥ 1.8 was used to identify sensitizers vs. nonsensitizers, compared to the traditional LLNA, accuracy was 93% (41/44), with a false positive rate of 25% (3/12), and a false negative rate of 0% (0/32). All three false positive substances were tested once in the LLNA: DA and had resulting maximum SI values between 1.8 and 2.5 (chlorobenzene maximum SI = 2.44; hexane maximum SI = 2.31; salicylic acid maximum SI = 2.00). Other available information, such as dose-response, evidence of systemic toxicity or excessive local irritation, and (where appropriate) statistical significance together with SI values should be considered to confirm that such borderline positive results are potential skin sensitizers. Consideration should also be given to various properties of the test substance, including whether it is structurally similar to known skin sensitizers. For example, peptide reactivity (Gerberick et al. 2007), could be used to interpret LLNA: DA results when borderline positive results (e.g., SI values between 1.8 and 2.5) are produced to confirm that such results are not false positive. Two of the three traditional LLNA nonsensitizers with positive LLNA: DA SI values in this range had minimal peptide reactivity and one did not have peptide reactivity data available. No unique characteristics were identified that could be used as rationale for excluding any particular types of substances from testing in the LLNA: DA.

An evaluation to determine the robustness of the optimum SI ≥ 1.8 criterion indicated that the SI was quite stable. Taking different samples of the data as training and validation sets had relatively little impact on the cutoff SI criterion or on the resulting number of false or false negative results.

Table 3-2 Performance of the LLNA: DA for 44 Substances Compared to the Traditional LLNA in Predicting Skin Sensitization Potential Using Alternative Decision Criteria Based on the Most Prevalent Outcome for Substances with Multiple Tests

Alternate Criterion	N[1]	Accuracy % (No.[2])	Sensitivity % (No.[2])	Specificity % (No.[2])	False Positive Rate % (No.[2])	False Negative Rate % (No.[2])	Positive Predictivity % (No.[2])	Negative Predictivity % (No.[2])
Statistics[3]	44	84 (37/44)	94 (30/32)	58 (7/12)	42 (5/12)	6 (2/32)	86 (30/35)	78 (7/9)
≥95% CI[4]	44	75 (33/44)	100 (32/32)	8 (1/12)	92 (11/12)	0 (0/32)	74 (32/43)	100 (1/1)
≥2 SD[5]	44	77 (34/44)	91 (29/32)	42 (5/12)	58 (7/12)	9 (3/32)	81 (29/36)	63 (5/8)
≥3 SD[6]	44	80 (35/44)	88 (28/32)	58 (7/12)	42 (5/12)	13 (4/32)	85 (28/33)	64 (7/11)
SI ≥ 5.0	44	57 (25/44)	41 (13/32)	100 (12/12)	0 (0/12)	59 (19/32)	100 (13/13)	39 (12/31)
SI ≥ 4.5	44	70 (31/44)	59 (19/32)	100 (12/12)	0 (0/12)	41 (13/32)	100 (19/19)	48 (12/25)
SI ≥ 4.0	44	84 (37/44)	78 (25/32)	100 (12/12)	0 (0/12)	22 (7/32)	100 (25/25)	63 (12/19)
SI ≥ 3.5	44	89 (39/44)	84 (27/32)	100 (12/12)	0 (0/12)	16 (5/32)	100 (27/27)	71 (12/17)
SI ≥ 3.0	*44*	*91 (40/44)*	*88 (28/32)*	*100 (12/12)*	*0 (0/12)*	*13 (4/32)*	*100 (28/28)*	*75 (12/16)*
SI ≥ 2.5	44	91 (40/44)	88 (28/32)	100 (12/12)	0 (0/12)	13 (4/32)	100 (28/28)	75 (12/16)
SI ≥ 2.0	44	91 (40/44)	97 (31/32)	75 (9/12)	25 (3/12)	3 (1/32)	91 (31/34)	90 (9/10)
SI ≥ 1.8	**44**	**93 (41/44)**	**100 (32/32)**	**75 (9/12)**	**25 (3/12)**	**0 (0/32)**	**91 (32/35)**	**100 (9/9)**
SI ≥ 1.5	44	89 (39/44)	100 (32/32)	58 (7/12)	42 (5/12)	0 (0/32)	86 (32/37)	100 (7/7)
SI ≥ 1.3	44	86 (38/44)	100 (32/32)	50 (6/12)	50 (6/12)	0 (0/32)	84 (32/38)	100 (6/6)

Italicized text indicates the decision criterion chosen by the LLNA: DA validation study team; Bolded text indicates the single decision criterion that had an overall increased performance in predicting skin sensitization potential when compared to the traditional LLNA.

Abbreviations: CI = confidence interval; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; No. = number; SD = standard deviation; SI = stimulation index.

[1] N = Number of substances included in this analysis.

[2] The proportion on which the percentage calculation is based.

[3] Analysis of variance for difference of group means when substances were tested at multiple doses or *t*-test when substances were tested at one dose. The ATP data were log-transformed prior to statistical analysis. For analysis of variance, significance at $p < 0.05$ was further tested by Dunnett's test.

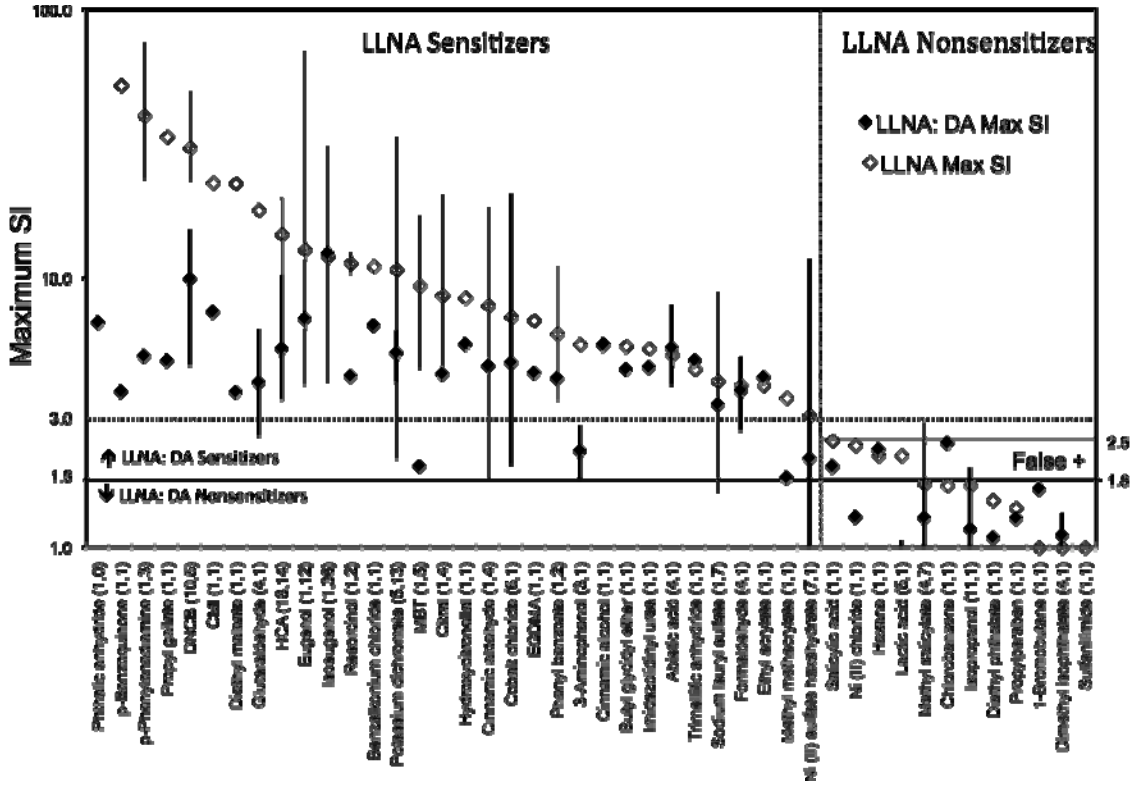
[4] The mean ATP of at least one treatment group was outside the 95% confidence interval for the mean ATP of the vehicle control group.

[5] The mean ATP of at least one treatment group was greater than 2 SD from the mean ATP of the vehicle control group.

[6] The mean ATP of at least one treatment group was greater than 3 SD from the mean ATP of the vehicle control group.

Figure 3-1 shows that SI values for the LLNA: DA are generally lower than those for traditional LLNA tests at similar test doses. SI values for substances with more than one test result are represented by the geometric mean with bars to show the overall range of individual study results used to calculate the geometric mean. The purpose of showing the geometric mean and associated ranges is to provide an assessment of variability among results, and the relative sensitivity of the traditional LLNA and LLNA: DA results. However, the accuracy analyses reported in the BRD are based on individual test results and not on a geometric mean. **Table 3-3** lists the maximum SI values for the substances included in **Figure 3-1**.

Figure 3-1 Comparison of LLNA: DA Stimulation Index with Traditional LLNA Stimulation Index[1]



Abbreviations: CMI = 5-chloro-2-methyl-4-isothiazolin-3-one; DNCB = 2,4-dinitrochlorobenzene; EGDMA = ethylene glycol dimethacrylate; HCA = hexyl cinnamic aldehyde; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; MBT = 2-mercaptobenzothiazole; Ni = nickel; False + = false positive results in the LLNA: DA based on majority call were in the SI range between 1.8 and 2.5; SI = stimulation index.

[1] LLNA: DA and traditional LLNA tests at similar doses are shown. Symbols show the maximum SI for substances with one test result or geometric mean maximum SI for substances with more than one test result. Bars show the range of values reported for multiple test results (heavy bars for LLNA: DA and light bars for traditional LLNA). Numbers in parentheses beside the substance names indicate the number of tests for the LLNA: DA followed by the traditional LLNA, which may differ from the total number of tests available since only tests with similar maximum doses were used in this figure. The accuracy analyses used individual test results rather than geometric mean SI values. Using individual test results, traditional LLNA nonsensitizers with at least one positive LLNA: DA test result in the SI range between 1.8 and 2.5 include salicylic acid, hexane, chlorobenzene, and isopropanol.

Table 3-3 Maximum SI Values of 44 Substances Evaluated in the LLNA: DA Compared to Traditional LLNA Tests with Similar Doses[1]

Substance Name[2]	Test Vehicle[3]	LLNA: DA Maximum SI Values[4]	Traditional LLNA Maximum SI Values
Sensitizers (LLNA: DA SI ≥ 1.8 and Traditional LLNA SI ≥ 3.0)			
Phthalic anhydride (1, 0)	AOO	6.85	NA
p-Benzoquinone (1, 1)	AOO	3.79	52.30
p-Phenylenediamine (1, 3)	AOO	5.14	23.30, 37.40, 75.30
Propyl gallate (1, 1)	AOO	4.95	33.60
DNCB (10, 5)	AOO	4.71, 7.86, 8.53, 9.23, 9.96, 10.89, 11.97, 12.60, 13.18, 15.14	23.00, 24.00, 26.80, 36.70, 49.60
CMI (1, 1)	DMF	7.50	22.70
Diethyl maleate (1, 1)	AOO	3.78	22.60
Glutaraldehyde (4, 1)	ACE	2.57, 3.39, 5.00, 6.45	18.00
HCA (18, 14)	AOO	3.51, 3.88, 3.92, 3.97, 4.44, 4.47, 4.82, 5.11, 5.41, 5.50, 5.71, 5.78, 6.45, 6.47, 7.09, 7.60, 8.42, 10.22	10.00, 11.60, 11.60, 13.40, 14.00, 14.00, 14.10, 14.50, 16.00, 17.00, 17.00, 17.00, 17.60, 20.00
Eugenol (1, 12)	AOO	7.07	4.01, 6.10, 9.30, 9.60, 10.20, 12.40, 14.10, 16.00, 16.10, 16.10, 17.00, 70.30
Isoeugenol (1, 36)	AOO	12.36	4.10, 4.90, 5.00, 5.60, 6.70, 6.80, 7.20, 7.20, 7.50, 7.50, 7.60, 8.70, 10.00, 11.00, 11.10, 11.80, 12.40, 13.80, 13.10, 13.10, 13.10, 14.10, 14.70, 14.70, 15.30, 17.00, 18.40, 19.00, 23.20, 19.20, 19.30, 23.20, 23.60, 24.40, 29.80, 31.00
Resorcinol (1, 2)	AOO	4.33	10.40, 12.50
Benzalkonium chloride (1, 1)	AOO / ACE	6.68	11.10
Potassium dichromate (5, 13)	DMSO	4.08, 4.78, 5.49, 6.01, 6.37	2.12, 5.40, 6.90, 10.10, 10.10, 10.40, 11.20, 13.00, 13.10, 16.10, 16.10, 19.10, 33.60
Citral (1, 4)	AOO	4.40	4.70, 6.20, 9.30, 20.50
Hydroxycitronellal (1, 1)	AOO	5.69	8.50
Cinnamic aldehyde (1, 4)	AOO	4.73	1.80, 7.60, 15.80, 18.40
EGDMA (1, 1)	MEK	4.45	7.00
Phenyl benzoate (1, 2)	AOO	4.24	3.50, 11.10

continued

Table 3-3 Maximum SI Values of 44 Substances Evaluated in the LLNA: DA Compared to Traditional LLNA Tests with Similar Doses[1] (continued)

Substance Name[2]	Test Vehicle[3]	LLNA: DA Maximum SI Values[4]	Traditional LLNA Maximum SI Values
Sensitizers (LLNA: DA SI ≥ 1.8 and Traditional LLNA SI ≥ 3.0)			
Cinnamic alcohol (1, 1)	AOO	5.66	5.70
Butyl glycidyl ether (1, 1)	AOO	4.59	5.60
Imidazolidinyl urea (1, 1)	DMF	4.67	5.50
Abietic acid (4, 1)	AOO	3.98, 4.64, 6.26, 7.96	5.20
Trimellitic anhydride (1, 1)	AOO	4.96	4.60
Sodium lauryl sulfate (1, 7)	DMF	3.39	1.60, 2.60, 4.10, 5.10, 5.10, 5.40, 8.90
Formaldehyde (4, 1)	ACE	2.69, 3.18, 4.84, 5.10	4.00
Ethyl acrylate (1, 1)	AOO	4.29	3.98
MBT (1, 5)	DMF	**2.00**	4.60, 9.10, 9.50, 10.80, 17.10
Cobalt chloride (6, 1)	DMSO	**2.01**, 2.54, 3.64, 4.25, 8.07, 20.55	7.21
3-Aminophenol (3, 1)	AOO	1.76, **2.38, 2.83**	5.70
Methyl methacrylate (1, 1)	AOO	**1.81**	3.60
Ni (II) sulfate hexahydrate (7, 1)	DMSO	0.79, 1.24, 1.52, 1.56, **2.13**, 3.49, 11.78	3.10
Traditional LLNA Nonsensitizers (SI < 3.0) with Borderline Positive SI Values in LLNA: DA (1.8 < SI <2.5; see bold text)			
Salicylic acid (1, 1)	AOO	**2.00**	2.50
Hexane (1, 1)	AOO	**2.31**	2.20
Chlorobenzene (1, 1)	AOO	**2.44**	1.70
Nonsensitizers (LLNA: DA SI < 1.8 and Traditional LLNA SI < 3.0)			
Ni (II) chloride (1, 1)	DMSO	1.30	2.40
Lactic acid (5, 1)	DMSO	0.91, 0.93, 0.97, 0.99, 1.06	2.20
Methyl salicylate (4, 7)	AOO	0.83, 1.20, 1.55, 1.77	0.90, 1.10, 1.72, 1.90, 2.10, 2.30, 2.90
Isopropanol (11, 1)	AOO	0.70, 0.76, 0.91, 1.01, 1.08, 1.21, 1.25, 1.45, 1.54, 1.57, **1.97**	1.70
Diethylphthalate (1, 1)	AOO	1.09	1.50
Propylparaben (1, 1)	AOO	1.28	1.40
1-Bromobutane (1, 1)	AOO	1.65	1.00

continued

Table 3-3 Maximum SI Values of 44 Substances Evaluated in the LLNA: DA Compared to Traditional LLNA Tests with Similar Doses[1] (continued)

Substance Name[2]	Test Vehicle[3]	LLNA: DA Maximum SI Values[4]	Traditional LLNA Maximum SI Values
Nonsensitizers (LLNA: DA SI < 1.8 and Traditional LLNA SI < 3.0)			
Dimethyl isophthalate (4, 1)	AOO	0.89, 1.00, 1.26, 1.34	1.00
Sulfanilimide (1, 1)	DMF	0.86	1.00

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); CMI = 5-Chloro-2-methyl-4-isothiazolin-3-one; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; DNCB = 2,4-dinitrochlorobenzene; EGDMA = ethylene glycol dimethacrylate; HCA = hexyl cinnamic aldehyde; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; MBT = 2-mercaptobenzothiazole; MEK = methyl ethyl ketone; NA = not available; Ni = nickel; SI = stimulation index.

[1] LLNA: DA and traditional LLNA tests at similar doses are shown and correspond to the same data depicted in **Figure 3-1**.

[2] Numbers in parentheses beside the substance names indicate the number of tests for the LLNA: DA followed by the traditional LLNA, which may differ from the total number of tests available since only tests with similar doses were included.

[3] The vehicle used was the same in LLNA: DA and traditional LLNA tests except for one substance, and in this case (for benzalkonium chloride) the first entry is the vehicle used for the LLNA: DA, and the second entry is for the traditional LLNA.

[4] The bold text indicates LLNA: DA tests with maximum SI values between 1.8 and 2.5.

3.5 Test Method Reliability (Intra- and Interlaboratory Reproducibility)

The BRD details the evaluation of intra- and interlaboratory reproducibility of the LLNA: DA test method (see **Section 7.0** of **Appendix C**). Intralaboratory reproducibility was assessed using a coefficient of variation (CV) analysis of EC3 (estimated concentration needed to produce an SI of 3.0) and EC1.8 values (estimated concentration needed to produce an SI of 1.8) for isoeugenol and eugenol (each substance was tested in three different experiments). The mean EC3 values and corresponding CVs for isoeugenol and eugenol were 2.74% ± 0.58% with a 21% CV, and 5.06% ± 0.55%, with an 11% CV, respectively. The mean EC1.8 values and corresponding CVs for isoeugenol and eugenol were 0.87% ± 0.31% (36% CV), and 3.38% ± 0.79% (23% CV), respectively.

Qualitative analyses of LLNA: DA reproducibility were conducted in both phases of an interlaboratory validation study, using SI ≥ 1.8 as the threshold to distinguish sensitizers from nonsensitizers. In the first phase (n = 12 substances [nine sensitizers and three nonsensitizers based on traditional LLNA test results] tested in three or 10 laboratories) there was 100% agreement among the laboratories for 10 substances (seven sensitizers and three nonsensitizers based on traditional LLNA test results). There was 67% (2/3) agreement among the tests for the remaining two traditional LLNA sensitizers. The interlaboratory CV values for the EC1.8 values for eight of the nine traditional LLNA sensitizers ranged from 15% to 140%. The interlaboratory CV value for the EC1.8 values for the traditional LLNA sensitizer nickel (II) sulfate hexahydrate could not be calculated since an EC1.8 value was only available from one of the three laboratories that tested it.

In the second phase (n = 5 substances [four sensitizers and one nonsensitizer based on traditional LLNA test results] tested in four or seven laboratories) there was 100% agreement among the

laboratories for four substances (three sensitizers and one nonsensitizer based on traditional LLNA results). There was 75% (3/4) agreement among the tests for the remaining traditional LLNA sensitizer. Interlaboratory CV values for the EC1.8 values of the four traditional LLNA sensitizers ranged from 14% to 93%.

There were 14 substances with multiple tests across the two phases of the interlaboratory validation study that could be used for analyses of reproducibility when using SI ≥ 1.8 to identify potential sensitizers. The SI results for 80% (8/10) of the sensitizers (based on traditional LLNA results) were 100% concordant in the LLNA: DA (i.e., all tests for that substance yielded maximum SI ≥ 1.8) (**Table 3-4**). The two traditional LLNA sensitizers with LLNA: DA tests that yielded maximum SI values less than 1.8 were 3-aminophenol and nickel (II) sulfate hexahydrate. The SI results for 75% (3/4) of the nonsensitizers (based on traditional LLNA results) were 100% concordant in the LLNA: DA (i.e., all tests for that substance yielded SI < 1.8). The concordance of the other nonsensitizer, isopropanol, was 91% (10/11).

Table 3-4 Concordance of LLNA: DA Tests for Substances with Multiple Tests Based on Maximum SI Category

Substance Name	LLNA: DA Nonsensitizers (Maximum SI < 1.8)[1]	LLNA: DA Sensitizers (SI ≥ 1.8)		Total Tests
		1.8 < Maximum SI < 2.5[1]	Maximum SI ≥ 2.5[1]	
Sensitizers[2]				
Abietic acid	0 (0%)	0 (0%)	4 (100%)	4
3-Aminophenol	1 (33.3%)	1 (33.3%)	1 (33.3%)	3
Cobalt chloride	0 (0%)	1 (12.5%)	7 (87.5%)	8
2,4-Dinitrochlorobenzene	0 (0%)	0 (0%)	11 (100%)	11
Formaldehyde	0 (0%)	0 (0%)	4 (100%)	4
Glutaraldehyde	0 (0%)	0 (0%)	4 (100%)	4
Hexyl cinnamic aldehyde	0 (0%)	0 (0%)	18 (100%)	18
Isoeugenol	0 (0%)	0 (0%)	4 (100%)	4
Nickel (II) sulfate hexahydrate	4 (50%)	2 (25%)	2 (25%)	8
Potassium dichromate	0 (0%)	0 (0%)	5 (100%)	5
Nonsensitizers[2]				
Dimethyl isophthalate	4 (100%)	0 (0%)	0 (0%)	4
Isopropanol	10 (91%)	1 (9%)	0 (0%)	11
Lactic acid	5 (100%)	0 (0%)	0 (0%)	5
Methyl salicylate	4 (100%)	0 (0%)	0 (0%)	4

Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.

[1] Numbers shown reflect number of tests. Percentage in parentheses reflects percentage of the total number of tests for each substance.

[2] Based on traditional LLNA test results.

3.6 Animal Welfare Considerations: Reduction, Refinement, and Replacement

The LLNA: DA will use the same number of animals as the updated ICCVAM-recommended traditional LLNA test method protocol (Appendix A of ICCVAM 2009a). However, since use of the traditional LLNA is restricted in some countries and institutions because of limitations on handling radioactivity, availability and use of the nonradioactive LLNA: DA may lead to further reduction in use of the guinea pig tests, which would provide for reduced animal use and increased refinement by avoiding the discomfort that can occur in the guinea pig tests when substances cause ACD. Additionally, the LLNA: DA test method protocol requires fewer mice per treatment group (a minimum of four animals per group) than either of the guinea pig tests (10-20 animals/group for the Buehler test and 5-10 animals/group for the GPMT).

4.0 ICCVAM Consideration of Independent Peer Review Panel Report and Other Comments

The ICCVAM evaluation process incorporates a high level of scientific peer review and transparency. The evaluation process for the LLNA: DA included two public review meetings by an independent scientific peer review panel, multiple opportunities for public comments (see **Section 1.0**), consideration of reports from an OECD Expert Consultation, and comments from the SACATM. ICCVAM and the IWG considered the Panel report, conclusions of the OECD Expert Consultation, the SACATM comments, and all public comments before finalizing the ICCVAM Test Method Evaluation Report and final BRD for the LLNA: DA. This section summarizes the ICCVAM consideration of these reports and comments. The Panel reports and public comments are provided in **Appendices D** and **F**.

4.1 ICCVAM Consideration of Independent Peer Review Panel Report and OECD Comments

4.1.1 Comments on Revised Draft ICCVAM Recommendations: Test Method Usefulness and Limitations

The Panel agreed that the available data and test method performance supported the use of the LLNA: DA to identify substances as potential sensitizers and nonsensitizers, with certain limitations. The Panel noted that the accuracy analysis they reviewed supported using two decision criteria (i.e., one to identify sensitizers and one to identify nonsensitizers). The Panel emphasized that the decision criteria were empirically derived from the data and produced the best combination of maximum accuracy coupled with the minimum number of results in the range of uncertainty (i.e., the range in which maximum SI results were between the decision criteria for sensitizers and nonsensitizers). Since using two decision criteria allows for a more definitive identification of sensitizers and nonsensitizers, this approach provides animal welfare benefits by reducing further tests that might be required in instances where the hazard classification of a substance is not as clear. In addition, one can use statistical analysis and/or other data and information (e.g., peptide reactivity, quantitative structure-activity relationships, skin penetration information) to provide more information on compounds that fall in the range of uncertainty. However, the Panel questioned how results in the range of uncertainty would be useful for regulatory purposes and emphasized that additional guidance would be needed on how to classify substances with SI values in the range of uncertainty.

The OECD Expert Consultation viewed that despite certain limitations, the LLNA: DA is useful as a modified LLNA test method that has the potential to reduce the number of animals required and refine the way in which animals are used for ACD testing. Like the Panel, OECD member country experts questioned the regulatory utility of the LLNA: DA since specific guidance on how to classify substances with SI values in the range of uncertainty has yet to be developed. Therefore, they recommended instead that a single decision criterion (as was originally proposed by ICCVAM and reviewed by the Panel in 2008) would be more useful to identify substances as potential sensitizers. They agreed with ICCVAM that SI $\geq$ 1.8 provided optimal test method performance by preventing false negative results. They also agreed with ICCVAM that users may want to consider additional information such as dose-response, evidence of systemic toxicity and/or excessive local skin irritation, and (where appropriate) statistical significance together with SI values to confirm borderline positive results (i.e., SI between 1.8 and 2.5) as potential skin sensitizers. Additionally, the OECD Expert Consultation agreed that the use of the LLNA: DA might not be appropriate for testing substances that affect ATP levels (e.g., substances that function as ATP inhibitors) or those that affect the accurate measurement of intracellular ATP (e.g., presence of ATP degrading enzymes, presence of extracellular ATP in the lymph node).

ICCVAM considered the Panel report and the OECD Expert Consultation recommendations, and concluded that the single SI decision criterion of SI ≥ 1.8 to classify sensitizers would avoid false negative results as well as indeterminate results, which are not useful for regulatory purposes. Borderline positive results that may occur between 1.8 and 2.5 could be evaluated using other information to confirm the result.

4.1.2 Comments on Revised Draft ICCVAM Recommendations: Test Method Protocol

The Panel concurred with ICCVAM that the validation studies indicated that the standardized protocol was sufficiently transferable and reproducible. The Panel agreed that laboratories should maintain a historical database of positive control SI values and some measure of variability over time. The evaluation of the variation in positive control responses over time has wide applicability to a broad range of test systems.

The Panel agreed with the ICCVAM-recommended protocol, which indicated that all existing toxicological information (e.g., acute toxicity and dermal irritation) and structural and physicochemical information on the test substance of interest (and/or structurally related test substances) should be considered, where available, in selecting three consecutive doses (see **Appendix D2**). The OECD Expert Consultation also agreed and emphasized that the highest dose should be the concentration that maximizes exposure while avoiding systemic toxicity and/or excessive local skin irritation after topical application in the mouse. In the absence of such information, and consistent with the updated ICCVAM-recommended protocol (ICCVAM 2009a), a prescreen test should be performed in order to define the appropriate dose level to test in the LLNA: DA. The Panel and the OECD Expert Consultation agreed in principle with ICCVAM that use of a reduced LLNA: DA test method protocol instead of the multi-dose LLNA: DA test method protocol has the potential to reduce the number of animals used in a test by omitting the middle and low dose groups. However, some members of the OECD Expert Consultation speculated that the reduced LLNA would have limited regulatory use and therefore the extent of potential animal savings is difficult to estimate.

4.1.3 Comments on Revised Draft ICCVAM Recommendations: Future Studies

The Panel concurred with ICCVAM's revised draft recommendations for future studies, emphasizing that additional decision criteria and guidance should be identified for substances that produce SI values in the range of uncertainty, and that the additional decision criteria be reassessed as additional discriminators and data become available (e.g., high-quality human ACD data). While the range of uncertainty is eliminated when using the single decision criterion of SI ≥ 1.8, the OECD Expert Consultation recommended that borderline positive results (i.e., SI values between 1.8 and 2.5) be further evaluated to determine if they are correctly identified as potential skin sensitizers.

The Panel recommended further consideration of statistical issues, including how to determine and evaluate classification methods (i.e., classification cutoff points). The Panel also recommended that future interlaboratory validation studies should simultaneously evaluate intralaboratory reproducibility, using appropriate statistics, to evaluate variation both within a laboratory and between laboratories.

ICCVAM considered the Panel report and the OECD Expert Consultation recommendations and concluded that efforts should be made to further characterize the sensitization potential of borderline positive substances that produce an SI between 1.8 and 2.5 in the LLNA: DA to confirm that such results are not false positive.

4.1.4 Comments on Revised Draft ICCVAM Recommendations: Performance Standards

The Panel agreed that the ICCVAM-recommended LLNA performance standards state the essential test method requirements, and that the LLNA: DA adheres to them such that it should be considered mechanistically and functionally similar. The only variation with the traditional LLNA is the means by which lymphocyte proliferation during the induction phase is evaluated. Likewise, the OECD Expert Consultation also considered the LLNA: DA to be mechanistically and functionally similar to the LLNA, and therefore agreed that the LLNA performance standards are applicable.

4.2 ICCVAM Consideration of Public and SACATM Comments

The ICCVAM evaluation process incorporates a high level of transparency. This process is designed to provide numerous opportunities for stakeholder involvement, including submitting written public comments and providing oral comments at ICCVAM independent peer review panel meetings and SACATM meetings. **Table 4-1** lists the 12 different opportunities for public comment that were provided during the ICCVAM evaluation of the validation status of new versions and applications of the LLNA. The number of public comments received in response to each of the opportunities is also indicated. A total of 49 comments were submitted. Comments received in response to or related to the FR notices are available on the NICEATM-ICCVAM website.[12] The following sections, delineated by FR notice, briefly discuss the public comments received.

Table 4-1 Opportunities for Public Comments

Opportunities for Public Comments	Date	Number of Public Comments Received
72 FR 27815: The Murine Local Lymph Node Assay: Request for Comments, Nominations of Scientific Experts, and Submission of Data	May 17, 2007	17
72 FR 52130: Draft Performance Standards for the Murine Local Lymph Node Assay: Request for Comments	September 12, 2007	4
73 FR 1360: Announcement of an Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents; Request for Comments	January 8, 2008	7
Independent Scientific Peer Review Panel Meeting Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay	March 4-6, 2008	16
73 FR 25754: Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)	May 7, 2008	1
73 FR 29136: Peer Review Panel Report on the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments	May 20, 2008	0

continued

[12] Available at http://ntp-apps.niehs.nih.gov/iccvampb/searchPubCom.cfm

Table 4-1 Opportunities for Public Comments (continued)

Opportunities for Public Comments	Date	Number of Public Comments Received
SACATM Meeting, Radisson Hotel, RTP, NC	June 18-19, 2008	0
74 FR 8974: Announcement of a Second Meeting of the Independent Scientific Peer Review Panel on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents (BRD); Request for Comments	February 27, 2009	1
Independent Scientific Peer Review Panel Meeting Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Evaluation of the Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay	April 28-29, 2009	2
74 FR 19562: Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)	April 29, 2009	0
74 FR 26242: Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments	June 1, 2009	1
SACATM Meeting, Hilton Arlington Hotel, Arlington, VA	June 25-26, 2009	0

4.2.1 Public Comments in Response to 72 FR 27815 (May 17, 2007): The Murine Local Lymph Node Assay: Request for Comments, Nominations of Scientific Experts, and Submission of Data

NICEATM requested the following:

1. Public comments on the appropriateness and relative priority of evaluation of the validation status of
    a. The LLNA as a stand-alone assay for determining potency (including severity) for the purpose of hazard classification
    b. The reduced LLNA approach (Kimber et al. 2006; ESAC 2007; ICCVAM 2009b)
    c. Nonradioactive LLNA methods
    d. The use of the LLNA for testing mixtures, aqueous solutions, and metals
    e. The current applicability domain
2. Nominations of expert scientists to consider as members of a possible peer review panel
3. Submission of data for the LLNA and/or modified versions of the LLNA

In response to this FR notice, NICEATM received 17 comments. Six comments included additional data and information, while two others offered data and information upon request. Three commenters nominated four potential panelists for consideration. Three commenters suggested reference publications for consideration during the Panel evaluation. The nominees were included in the database of experts from which the Panel was selected. The data and suggested references were included in the ICCVAM draft review documents that were provided to the Panel at the March 2008 meeting.

1. A commenter suggested rearranging the priority sequence of test method evaluation from most to least pressing: a, e, d, b, and c (see list above).

- ICCVAM did not establish a relative priority for these activities because they were all considered to be high-priority activities. Accordingly, all LLNA-related activities described above were discussed at the March 2008 Panel meeting.

One comment pertained to the LLNA: DA.

1. One commenter indicated that several nonradioactive detection methods for the LLNA (e.g., bromodeoxyuridine [BrdU] incorporation, methods measuring the release of various cytokines, methods using fluorescent markers, and quantification by flow cytometry) have been developed and shown to be as sensitive as protocols involving radiolabeling. The commenter indicated that since both ECVAM and JaCVAM were reviewing some of these types of nonradioactive methods that ICCVAM should collaborate with these ongoing efforts rather than initiate a comprehensive independent review.

- In 2007, the CPSC requested that ICCVAM evaluate several modifications of the LLNA, which included the LLNA: DA. After considering comments from the public and the SACATM, ICCVAM assigned the activity a high priority. Scientists from ECVAM and JaCVAM served as liaisons to the IWG during the evaluation of the LLNA: DA and actively participated in the review. Both liaisons nominated scientists to the peer review panel and the JaCVAM liaison provided much of the validation data for the review.

4.2.2 Public Comments in Response to 72 FR 52130 (September 12, 2007): Draft Performance Standards for the Murine Local Lymph Node Assay: Request for Comments

NICEATM requested public comments on the September 2007 draft ICCVAM-recommended LLNA performance standards developed to facilitate evaluation of modified LLNA test method protocols with regard to the traditional LLNA. In response to this FR notice, NICEATM received four comments, two of which suggested clarifications to the text. Another comment recommended that test substances chosen for testing in the various LLNA methods should be pure, with conclusive structures, and should not be mixtures. Most comments specifically addressed the LLNA performance standards, although one comment pertained to the LLNA in general.

1. One commenter supported the development of performance standards that expedite the validation of new protocols similar to previously validated methods but was disappointed that NICEATM-ICCVAM had chosen to develop performance standards for such a narrow scope of applicability (i.e., modifications of the standard LLNA that involve incorporation of nonradioactive methods of detecting lymphocyte proliferation). The commenter suggested that limited resources available to NICEATM-ICCVAM would be better spent on activities that would have greater impact on the reduction, refinement, or replacement of animal use, such as evaluating the use of human cell lines or *in vitro* skin models as a replacement for the LLNA.

- ICCVAM considered the comment and concluded that the proposed modifications to the LLNA test method protocol and expanded applications have the potential to further reduce and refine animal use. ICCVAM is committed to identifying *in vitro* models and non-animal approaches for assessing ACD and is engaged with ECVAM and JaCVAM in the development of validation studies for such methods.

There were no comments that specifically addressed the LLNA: DA.

4.2.3 Public Comments in Response to 73 FR 1360 (January 8, 2008): Announcement of an Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents; Request for Comments

NICEATM requested public comments on the January 2008 draft BRDs, draft ICCVAM test recommendations, draft test method protocols, and revised draft LLNA performance standards for an international independent scientific peer review panel meeting to evaluate modifications and new applications for the LLNA. NICEATM received 23 comments in response to this FR notice; seven written comments were received in advance of the meeting, and 16 oral comments were offered at the Panel meeting.

One written comment was relevant to the LLNA: DA.

1. The commenter indicated that beyond the method to assess lymph node cell proliferation, the test method protocol for the LLNA: DA contained several key deviations from the OECD TG 429 recommended protocol and the essential test method components as described in the January 2008 draft ICCVAM-recommended LLNA performance standards (i.e., major modifications from the traditional LLNA in both the test substance treatment and sampling schedule). The commenter viewed that the LLNA: DA should not be considered for validation as an alternative to the traditional LLNA since the modifications extended beyond the specifications in the January 2008 draft ICCVAM-recommended LLNA performance standards.

- The validation studies for the LLNA: DA test method were completed prior to the development of LLNA performance standards and thus, the ICCVAM-recommended LLNA performance standards were not used to evaluate the LLNA: DA. Further, despite the differences between the LLNA: DA test method protocol and the traditional LLNA test method protocol, ICCVAM concurs with the Panel that the LLNA: DA is mechanistically and functionally similar to the traditional LLNA and therefore the LLNA performance standards would otherwise be applicable.

Two oral comments were relevant to the LLNA: DA.

1. One commenter agreed with ICCVAM that the LLNA: DA (and also the LLNA: BrdU by enzyme-linked immunosorbent assay [ELISA]) should be evaluated separately because of different treatment schedules. The commenter also questioned whether the extra topical dose in the LLNA: DA was necessary, and expressed concern that additional doses may cause skin irritation. For this reason, the commenter suggested that the SI should be evaluated at earlier sample times and without SLS pretreatment.

- Yamashita et al. (2005) examined the effect of various dosing regimens on the SI value produced in the LLNA: DA. The fourth topical application of test substance was required for sensitizers to produce SI $\geq$ 3.0.
- The effect of SLS pretreatment on the SI values of selected substances is presented in the final BRD (**Annex I** of **Appendix C**) and Idehara et al. (2008). Briefly, the data indicated that the calculated EC3 values were lower for substances pretreated with an aqueous solution of 1% SLS than for substances not pretreated with an aqueous solution of 1% SLS. This included some weak sensitizers for which an enhanced response would be important to detect.
- The SLS pretreatment constitutes application of a 1% aqueous solution, which does not induce excessive local skin irritation. SLS is an irritant in mice at 10% in *N,N,*-dimethylformamide (Antonopoulos et al. 2008).

2. Another commenter cited data from Ullmann (2002) that indicates differences in the responsiveness of six different mouse strains (CBA/CaOlaHsd, CBA/Ca [CruBR], CBA/Jlbm [SPF], CBA/JNCrj, BALB/c, and NMRI) to 25% 2-mercaptobenzothiazole. The data showed that CBA/JNCrj mice had markedly lower responses compared to the other strains tested, which may explain the negative result for 2-mercaptobenzothiazole produced by the LLNA: DA test method.

- Validation studies for the LLNA: DA were conducted exclusively with the CBA/JNCrlj strain, which is therefore considered the preferred strain. There were insufficient LLNA: DA data in multiple strains to allow for an evaluation of potential strain differences.

4.2.4 Public Comments in Response to 73 FR 25754 (May 7, 2008): Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)

NICEATM announced the SACATM meeting and requested written and public oral comment on the agenda topics. One public comment was received in response to this FR notice. The commenter made a general comment that the members of SACATM do not represent a cross-section of the American public.

- The SACATM charter indicates that the Committee shall consist of 15 members, including the Chair. Voting members shall be appointed by the Director, NIEHS, and include representatives from an academic institution, a State government agency, an international regulatory body, or any corporation developing or marketing new or revised or alternative test methodologies, including contract laboratories. Knowledgeable representatives from public health, environmental communities, or organizations using new or alternative test methodologies may be included as appropriate. There shall be at least one knowledgeable representative having a history of expertise, development, or evaluation of new or revised or alternative test methods from each of the following categories: (1) personal care, pharmaceutical, industrial chemicals, or agricultural industry; (2) any other industry that is regulated by one of the Federal agencies on ICCVAM; and (3) a national animal protection organization established under section 501(c)(3) of the Internal Revenue Code of 1986. The Director, NIEHS, shall select the Chair from among the appointed members of SACATM.

4.2.5 Public Comments in Response to 73 FR 29136 (May 20, 2008): Peer Review Panel Report on the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments

NICEATM requested submission of written public comments on the Independent Scientific Peer Review Panel Assessment. No public comments were received in response to this FR notice.

4.2.6 Public and SACATM Comments: SACATM Meeting on June 18-19, 2008

The June 18-19, 2008, SACATM meeting included a discussion of the ICCVAM review of the LLNA test method (**Appendix F3**).

There were no public comments specific to the LLNA: DA.

Regarding the LLNA: DA, one SACATM member indicated that it was uncertain whether the test method would perform well for mixtures, metals, or aqueous solutions.

- As outlined in the test method recommendations, ICCVAM considers the applicability domain for the LLNA: DA to be the same as the traditional LLNA unless there are properties associated with a class of materials that may interfere with the accuracy of the LLNA: DA. However, inconsistent results for nickel sulfate in the LLNA: DA suggest that the LLNA: DA may not be suitable for testing nickel compounds. Therefore, ICCVAM recommends the accrual of additional data from LLNA: DA studies on such nickel compounds with comparative human and/or guinea pig data in order to more comprehensively evaluate the suitability of the LLNA: DA for testing nickel compounds.

4.2.7 Public Comments in Response to 74 FR 8974 (February 27, 2009): Announcement of a Second Meeting of the Independent Scientific Peer Review Panel on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents (BRD); Request for Comments

NICEATM requested public comments on the revised draft BRDs, revised draft ICCVAM test recommendations, and revised draft test method protocols for the second international independent scientific peer review panel meeting to evaluate modifications and new applications for the LLNA. NICEATM received three comments in response to this FR notice: one written comment and two oral comments offered at the Panel meeting.

1. There was a general comment expressing concern that the extensive time and resources that ICCVAM has devoted to this evaluation has detracted from focus on promising *in vitro* methods with potential to have a much greater impact on animal use.

- ICCVAM considers that the evaluations conducted to date have significant potential to further reduce and refine animal use, particularly where the use of the LLNA is precluded due to restrictions associated with the use of radioactivity. ICCVAM is also committed to identifying *in vitro* models and non-animal approaches for assessing ACD and is engaged with ECVAM and JaCVAM in the development of validation studies for such methods.

The commenter further made one written comment relevant to the LLNA: DA.

1. The commenter supported the revised draft ICCVAM recommendation that the LLNA: DA can be used for ACD testing with specific defined limitations in the decision criteria. The commenter viewed that substances falling within the intermediate SI (i.e., when maximum SI results were between the SI decision criteria for sensitizers and nonsensitizers) would be subjected to an integrated decision strategy in conjunction with all other available information (e.g., dose-response information, statistical analyses of treated vs. control animals, peptide reactivity, molecular weight, results from related chemicals, other testing data). While the commenter offered general support for this use, they emphasized that it should be made clear that "other testing data" refers to retrospective analyses rather than initiation of additional tests in animals.

- ICCVAM agrees that additional animal tests should be avoided whenever possible. The intermediate SI range was discarded because it was irrelevant for ICCVAM's final recommendation to use a single decision criterion, SI $\geq$ 1.8, to classify potential sensitizers. However, ICCVAM recommends that borderline positive results (i.e., SI values between 1.8 and 2.5) should be evaluated with other available information (e.g., dose-response information, evidence of systemic toxicity and/or excessive local skin irritation, statistical comparison of treated vs. vehicle control groups [where appropriate], peptide reactivity, molecular weight, results from related substances, other testing data) to confirm that such results are positive.

The commenter further noted that the Panel recommended that the LLNA: DA and the two other nonradioactive methods should be evaluated for their ability to assess mixtures, metals, and aqueous solutions concurrently with the assessment of these substances in the traditional LLNA. The commenter viewed that since the only difference between these methods and the traditional LLNA is the method of detection, it is unlikely that there will be any differences in the applicability of these methods and the traditional LLNA with regard to mixtures, metals, and aqueous solutions. Therefore, it would be highly inappropriate to perform these redundant studies.

- As outlined in the test method recommendations, ICCVAM considers the applicability domain for the LLNA: DA to be the same as the traditional LLNA unless there are properties associated with a class of materials that may interfere with the accuracy of the LLNA: DA. However, inconsistent results for nickel sulfate in the LLNA: DA suggest that the LLNA: DA may not be suitable for testing nickel compounds. Therefore, ICCVAM recommends the accrual of additional data from LLNA: DA studies on such nickel compounds with comparative human and/or guinea pig data in order to more comprehensively evaluate the suitability of the LLNA: DA for testing nickel compounds.

One oral comment was relevant to the LLNA: DA.

1. One commenter stated that the nonradiolabeled LLNA methods should not be held to a higher standard than the traditional LLNA.

- ICCVAM evaluated the LLNA: DA test method based on the applicable criteria for validation and acceptance of toxicological test methods in the ICCVAM submission guidelines (ICCVAM 2003). ICCVAM is committed to ensuring that new methods are equivalent to or better than the currently accepted toxicological methods in order to protect public health.

4.2.8 Public Comments in Response to 74 FR 19562 (April 29, 2009): Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)

NICEATM announced the SACATM meeting and requested written and public oral comment on the agenda topics. No public comments were received in response to this FR notice.

4.2.9 Public Comments in Response to 74 FR 26242 (June 1, 2009): Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments

NICEATM requested submission of written public comments on the Independent Scientific Peer Review Panel Assessment. One comment was received in response to this FR notice.

The commenter made one comment relevant to the LLNA: DA.

1. The commenter did not consider the nonradioactive LLNA methods to provide significant advantages to the traditional LLNA.

- The ICCVAM recommendations for the nonradioactive test methods state that the proposed nonradioactive modifications to the LLNA test method protocol have significant potential to further reduce and refine animal use, given that they will likely increase the use of the LLNA instead of guinea pig test methods where radioactivity is prohibited.

The commenter also indicated that for the LLNA: DA an explanation of the use of SLS was needed.

- As indicated in Section 2.0 of the final ICCVAM BRD (**Appendix C**), 1% SLS pretreatment is used in the LLNA: DA because various researchers have shown that an aqueous solution of 1% SLS does not elicit a positive response in the traditional LLNA but when applied prior to test substance administration there is generally an increased response compared to the test substance alone (van Och et al. 2000; De Jong et al. 2002).

4.2.10 Public and SACATM Comments: SACATM Meeting on June 25-26, 2009

The June 25-26, 2009, SACATM meeting included a discussion of the ICCVAM review of the LLNA test method (**Appendix F4**).

There were no public comments specific to the LLNA: DA.

In general, SACATM was supportive of the Panel report. However, there was general concern regarding the potential for over-labeling substances that may occur by using LLNA test results. They emphasized the need for developing non-animal test methods for identifying potential skin sensitizers.

Regarding the LLNA: DA, one SACATM member did not consider ATP content to be an accurate measure of lymphocyte proliferation and therefore considered methods that use BrdU incorporation (i.e., LLNA: BrdU-ELISA and LLNA: BrdU by flow cytometry) to be higher priority for moving forward.

- Measuring ATP content by bioluminescence, as is done in the LLNA: DA by the luciferin-luciferase assay, is known to correlate with living cell number (Crouch et al. 1993) and therefore indicates an increased number of proliferating cells in the draining auricular lymph nodes (Ishizaka et al. 1984; Dexter et al. 2003). As indicated in Section 2.0 of the final ICCVAM BRD (**Appendix C**), the emitted light intensity (measured using a luminometer) is linearly related to the ATP concentration and the luciferin-luciferase assay is a sensitive method for ATP quantitation used in a wide variety of applications (Lundin 2000).

Another SACATM member asked if the SLS pretreatment had ever been validated.

- Annex I of the final ICCVAM BRD (**Appendix C**) and Idehara et al. (2008) provide comparative results in the LLNA: DA for a number of substances tested both with and without SLS pretreatment. Briefly, the data indicate that the calculated EC3 values were lower for substances pretreated with SLS than for substances not pretreated with SLS. This included some weak sensitizers for which an enhanced response would be important to detect.

Another SACATM member indicated that the use of two SI decision criteria in the LLNA: DA (i.e., one for determining sensitizers and one for determining nonsensitizers) could potentially place many compounds in the range of uncertainty (i.e., the range in which maximum SI results were between the SI decision criteria for sensitizers and nonsensitizers), so the decision criteria should be reassessed as more data are obtained.

- The final ICCVAM recommendations state that a single decision criterion of SI $\geq$ 1.8 be used to classify substances as potential sensitizers since there were no false negatives in the current validation database, relative to the traditional LLNA, when this criterion is used. However, using an SI $\geq$ 1.8 as the decision criterion results in a false positive rate of 25% (3/12) compared to the traditional LLNA. Since the three false positive substances in the LLNA: DA produced SI values between 1.8 and 2.5, users may want to consider additional information (e.g., dose-response information, evidence of systemic toxicity and/or excessive local skin irritation, statistical comparison of treated vs. vehicle

control groups [where appropriate], peptide reactivity, molecular weight, results from related substances, other testing data) to confirm that results in this SI range are positive.

Another SACATM member commented that many laboratories had moved away from using the LLNA because it used radioactivity. Therefore, the option of LLNA test method protocols that do not use radioactivity would likely increase use of the LLNA.

5.0 References

Antonopoulos C, Cumberbatch M, Mee JB, Dearman RJ, Wei XQ, Liew FY, et al. 2008. IL-18 is a key proximal mediator of contact hypersensitivity and allergen-induced Langerhans cell migration in murine epidermis. J Leukoc Biol 83:361-367.

Basketter DA, Clapp C, Jefferies D, Safford B, Ryan CA, Gerberick F, et al. 2005. Predictive identification of human skin sensitization thresholds. Contact Dermatitis 53:260-267.

Basketter DA, Scholes EW, Wahlkyist H, Montelius J. 1995. An evaluation of the suitability of benzocaine as a positive control skin sensitizer. Contact Dermatitis 33:28-32.

Basketter DA, Sanders D, Jowsey IR. 2007. The skin sensitization potential of resorcinol: experience with the local lymph node assay. Contact Dermatitis 56:196-200.

Betts CJ, Dearman RJ, Heylings JR, Kimber I, Basketter DA. 2006. Skin sensitization potency of methyl methacrylate in the local lymph node assay: comparisons with guinea-pig data and human experience. Contact Dermatitis 55:140-147.

Buehler EV. 1965. Delayed contact hypersensitivity in the guinea pig. Arch Dermatol 91:171-177.

Crouch SP, Kozlowski R, Slater KJ, Fletcher J. 1993. The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. J Immunol Meth 160:81-88.

Dean JH, Twerdok LE, Tice RR, Sailstad DM, Hattan DG, Stokes WS. 2001. ICCVAM evaluation of the murine local lymph node assay. Conclusions and recommendations of an independent scientific peer review panel. Regul Toxicol Pharmacol 34:258-273.

De Jong WH, Tentij M, Spiekstra SW, Vandebriel RJ, Van Loveren H. 2002. Determination of the sensitising activity of the rubber contact sensitisers TMTD, ZDMC, MBT and DEA in a modified local lymph node assay and the effect of sodium dodecyl sulfate pretreatment on local lymph node responses. Toxicology 176:123-134.

Dexter SJ, Cámara M, Davies M, Shakesheff KM. 2003. Development of a bioluminescent ATP assay to quantify mammalian and bacterial cell number from a mixed population. Biomat 24:27-34.

ESAC. 2007. Statement on the Reduced Local Lymph Node Assay (rLLNA). European Commission Directorate General, Joint Research Centre, Institute for Health and Consumer Protection, European Centre for the Validation of Alternative Methods. Available: http://ecvam.jrc.it/ft_doc/ESAC26_statement_rLLNA_20070525-1.pdf.

Gerberick GF, House RV, Fletcher ER, Ryan CA. 1992. Examination of the local lymph node assay for use in contact sensitization risk assessment. Fundam Appl Toxicol 19:438-445.

Gerberick GF, Ryan CA, Kern PS, Dearman RJ, Kimber I, Patlewicz GY, et al. 2004. A chemical dataset for evaluation of alternative approaches to skin-sensitization testing. Contact Dermatitis 50:274-288.

Gerberick GF, Ryan CA, Kern PS, Schlatter H, Dearman RJ, Kimber I, et al. 2005. Compilation of historical local lymph node data for evaluation of skin sensitization alternative methods. Dermatitis 16:157-202.

Gerberick GF, Vassallo JD, Foertsch LM, Price BB, Chaney JG, Lepoittevin JP. 2007. Quantification of chemical peptide reactivity for screening contact allergens: a classification tree model approach. Toxicol Sci 97:417-27.

Haneke KE, Tice RR, Carson BL, Margolin BH, Stokes WS. 2001. ICCVAM evaluation of the murine local lymph node assay. Data analyses completed by the National Toxicology Program

Interagency Center for the Evaluation of Alternative Toxicological Methods. Regul Toxicol Pharmacol 34:274-286.

Hilton J, Dearman RJ, Harvey P, Evans P, Basketter DA, Kimber I. 1998. Estimation of relative skin sensitizing potency using the local lymph node assay: a comparison of formaldehyde with glutaraldehyde. Am J Contact Dermat 9:29-33.

Hutchings CV, Shum KW, Gawkrodger DJ. 2001. Occupational contact dermatitis has an appreciable impact on quality of life. Contact Dermatitis 45:17-20.

ICCVAM. 1999. The Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemical/Compounds. NIH Publication No. 99-4494. Research Triangle Park, NC:National Institute of Environmental Health Sciences. Available: http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf.

ICCVAM Authorization Act. 2000. Public Law 106-545.

ICCVAM. 2003. ICCVAM Guidelines for the Nomination and Submission of New, Revised, and Alternative Test Methods. NIH Publication No. 03-4508. Research Triangle Park, NC:National Institute of Environmental Health Sciences.

ICCVAM. 2009a. Recommended Performance Standards: Murine Local Lymph Node Assay. NIH Publication No. 09-7357. Research Triangle Park, NC:National Institute of Environmental Health Sciences. Available: http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm.

ICCVAM. 2009b. ICCVAM Test Method Evaluation Report. The Reduced Murine Local Lymph Node Assay: An Alternative Test Method Using Fewer Animals to Assess the Allergic Contact Dermatitis of Potential of Chemicals and Products. NIH Publication No. 09-6439. Research Triangle Park, NC:National Institute of Environmental Health Sciences. Available: http://iccvam.niehs.nih.gov/methods/immunotox/LLNA-LD/TMER.htm.

Idehara K, Yamagishi G, Yamashita K, Ito M. 2008. Characterization and evaluation of a modified local lymph node assay using ATP content as a non-radio isotopic endpoint. J Pharmacol Toxicol Methods 58:1-10.

Ishizaka A, Tono-oka T, Matsumoto S. 1984. Evaluation of the proliferative response of lymphocytes by measurement of intracellular ATP. J Immunol Meth 72:127-132.

ISO. 2002. Biological evaluation of medical devices – 10993 Part 10: Tests for irritation and delayed-type hypersensitivity. Available for purchase at: http://www.iso.org/iso/home.htm.

Kimber I, Dearman RJ, Betts CJ, Gerberick GF, Ryan CA, Kern PS, et al. 2006. The local lymph node assay and skin sensitization: a cut-down screen to reduce animal requirements? Contact Dermatitis 54:181-185.

Lundin A. 2000. Use of firefly luciferase in ATP-related assays of biomass, enzymes, and metabolites. Meth Enzymol 305:346-370.

Magnusson B, Kligman AM. 1970. Allergic Contact Dermatitis in the Guinea Pig. Springfield, IL:Charles C. Thomas.

OECD. 2002. Test Guideline 429. Skin Sensitisation: Local Lymph Node Assay, adopted April 24, 2002. In: OECD Guidelines for Testing of Chemicals. Paris:OECD. Available: http://titania.sourceoecd.org/vl=7033968/cl=16/nw=1/rpsv/cw/vhosts/oecdjournals/1607310x/v1n4/contp1-1.htm.

Omori T, Idehara K, Kojima H, Sozu T, Arima K, Goto H, et al. 2008. Interlaboratory validation of the modified murine local lymph node assay based on adenosine triphosphate measurement. J Pharmacol Toxicol Methods 58:11-26.

Ryan CA, Cruse LW, Skinner RA, Dearman RJ, Kimber I, Gerberick GF. 2002. Examination of a vehicle for use with water soluble materials in the murine local lymph node assay. Food Chem Toxicol 40:1719-1725.

Sailstad DM, Hattan D, Hill RN, Stokes WS. 2001. ICCVAM evaluation of the murine local lymph node assay: the ICCVAM review process. Regul Toxicol Pharmacol 34:249-257.

Skoet R, Zachariae R, Agner T. 2003. Contact dermatitis and quality of life: a structured review of the literature. Br J Dermatol 149:452-456.

Ullmann L. 2002. Comparison of the allergic potency of alpha-hexyl cinnamaldehyde (HCA) and 2-mercaptobenzothiazole (MBT) as positive control substances in the murine local lymph node assay (LLNA). J Toxicol Sci 27:404.

Van Och FM, Slob W, de Jong WH, Vandebriel RJ, van Loveren H. 2000. A quantitative method for assessing the sensitizing potency of low molecular weight chemicals using a local lymph node assay: employment of a regression method that includes determination of the uncertainty margins. Toxicology 146:49-59.

Yamashita K, Idehara K, Fukuda N, Yamagishi G, Kawada N. 2005. Development of a modified local lymph node assay using ATP measurement as an endpoint. Alternatives to Animal Testing and Experimentation 11:136-144.