新規試験法提案書

皮膚感作性試験代替法（LLNA-BrdU-ELISA 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. 2012-04

皮膚感作性試験代替法 Local Lymph Node Assay (LLNA): BrdU-ELISA 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): BrdU-ELISA 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: BrdU-ELISA Evaluation Report (2010)、LLNA: BrdU-ELISAのJaCVAM評価報告（2011）およびOECD Test Guideline (TG) 442B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:BrdU-ELISA 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）
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任期：平成 24 年 4 月 1 日～平成 26 年 3 月 31 日

JaCVAM運営委員会

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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): BrdU-ELISA 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: BrdU-ELISA 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. 442B, it is concluded that the LLNA: BrdU-ELISA 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):
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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）
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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: BrdU-ELISA 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: BrdU-ELISA 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): BrdU-ELISA 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、bromodeoxyuridine (BrdU)の DNA への取込みを指標とし、BrdU 量を酵素免疫測定法（ELISA）により吸光度として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 10 物質の試験成績を含む 43 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$\geq$1.6 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: BrdU-ELISA 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 10 物質を含む 43 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≧2.0 から≧1.6 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、BrdU 測定に市販の ELISA キットを使用することから、操作の変更（例えば、プレートの洗浄および乾燥、二次抗体の反応時間等）は測定値が変動する要因になりうる。しかし、実施に当たっては、ICCVAM の報告書を参照して施設毎に試験プロトコルを確立し、そのプロトコルを忠実に守ることが必要である。

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 を必要としないことより、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（LLNA：BrdU 法）の評価会議報告書、JaCVAM 評価会議（平成 22 年 5 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): BrdU-ELISA の第三者評価報告（平成 24 年 7 月）

3) Hajime Kojima, et al. Inter-laboratory validation of the modified murine local lymph node assay based on 5-bromo-2'-deoxyuridine incorporation. J. Appl. Toxicol. **31**: 63-74 (2011)

4) ICCVAM (2010). ICCVAM Test Method Evaluation Report on the Murine Local Lymph Node Assay: BrdU-ELISA, a Nonradioactive Alternative Test Method to Assess the Allergic Contact Dermatitis Potential of Chemicals and Products. NIH Publication No. 10-7552. Research Triangle Park, NC: National Institute of Environmental Health Sciences.

Local Lymph Node Assay: BrdU-ELISA (LLNA: BrdU-ELISA、局所リンパ節試験: BrdU-ELISA)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: BrdU-ELISA は、放射性物質の ^{3}H-TdR の代わりに、bromodeoxyuridine (BrdU)の取り込み量を酵素免疫測定法により吸光度として測定し、細胞増殖の指標にしたものであり、原法の LLNA と同じ原理による試験法である。本試験法の原理並び簡便性は、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 BrdU の取込量の比(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:BrdU-ELISA の検証報告（2008）と ICCVAM の LLNA：BrdU-ELISA 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 1.6 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：BrdU-ELISA 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 1.6 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 1.6 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 1.6 から 2 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: BrdU-ELISA は、放射性物質の ^{3}H-TdR の代わりに、bromodeoxyuridine (BrdU)の取り込み量を酵素免疫測定法により吸光度として測定し、細胞増殖の指標にしたものであり、原法の LLNA と同じ原理による試験法である。本試験法の原理並び簡便性は、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のカットオフ値［溶媒処置群（陰性対照群）に対する被験物質処置群の BrdU の取込量の比

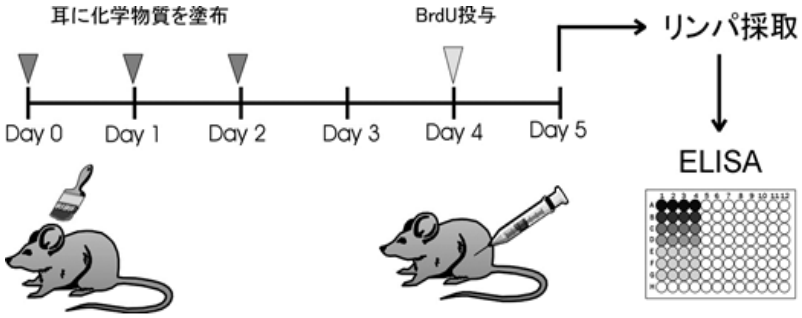
(Stimulation index、SI 値)] に本邦と海外において違いが生じている。

本報告では、JaCVAM で実施された LLNA: BrdU-ELISA の検証報告(2011)[2]と ICCVAM の LLNA: BrdU-ELISA 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: BrdU-ELISA（図1）の試験法の原理は、原法のLLNAと同じである。LLNA: BrdU-ELISAでは、LLNA同様に3日間連続して被験物質を耳介に経皮投与し、5日目に ^{3}H-TdR の静脈内投与の代わりにBrdUを腹腔内投与し、6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: BrdU-ELISAでは、BrdUのDNAへの取込を指標とし、BrdU量をEnzyme Linked Immuno Solvent Assay（ELISA）により検出するものである。

図１　LLNA: BrdU-ELISA の概略



２．JaCVAM の LLNA: BrdU-ELISA 検証試験における SI 値

JaCVAM の行った検証試験では、7 施設が参加し、10 化合物（2,4-dinitrochlorobenzene、eugenol、formaldehyde、glutaraldehyde、hexylcinnamic aldehyde、isopropanol、lactic acid、methyl salicylate、nickel sulfate、および *trans*-cinnamic aldehyde）について盲検下にて試験された。

検証試験では、3 化合物（isopropanol、2,4-dinitrochlorobenzene および hexylcinnamic aldehyde）が全施設で検証され、7 化合物（eugenol、formaldehyde、glutaraldehyde、lactic

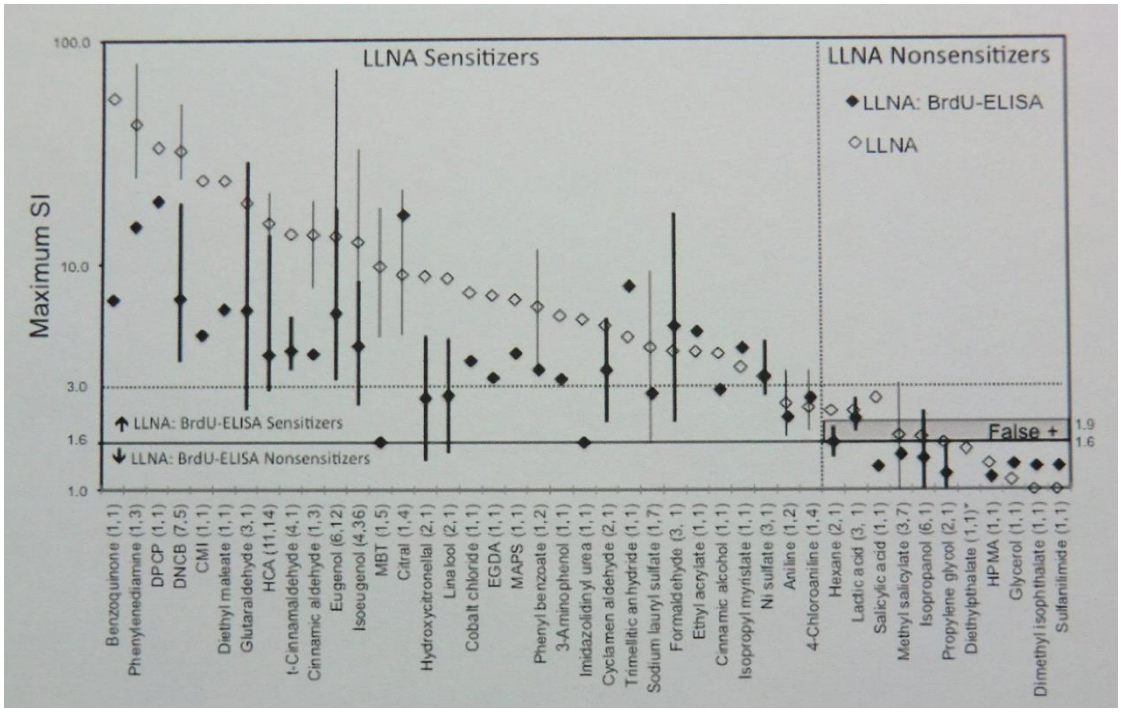
acid、methyl salicylate、nickel sulfate、および *trans*-cinnamic aldehyde）が 3 施設で検証された。試験結果は、各々の化合物処置群について抗 BrdU 抗体を用いた ELISA 法による吸光度として示され、各々の化合物処置群の SI 値が、同時に測定された溶媒対照群に対する BrdU の取込量の比として求められた。その結果、皮膚感作性陽性を示す SI 値は、≥2 と設定された。

この検討で 10 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。SI 値≥2 における LLNA: BrdU-ELISA の GPMT/BT に対する感度、特異性および精度は、各々7/7 (100%)、3/3 (100%)および 10/10 (100%) であった。

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

ICCVAM は、次のように結論している。即ち、LLNA: BrdU-ELISA 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: BrdU-ELISA を支持するものである。JaCVAM の検証試験で得られた 10 化合物のデータを含む 43 化合物の検証データベースの retrospective 解析において、LLNA: BrdU-ELISA は、LLNA で皮膚感作性物質と評価された 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 11 化合物の内 9 化合物（18%[2/11]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 1.6 を使用することを勧告した。ICCVAM のこの勧告は、SI 値≥1.6 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。

図 2　LLNA: BrdU-ELISA 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: BrdU-ELISA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。）

LLNA: BrdU-ELISAの欠点として、1.6から1.9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のあることが挙げられている。ICCVAMは、この点について次のように報告している。即ち、皮膚感作性物質がLLNA:BrdU-ELISAの精度に干渉するある種の特性を持たない限り、LLNA:BrdU-ELISAの適用領域は原法のLLNAと同じ筈である。1つの例外は、nickel化合物である。原法のLLNAと異なり、LLNA:BrdU-ELISAは、それらを潜在的な皮膚感作物質として正確に同定する能力を有し、nickel化合物の皮膚感作性を評価できる。

LLNA:BrdU-ELISA 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:BrdU-ELISA 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 1.6 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 18% (2/11)、偽陰性率 0% (0/32)の 95%（41/43）であった。LLNA:BrdU-ELISA において SI 値≧1.6 を使用すると、1.6 から 1.9 の間の SI 値を示す 2 偽陽性化合物（hexane および lactic acid）が生じた。それ故、1.6 から 1.9 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を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 1.6 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 1.6 を使用すると、原法の LLNA と比較して 18% (2/11)の偽陽性が生じる。LLNA:BrdU-ELISA において 2 偽陽性物質が 1.6 から 1.9 の間の SI 値を示したことから、この範囲での成績については、真に陽性であること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: BrdU-ELISA の検証の比較

研究組織	LLNA: BrdU-ELISA	
	検証に使用された化合物数	陽性判定の SI 値
ICCVAM	43	≧ 1.6
JaCVAM	10	≧ 2

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＜2 の皮膚感作性物質は、僅か 2 化合物（2-mercaptobenzothiazole [MBT]および imidazolidinyl urea）である。また、ICCVAM の基準である SI 値≧1.6 においては、11 非感作性物質の内偽陽性を示す 2 化合物（hexane および lactic acid）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できるカットオフ値 1.6 を判定基準として採用することが妥当であると考える。また、LLNA: BrdU-ELISA 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 1.6 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 1.6 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≧1.6 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) Hajime Kojima, et al. Inter-laboratory validation of the modified murine local lymph node assay based on 5-bromo-2'-deoxyuridine incorporation. J. Appl. Toxicol. **31**: 63-74 (2011)

3) ICCVAM (2010). ICCVAM Test Method Evaluation Report on the Murine Local Lymph Node Assay: BrdU-ELISA, a Nonradioactive Alternative Test Method to Assess the Allergic Contact Dermatitis Potential of Chemicals and Products. NIH Publication No. 10-7552. Research Triangle Park, NC: National Institute of Environmental Health Sciences.

4) OECD Guideline for the Testing of Chemicals: Skin Sensitization: Local Lymph Node Assay: BrdU-ELISA (TG 442B), 22 July (2010)

OECD/OCDE **442B**

Adopted:
22 July 2010

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Skin Sensitization: Local Lymph Node Assay: BrdU-ELISA

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: BrdU-ELISA [Enzyme-Linked Immunosorbent Assay] is a non-radioactive modification to the LLNA test method, which utilises non-radiolabelled 5-bromo-2-deoxyuridine (BrdU) (Chemical Abstracts Service [CAS] No 59-14-3) in an ELISA-based test system to measure lymphocyte proliferation. The LLNA: BrdU-ELISA has been validated and reviewed and recommended by an international independent scientific peer review panel as considered useful for identifying skin sensitizing and non-sensitizing test substances, with certain limitations (10) (11) (12). 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 (13). The LLNA (TG 429) and the two non-radioactive modifications, LLNA: BrdU-ELISA (TG 442 B) and LLNA: DA (TG 442 A), all provide an advantage over the guinea pig tests in TG 406 (13) in terms of reduction and refinement of animal use.

2. Similar to the LLNA, the LLNA: BrdU-ELISA 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) (13).

DEFINITIONS

3. Definitions used are provided in Annex 1.

INITIAL CONSIDERATIONS AND LIMITATIONS

4. The LLNA: BrdU-ELISA 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: BrdU-ELISA should be used in place of the LLNA or guinea pig tests (*i.e.* TG 406) (13), 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: BrdU-ELISA is

appropriate for the test substance (given the incompatibility of limited types of test substances with the LLNA: BrdU-ELISA [see paragraph 5]) and to aid in dose selection.

5. The LLNA: BrdU-ELISA 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 the animal use for this purpose when compared to the guinea pig tests (TG 406) (13). Moreover, the LLNA: BrdU-ELISA offers a substantial refinement of the way in which animals are used for allergic contact sensitization testing, since unlike the TG 406, the LLNA: BrdU-ELISA does not require that challenge-induced dermal hypersensitivity reactions be elicited. Furthermore, the LLNA: BrdU-ELISA does not require the use of an adjuvant, as is the case for the guinea pig maximisation test (13). Thus, the LLNA: BrdU-ELISA reduces animal distress. Despite the advantages of the LLNA: BrdU-ELISA over TG 406 (13), 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 (15) may necessitate the use of guinea pig tests (*i.e.* TG 406 (13)). Limitations that have been identified for the LLNA (1) have been recommended to apply also to the LLNA: BrdU-ELISA (10). Other than such identified limitations, the LLNA: BrdU-ELISA 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: BrdU-ELISA. In addition, consideration should be given to the possibility of borderline positive results when Stimulation Index (SI) values between 1.6 and 1.9 are obtained (see paragraphs 31-32). This is based on the validation database of 43 substances using an SI $\geq$ 1.6 (see paragraph 6) for which the LLNA: BrdU-ELISA correctly identified all 32 LLNA sensitizers, but incorrectly identified two of 11 LLNA non-sensitizers with SI values between 1.6 and 1.9 (*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: BrdU-ELISA 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 group (VC). 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 $\geq$1.6 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 BrdU content to indicate an increased number of proliferating cells in the draining auricular lymph nodes. BrdU is an analogue of thymidine and is similarly incorporated into the DNA of proliferating cells. The incorporation of BrdU is measured by ELISA, which utilises an antibody specific for BrdU that is also labelled with peroxidase. When the substrate is added, the peroxidase reacts with the substrate to produce a coloured product that is quantified at a specific absorbance using a microtiter plate reader.

DESCRIPTION OF THE ASSAY

Selection of animal species

7. The mouse is the species of choice for this test. Validation studies for the LLNA: BrdU-ELISA were conducted exclusively with the CBA/JN strain, which is therefore considered the preferred strain (10) (12). 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: BrdU-ELISA response do not exist.

Housing and feeding conditions

8. Mice should be group-housed (16), 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 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: BrdU-ELISA. 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: BrdU-ELISA response at an exposure level expected to give an increase in the SI≥ 1.6 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 > 14 would be considered excessive). Preferred PC test substances are 25% hexyl cinnamic aldehyde (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: BrdU-ELISA regularly (*i.e.* conduct the LLNA: BrdU-ELISA 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: BrdU-

ELISA 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: BrdU-ELISA (*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 (17).

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 (18). 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 test 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: BrdU-ELISA;
- 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 group (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 the references 2 and 19. 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 (19)(20). 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 (17). 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: BrdU-ELISA. The purpose of the pre-screen test is to provide guidance for selecting the maximum dose level to use in the main LLNA: BrdU-ELISA 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 a concentration of 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: BrdU-ELISA 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 6). Both ears of each mouse are observed for erythema and scored using Table 1 (20). 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), and Day 6. Additionally, on Day 6, 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 (21) (22). The highest dose selected for the main LLNA: BrdU-ELISA 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 (21) (22), 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 (22) (23) (24) (25) (26) (27) (28). However, while statistically significant increases can occur when ear thickness is less than 25% they have not been associated specifically with excessive irritation (25) (26) (27) (28) (29).

24. The following clinical observations may indicate systemic toxicity (30) when used as part of an integrated assessment and therefore may indicate the maximum dose level to use in the main LLNA: BrdU-ELISA: 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 6 and mortality should be considered in the evaluation. Moribund animals or animals showing signs of severe pain and distress should be humanely killed (31).

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 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 and 3:*

  Repeat the application procedure carried out on Day 1.

- *Days 4:*

  No treatment.

- *Days 5:*

  Inject 0.5 mL (5 mg/mouse) of BrdU (10 mg/mL) solution inter-peritoneally.

- *Day 6:*

  Record the weight of each animal and any clinical observation. Approximately 24 hours (24 h) after BrdU injection, 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 (17). 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 into 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 gentle mechanical disaggregation through 200 micron-mesh stainless steel gauze or another acceptable technique for generating a single-cell suspension (*e.g.* use of a disposable plastic pestle to crush the lymph nodes followed by passage through a #70 nylon mesh). The procedure for preparing the LNC suspension is critical in this assay and therefore every operator should establish the skill in advance. Further, the lymph nodes in NC animals are small, so careful operation is important to avoid any artificial effects on SI values. In each case, the target volume of the LNC suspension should be adjusted to a determined optimised volume (approximately 15 mL). The optimised volume is based on achieving a mean absorbance of the NC group within 0.1- 0.2.

Determination of cellular proliferation (measurement of BrdU content in DNA of lymphocytes)

27. BrdU is measured by ELISA using a commercial kit (*e.g.* Roche Applied Science, Mannheim, Germany, Catalogue Number 11 647 229 001). Briefly, 100 μL of the LNC suspension is added to the wells of a flat-bottom microplate in triplicate. After fixation and denaturation of the LNC, anti-BrdU antibody is added to each well and allowed to react. Subsequently the anti-BrdU antibody is removed by washing and the substrate solution is then added and allowed to produce chromogen. Absorbance at 370 nm with a reference wavelength of 492 nm is then measured. In all cases, assay test conditions should be optimised (see paragraph 26).

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 (31).

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 BrdU labelling index/mouse within each test substance group and the PC group by the mean BrdU labelling index for the solvent/VC group. The average SI for the VCs is then one.

The BrdU labelling index is defined as:

$$\text{BrdU labelling index} = (ABS_{em} - \text{ABS blank}_{em}) - (ABS_{ref} - \text{ABS blank}_{ref})$$

Where; em = emission wavelength; and ref = reference wavelength.

31. The decision process regards a result as positive when SI $\geq$ 1.6 (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 1.6 and 1.9) is declared positive (3) (6) (32).

32. For a borderline positive response between an SI of 1.6 and 1.9, 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 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 BrdU labelling index values, the group mean BrdU labelling index/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 substance:

- 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:

- source, lot number, and manufacturer's quality assurance/quality control data (antibody sensitivity and specificity and the limit of detection) for the ELISA 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 humane 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;
- a table of individual mouse BrdU labelling indices and SI values for each treatment group;
- mean and associated error term (*e.g.* SD, SEM) for BrdU labelling index/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.

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ANNEX 1

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 (33).

Benchmark test 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 test substance incorrectly identified as negative or non-active by a test method, when in fact it is positive or active (33).

False positive: A test substance incorrectly identified as positive or active by a test, when in fact it is negative or non-active (33).

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 substance, 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 (33).

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 (33).

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 (33).

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: BrdU-ELISA
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
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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	Dinitrochlorobenzene
DPCP	Diphenylcyclopropanone
dpm	Disintegrations per minute
EC1.6	Estimated concentration needed to produce a stimulation index of 1.6
EC3	Estimated concentration needed to produce a stimulation index of 3
ECVAM	European Centre for the Validation of Alternative Methods
EGDA	Ethylene glycol dimethacrylate
ELISA	Enzyme-linked immunosorbent assay
EPA	U.S. Environmental Protection Agency
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
LLNA	Murine local lymph node assay
LLNA: BrdU-ELISA	Murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine
LNC	Lymph node cells
MAPS	4-methyl aminophenol sulfate
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
SACATM	Scientific Advisory Committee on Alternative Toxicological Methods
SD	Standard deviation
SEM	Standard error of the mean
SI	Stimulation index
TG	Test Guideline
U.K.	United Kingdom
U.S.	United States
U.S.C.	United States Code

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[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.*

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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 that measures bromodeoxyuridine (BrdU) incorporation into proliferating lymphocytes by an enzyme-linked immunosorbent assay (ELISA) (referred to hereafter as the "LLNA: BrdU-ELISA"), instead of using a radioactive marker to measure lymphocyte proliferation. The BrdU-ELISA was developed by Dr. Masahiro Takayoshi at the Chemicals Evaluation and Research Institute in Saitama, Japan and 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 the Validation of Alternative Methods (ECVAM) and JaCVAM served as liaisons to the ICCVAM Immunotoxicity Working Group (IWG). A detailed timeline of the LLNA: BrdU-ELISA evaluation is included with this report.

This Test Method Evaluation Report provides ICCVAM's recommendations regarding the LLNA: BrdU-ELISA for assessing the ACD potential of chemicals and products. Since the LLNA: BrdU-ELISA does not require 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 discourage or severely limit the use of radioactive materials. The report also summarizes the validation status of the LLNA: BrdU-ELISA and provides the ICCVAM-recommended LLNA: BrdU-ELISA 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: BrdU-ELISA that was circulated in July 2009 to the 30 OECD member countries

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

[2] The "traditional LLNA" refers to the validated 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).

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

ICCVAM solicited and considered public comments and stakeholder involvement throughout the LLNA: BrdU-ELISA 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: BrdU-ELISA. The recommendations and the background review document (BRD), 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, 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 (Consumer Product Safety Commission) 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: BrdU-ELISA 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 for ACD testing while continuing to support the protection of human health.

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[3] Available at http://iccvam.niehs.nih.gov/methods/immunotox/llna-ELISA/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: BrdU-ELISA. 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: BrdU-ELISA uses bromodeoxyuridine (BrdU) uptake to measure proliferating lymphocytes. The BrdU in this version is quantified with an enzyme-linked immunosorbent assay (ELISA) kit, 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: BrdU-ELISA as an alternative to the traditional LLNA. The report includes the ICCVAM-recommended LLNA: BrdU-ELISA test method protocol, the final LLNA: BrdU-ELISA 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: BrdU-ELISA 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 to the public for comment. The Panel met twice in public session to review the initial and revised draft BRD and draft ICCVAM recommendations. The initial draft BRD evaluated data for 24 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: BrdU-ELISA 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 31 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: BrdU-ELISA 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: BrdU-ELISA. 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: BrdU-ELISA, proposed responses to comments from member countries, and evaluated LLNA: BrdU-ELISA results for 12 additional substances tested and submitted to NICEATM after the April 2009 Panel evaluation. 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

[4] *The traditional LLNA* refers to the validated 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).

the Test Guidelines Programme, which approved the LLNA: BrdU ELISA as TG442B 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.

ICCVAM Recommendations: Test Method Usefulness and Limitations

ICCVAM concludes that the accuracy and reliability of the LLNA: BrdU-ELISA support use of the test method to identify substances as potential skin sensitizers or nonsensitizers. For the validation database of 43 substances, the LLNA: BrdU-ELISA correctly identified all 32 LLNA sensitizers (0% [0/32] false negatives), and nine of the 11 LLNA nonsensitizers (18% [2/11] false positives). ICCVAM recommends that a stimulation index (SI) $\geq$ 1.6 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 SI $\geq$ 1.6 is used.

A limitation of the LLNA: BrdU-ELISA is the potential for false positive results when borderline positive responses between an SI of 1.6 and 1.9 are obtained (see **Section 3.4**). ICCVAM considers the applicability domain for the LLNA: BrdU-ELISA 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: BrdU-ELISA. One exception would be nickel compounds. Unlike the traditional LLNA, the LLNA: BrdU-ELISA can be used for testing nickel compounds based on its ability to correctly identify them as potential sensitizers.

ICCVAM Recommendations: Test Method Protocol

ICCVAM recommends a LLNA: BrdU-ELISA test method protocol that is based on the protocol developed by Takeyoshi et al. (2001) and refined during an interlaboratory validation study (Kojima et al. 2008). The ICCVAM-recommended LLNA: BrdU-ELISA protocol incorporates all aspects of the ICCVAM-recommended traditional LLNA test method protocol, except for those procedures unique to the conduct of the LLNA: BrdU-ELISA. In testing situations where dose-response information is not required, or negative results are anticipated, ICCVAM recommends that the reduced LLNA: BrdU-ELISA should be considered and used where determined appropriate. The reduced LLNA tests only the high dose, thus further reducing animal use by up to 40%.

ICCVAM Recommendations: Future Studies

ICCVAM recommends the following future studies to further characterize the usefulness and limitations of the LLNA: BrdU-ELISA 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 post-marketing 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: BrdU-ELISA.
- Efforts should be made to further characterize the sensitization potential of borderline positive substances (those that produce an SI between 1.6 and 1.9) in the LLNA: BrdU-ELISA 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.

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: BrdU-ELISA. The ICCVAM-recommended performance standards for the traditional LLNA apply to the LLNA: BrdU-ELISA because the test method is functionally and mechanistically similar to the traditional LLNA.

Validation Status of the LLNA: BrdU-ELISA

The mechanistic basis of the LLNA: BrdU-ELISA 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 more than three-fold or higher than the vehicle control is considered a positive response indicative of a skin sensitizing substance. The only difference between the test method protocols for the traditional LLNA and the LLNA: BrdU-ELISA is the procedure for measuring lymphocyte proliferation. The traditional LLNA assesses lymphocyte proliferation by measuring the incorporation of radioactivity into the DNA of dividing cells in the draining auricular lymph nodes. The LLNA: BrdU-ELISA assesses cell proliferation by measuring the incorporation of a nonradioactive thymidine analog, BrdU, into the DNA of dividing cells using an ELISA.

The accuracy of the LLNA: BrdU-ELISA was compared to that of the traditional LLNA using the current validation database of 43 test substances. Optimal LLNA: BrdU-ELISA performance was achieved using SI $\geq$ 1.6 to classify sensitizers versus nonsensitizers. Compared to the traditional LLNA, accuracy was 95% (41/43), with a false positive rate of 18% (2/11) and a false negative rate of 0% (0/32). The two false positive substances produced SI values between 1.6 and 1.9 in the LLNA: BrdU-ELISA. 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 SI $\geq$ 1.6 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 criteria or on the resulting number of false positives or false negatives.

ICCVAM concludes that the reproducibility of the LLNA: BrdU-ELISA 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. One study was conducted to assess interlaboratory reproducibility.

In a qualitative analysis of intralaboratory reproducibility, two to six LLNA: BrdU-ELISA tests yielded 100% concordance for sensitizer/nonsensitizer outcomes for 10/12 substances (10 sensitizers and two nonsensitizers). One of the nonsensitizers with 100% concordance, however, produced false positive results in 2/2 tests. The two discordant substances were traditional LLNA sensitizers that yielded one test with SI < 1.6 and another test with SI > 1.6. Quantitative analyses of EC1.6 values (estimated concentration needed to produce an SI of 1.6) were performed for four substances tested two to five times. The analyses produced coefficient of variation (CV) values from 37% to 118%.

The qualitative interlaboratory reproducibility analysis of 10 substances (seven sensitizers and three nonsensitizers) tested in three to seven laboratories indicated 100% interlaboratory agreement (3/3, 6/6, or 7/7) for nine substances (seven sensitizers and two nonsensitizers). One of the nonsensitizers with 100% concordance, however, produced false positive results in 3/3 laboratories. There was 67% (4/6) agreement among the tests for the remaining nonsensitizer. Interlaboratory CV values for the EC1.6 values of the seven sensitizers ranged from 31% to 93%.

Reproducibility of results for the 18 substances (13 LLNA sensitizers and 5 LLNA nonsensitizers) that had two to 12 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 ≥ 1.6 decision criterion was used to classify sensitizers and nonsensitizers, the results for 78% (14/18) of the substances were 100% concordant. The results for 85% (11/13) of the LLNA sensitizers were 100% concordant (i.e., all yielded SI ≥ 1.6) for two to 12 tests. The results for 60% (3/5) of the nonsensitizers were 100% concordant for two to three tests. All (3/3) tests for two nonsensitizers had SI < 1.6. All (2/2) tests for the third nonsensitizer yielded SI values between 1.6 and 1.9, the narrow region in which false positive results occurred.

The Panel agreed with ICCVAM that the reproducibility of the LLNA: BrdU-ELISA supported the use of the method to identify substances as potential skin sensitizers and nonsensitizers.

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: BrdU-ELISA included two public review meetings by an independent scientific peer review panel, multiple opportunities for public comments, consideration of the OECD Expert Consultation on the LLNA, 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: BrdU-ELISA.

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 (GP) 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 GP tests for most testing situations.

The LLNA with detection of bromodeoxyuridine (BrdU) incorporation by an enzyme-linked immunosorbent assay (ELISA) (referred to hereafter as the "LLNA: BrdU-ELISA") 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 using the incorporation of BrdU into newly synthesized DNA rather than by quantifying the incorporation of ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine. The increase in BrdU in lymph nodes from test animals compared to vehicle controls is then quantified using an ELISA kit. The LLNA: BrdU-ELISA can reduce the use of animals for skin sensitization testing when it is used in place of GP 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 evaluation 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: BrdU-ELISA should have a high priority for evaluation. A detailed timeline of the LLNA: BrdU-ELISA evaluation is provided in **Appendix A**. The ICCVAM-recommended LLNA: BrdU-ELISA test method protocol and the final LLNA: BrdU-ELISA 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: BrdU-ELISA 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 for the IWG.

To facilitate peer review of the LLNA: BrdU-ELISA 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: BrdU-ELISA data and three individuals or organizations nominated members to the Panel (see **Section 4.0**).

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[1] The "traditional LLNA" refers to the validated 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 24 substances (16 sensitizers and eight nonsensitizers, as classified by the traditional LLNA) that were tested in a single laboratory, with results reported among six published studies and one platform presentation. 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: BrdU-ELISA (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: BrdU-ELISA and the completeness of the ICCVAM draft BRD (see **Appendix D1**). The Panel evaluated (1) the extent to which the draft BRD addressed established validation and acceptance criteria and (2) the extent to which the 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: BrdU-ELISA 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: BrdU-ELISA 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 on a larger set of balanced reference substances with respect to physicochemical properties and sensitization potency; and 3) an evaluation of interlaboratory reproducibility. On May 20, 2008, ICCVAM posted a report of the Panel's recommendations[6] (see **Appendix D2**) 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 31 substances (22 sensitizers and nine 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: BrdU-ELISA. 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: BrdU-ELISA (see **Appendix D3**). 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

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[4] Available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_25553.pdf
[5] 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
[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

Appendix D4) 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 Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) for the LLNA: BrdU-ELISA 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, the Environmental Protection Agency, the Food and Drug Administration, and 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: BrdU-ELISA, proposed responses to comments from member countries, and evaluated additional LLNA: BrdU-ELISA results for substances tested and submitted to NICEATM after the Panel evaluation. The expert group convened a subsequent teleconference on December 1, 2009, to discuss outstanding issues identified at the October meeting. A revised TG was again distributed to the 30 OECD member countries in December 2009 for review and comment by national experts and interested stakeholders. A final teleconference of the 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: BrdU-ELISA. The recommendations (**Section 2**) and the final BRD **(Appendix C)** 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 ICCVAM test method recommendations. ICCVAM recommendations are available to the public on the NICEATM-ICCVAM website, and agency responses also will be made available on the website as they are received.

[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: BrdU-ELISA Test Method

ICCVAM evaluated the validation status of the LLNA: BrdU-ELISA as a nonradioactive modification of the traditional LLNA (ICCVAM 1999; Sailstad et al. 2001; Dean et al. 2001 Haneke et al. 2001) to identify substances that may cause ACD for regulatory hazard classification and labeling purposes. While the traditional LLNA assesses cellular proliferation by measuring the incorporation of ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine into the DNA of dividing lymph node cells, the LLNA: BrdU-ELISA assesses cellular proliferation by measuring the incorporation of the thymidine analog BrdU using ELISA detection (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: BrdU-ELISA supports the use of the test method to identify substances as potential skin sensitizers and nonsensitizers. For the validation database of 43 substances,[11] the LLNA: BrdU-ELISA correctly identified all 32 LLNA sensitizers (0% [0/32] false negatives), and nine of the 11 LLNA nonsensitizers (18% [2/11] false positives). ICCVAM recommends that a stimulation index (SI) $\geq$ 1.6 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 $\geq$ 1.6 is used.

A limitation of the LLNA: BrdU-ELISA is the potential for false positive results when borderline positive responses between an SI of 1.6 and 1.9 are obtained (see **Section 3.4**). ICCVAM considers the applicability domain for the LLNA: BrdU-ELISA 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: BrdU-ELISA. One exception would be nickel compounds where, unlike the traditional LLNA, the LLNA: BrdU-ELISA can be used for testing nickel compounds based on its ability to correctly identify them as potential sensitizers.

2.2 ICCVAM Recommendations: Test Method Protocol

ICCVAM recommends a LLNA: BrdU-ELISA test method protocol (**Appendix B**) that was based on the protocol developed by Takeyoshi et al. (2001) and refined during an interlaboratory validation study (Kojima et al. 2008). The ICCVAM-recommended LLNA: BrdU-ELISA 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: BrdU-ELISA. Key aspects included in the ICCVAM-recommended protocol include the following:

- 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.

[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 18 substances tested with the LLNA: BrdU-ELISA.

- Inclusion of a concurrent vehicle control and concurrent positive control in each study is recommended.

Additionally, ICCVAM recommends 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: BrdU-ELISA should be considered and used where determined appropriate. The reduced LLNA: BrdU-ELISA 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: BrdU-ELISA 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 post-marketing 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: BrdU-ELISA.
- Efforts should be made to further characterize the sensitization potential of borderline positive substances (those that produce an SI between 1.6 and 1.9) in the LLNA: BrdU-ELISA 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: BrdU-ELISA. The ICCVAM-recommended performance standards for the traditional LLNA apply to the LLNA: BrdU-ELISA 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: BrdU-ELISA are not proposed at this time.

3.0 Validation Status of the LLNA: BrdU-ELISA Test Method

The ICCVAM BRD for the LLNA: BrdU-ELISA test method (**Appendix C**) provides a comprehensive review of the current validation status of the LLNA: BrdU-ELISA test method, including its accuracy and reliability, the substances tested, the rationale for the standardized 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: BrdU-ELISA test method.

3.1 Test Method Description

Originally developed by Takeyoshi et al. (2001) and refined during an interlaboratory validation study (Kojima et al. 2008), the purpose of the LLNA: BrdU-ELISA 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: BrdU-ELISA 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 three consecutive days to the ears of mice at a concentration that provides maximum solubility of the test substance without systemic toxicity and/or excessive local irritation. Two days after the final application of the test substance, 10 mg/mL BrdU, a thymidine analog, in 0.5 mL physiological saline is administered via intraperitoneal injection to each mouse. Approximately 24 hours later, the draining auricular lymph nodes are excised, and a single-cell suspension from the lymph nodes of each animal is prepared for quantifying the incorporation of BrdU, which correlates with lymph node cell proliferation.

The incorporation of BrdU for each mouse is measured using an ELISA and is expressed in absorbance units. The SI is calculated as the ratio of the mean absorbance/mouse for each treatment group against the mean absorbance/mouse for the vehicle control group. Substances producing an SI greater than a specified threshold are considered to be sensitizers. Based on the accuracy evaluation described in **Section 3.4**, the optimum accuracy was produced by SI $\geq$ 1.6.

3.1.2 Similarities and Differences Between the Protocols for the Traditional LLNA and the LLNA: BrdU-ELISA

The differences between the traditional LLNA (Dean et al. 2001; Sailstad et al. 2001; ICCVAM 1999) and the LLNA: BrdU-ELISA include the marker used to detect lymphocyte proliferation, the route of administration of the marker, and time of lymph node excision. In the traditional LLNA, 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. As noted above, in the LLNA: BrdU-ELISA, a BrdU solution is injected intraperitoneally to each mouse, and the draining auricular lymph nodes are excised 24 hrs later. All other procedures for the two methods are identical.

3.2 Validation Database

The current validation database for the LLNA: BrdU-ELISA includes results from studies of 43 substances that had previously been tested in the traditional LLNA. These results were obtained from six published studies (Takeyoshi et al. 2003; 2004a; 2004b; 2005; 2006; 2007a), several unpublished studies (Takeyoshi M, unpublished data), one platform presentation (Takeyoshi 2007b), and one poster presentation (Kojima et al. 2008). The data from Takeyoshi et al. were generated in a

single laboratory while the data from Kojima et al. were generated in multiple laboratories during an interlaboratory validation study. Data for 31 substances were available and reviewed by the independent peer review panel in April 2009. Data for 12 additional substances and additional results for four previously tested substances were submitted after the Panel review. ICCVAM and the OECD Expert Consultation considered these additional data and the LLNA: BrdU-ELISA BRD was updated to include the additional data.

The reference test data for the 43 substances were obtained from traditional LLNA tests. Of the 43 substances, 32 were classified by the traditional LLNA as skin sensitizers and 11 were classified as nonsensitizers. GP skin sensitization data were available for 35 substances and human skin sensitization test data or clinical case report information was available for 41 substances (see **Appendix C, Annex III-1**).

Table 3-1 lists the 43 substances, uses, chemical classifications, traditional LLNA EC3 and maximum stimulation index (SI) values, and LLNA: BrdU-ELISA EC1.6 and maximum SI values. Nineteen chemical classes were represented by the substances tested in the LLNA: BrdU-ELISA; 11 substances were classified in more than one chemical class. The classes with the highest number of substances were carboxylic acids (13 substances) and aldehydes (six substances). 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. Fifteen of these classes were also represented in the LLNA: BrdU-ELISA database (only amides, ethers, ketones, macromolecular substances, and polycyclic compounds 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: BrdU-ELISA database. Nevertheless, the Panel considered the database of substances tested in the LLNA: BrdU-ELISA 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 SI = 3) for the 33 sensitizers ranged from 0.009% to 47.5%.

Physicochemical characteristics for the 43 substances are provided in **Appendix C, Annex II**. Molecular weights ranged from 30.03 to 388.29 g/mole. Twenty-five substances are liquids and 18 substances are solids. Log octanol: water partition coefficients, which were available for 41 substances, ranged from -3 to 3.88. Peptide reactivity, which was available for 22 substances, ranged from high to minimal (Gerberick et al. 2007).

Table 3-1 Product Use and Chemical Classification, Traditional LLNA EC3 Values, LLNA: BrdU-ELISA EC1.6 Values, and Maximum SI Values for 43 Tested Substances

Substance Name	Product Use[1]	Chemical Class[2]	Traditional LLNA EC3 (Maximum SI)[3]	LLNA: BrdU-ELISA EC1.6 (Maximum SI)[3]
5-Chloro-2-methyl-4-isothaizolin-3-one*	Cosmetics; Manufacturing; Pesticides	Sulfur Compounds; Heterocyclic Compounds	0.009 (27.7)	0.065 (4.8)
p-Benzoquinone	Manufacturing; Pesticides; Pharmaceuticals	Quinones	0.010 (52.3)	0.150 (6.9)
2,4-Dinitrochlorobenzene*	Manufacturing; Pesticides	Hydrocarbon, Halogenated; Nitro Compounds; Hydrocarbons, Cyclic	0.049 (43.9)	0.032 (18.8)
Diphenylcyclopropenone	Pharmaceuticals	Hydrocarbons, Cyclic	0.050 (NA)	0.450 (19.1)
Glutaraldehyde	Cosmetics; Disinfectant; Manufacturing; Pesticides	Aldehydes	0.083 (18.0)	0.115 (28.6)
4-Phenylenediamine*	Intermediate in chemical synthesis; Manufacturing	Amines	0.11 (26.4)	0.285 (14.7)
Formaldehyde	Disinfectant; Manufacturing	Aldehydes	0.50 (4.0)	0.163 (16.6)
Cobalt chloride*	Manufacturing; Pesticides	Inorganic Chemical, Elements; Inorganic Chemical, Metals	0.66 (7.2)	0.316 (3.7)
4-Methylaminophenol sulfate	Manufacturing	Amines; Phenols	0.8 (6.7)	1.081 (4.0)
trans-Cinnamaldehyde	Food additive; Fragrance agent	Aldehydes	1.4 (13.1)	1.530 (5.9)
Isoeugenol*	Food additive; Fragrance agent	Carboxylic Acids	1.5 (31.0)	5.156 (8.4)
2-Mercaptobenzothiazole*	Manufacturing; Pesticides	Heterocyclic Compounds	1.7 (8.6)	12.097 (1.6)
Cinnamic aldehyde	Cosmetics; Food additive; Fragrance agent; Intermediate in chemical synthesis; Personal care products; Pesticides	Aldehydes	1.9 (18.4)	4.808 (4.0)
3-Aminophenol	Cosmetics; Pharmaceuticals	Amines; Phenols	3.2 (5.7)	2.990 (3.1)

Substance Name	Product Use[1]	Chemical Class[2]	Traditional LLNA EC3 (Maximum SI)[3]	LLNA: BrdU-ELISA EC1.6 (Maximum SI)[3]
Diethyl maleate	Food additive; Intermediate in chemical synthesis	Carboxylic Acids	3.6 (22.6)	8.049 (6.3)
Trimellitic anhydride	Manufacturing	Anhydrides; Carboxylic Acids	4.7 (4.6)	0.862 (7.9)
Nickel sulfate	Manufacturing	Inorganic Chemicals, Metals; Inorganic Chemicals, Elements	4.8 (3.1)	1.027 (4.5)
4-Chloroaniline	Intermediate in chemical synthesis; Manufacturing; Pesticides; Pharmaceuticals	Amines	9.00 (3.3)	11.029 (2.5)
Sodium lauryl sulfate*	Cosmetics; Food additive; Manufacturing; Personal care products; Pesticides; Pharmaceuticals	Alcohols; Sulfur Compounds; Lipids	8.1 (8.9)	13.334 (2.6)
Citral*	Fragrance agent	Hydrocarbons, Other	9.2 (20.5)	7.143 (16.4)
Hexyl cinnamic aldehyde*	Food additive; Fragrance agent	Aldehydes	9.7 (20.0)	12.920 (13.5)
Eugenol*	Cosmetics; Food additive; Intermediate in chemical synthesis; Manufacturing; Personal care products; Pharmaceuticals	Carboxylic Acids	10.1 (17.0)	8.851 (17.7)
Phenyl benzoate*	Manufacturing; Pesticides	Carboxylic Acids	13.6 (11.1)	16.954 (3.4)
Cinnamic alcohol*	Cosmetics; Food additive; Fragrance agent; Intermediate in chemical synthesis; Personal care products	Alcohols	21.0 (5.7)	24.091 (2.7)
Cyclamen aldehyde	Food additive; Fragrance agent	Aldehydes	22.3 (5.2)	41.496 (5.7)
Hydroxycitronellal	Food additive; Fragrance agent; Personal care products	Hydrocarbons, Other	24.0 (8.5)	13.636 (4.8)
Imidazolidinyl urea*	Cosmetics; Personal care products; Pesticides	Urea	24.0 (5.5)	49.545 (1.6)
Ethylene glycol dimethacrylate*	Manufacturing	Carboxylic Acids	28.0 (7.0)	31.751 (3.1)
Linalool	Cosmetics; Food additive; Fragrance agent; Personal care products; Pesticides	Hydrocarbons, Other	30.0 (8.3)	27.596 (4.7)

Substance Name	Product Use[1]	Chemical Class[2]	Traditional LLNA EC3 (Maximum SI)[3]	LLNA: BrdU-ELISA EC1.6 (Maximum SI)[3]
Ethyl acrylate	Manufacturing	Carboxylic Acids	32.8 (4.0)	33.333 (5.0)
Isopropyl myristate	Cosmetics; Personal care products; Pharmaceuticals	Lipids	44.0 (3.4)	9.404 (4.2)
Aniline	Food additive; Manufacturing; Personal care products; Pesticides; Pharmaceuticals	Amines	47.5 (4.4)	73.596 (2.1)
2-Hydroxypropyl methacrylate	Intermediate in chemical synthesis; Manufacturing	Carboxylic Acids	NC (1.3)	NC (1.1)
Diethyl phthalate	Cosmetics; Manufacturing; Personal care products; Pesticides; Pharmaceuticals	Carboxylic Acids	NC (1.5)	NC (0.9)
Dimethyl isophthalate	Manufacturing; Fragrance agent	Carboxylic Acids	NC (1.0)	NC (1.3)
Glycerol	Cosmetics; Food additive; Intermediate in chemical synthesis; Manufacturing; Personal care products; Pharmaceuticals; Solvent	Alcohols; Carbohydrates	NC (1.1)	NC (1.3)
Hexane	Manufacturing; Solvent	Hydrocarbons, Acyclic	NC (2.2)	56.328 (1.9)
Isopropanol*	Cosmetics; Disinfectant; Food additive; Intermediate in chemical synthesis; Manufacturing; Personal care products; Pharmaceuticals; Solvent	Alcohols	NC (1.7)	5.344 (2.2)[4]
Lactic acid*	Food additive; Manufacturing; Pharmaceuticals	Carboxylic Acids	NC (2.2)	15.177 (2.5)
Methyl salicylate*	Cosmetics; Food additive; Fragrance agent; Personal care products; Pharmaceuticals; Solvent	Carboxylic Acids	NC (2.9)	NC (1.4)
Salicylic acid*	Food additive; Manufacturing; Pharmaceuticals	Phenols; Carboxylic Acids	NC (2.5)	NC (1.3)
Sulfanilamide	Pharmaceuticals	Hydrocarbons, Cyclic; Sulfur Compounds	NC (1.0)	NC (1.3)
Propylene glycol	Cosmetics; Food additive; Intermediate in chemical synthesis; Personal care products; Pharmaceuticals; Solvent	Alcohols	NC (1.6)	NC (1.6)

Abbreviations: EC3 = estimated concentration (expressed as percentage) needed to produce SI = 3; EC1.6 = estimated concentration (expressed as percentage) needed to produce SI = 1.6; LLNA = murine local lymph node assay; LLNA: BrdU-ELISA= local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; NA = not available; NC = not calculated since maximum SI < 3.0 for the traditional LLNA or maximum SI < 1.6 for the LLNA: BrdU-ELISA; SI = stimulation index.

* Reference substance from ICCVAM (2009a).

[1] Information gathered from the following databases:
Hazardous Substances Database (http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB)
Haz-Map (http://hazmap.nlm.nih.gov/)
Household Products Database (http://hpd.nlm.nih.gov/index.htm)
International Programme on Chemical Safety INCHEM database (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, developed by the National Library of Medicine (http://www.nlm.nih.gov/mesh/meshhome.html).

[3] Mean EC3 (expressed as percent concentration) and maximum SI values are from the NICEATM database of traditional LLNA studies. EC1.6 and SI values for individual LLNA: BrdU-ELISA tests are provided in Annex IV of the BRD (**Appendix C**).

[4] Highest SI of seven tests. Because the majority (five) of the seven tests, had SI values < 1.6, isopropanol is considered to be a nonsensitizer in the LLNA: BrdU-ELISA.

3.3 Reference Test Method Data

Thirty-five of the 43 substances that were tested in the traditional LLNA were considered in the original evaluation of the LLNA by ICCVAM (ICCVAM 1999). The traditional LLNA reference data used for the accuracy evaluation were obtained from ICCVAM (1999) for 33 of these substances. Data for two substances which were negative in the original LLNA evaluation (ICCVAM 1999), aniline and nickel sulfate, were obtained from more recent sources that tested higher concentrations and obtained positive results. The traditional LLNA data for the remaining eight substances that were not considered in the original ICCVAM evaluation were obtained from the scientific literature. The reference data for GP tests (GPMT or Buehler test) and human tests (human maximization test, human patch test allergen, or other human data) were also obtained from the original LLNA evaluation (ICCVAM 1999) and the scientific literature. The LLNA, GP, and human reference data and sources for the 43 substances evaluated are provided in Annex III of the BRD (**Appendix C**).

3.4 Test Method Accuracy

The ICCVAM evaluation of the LLNA: BrdU-ELISA included an assessment of multiple decision criteria including SI ≥ 2.0, the threshold for distinguishing sensitizers and nonsensitizers that was used in the protocol for the interlaboratory validation study (Kojima et al. 2008) (**Table 3-2**). When the optimal decision criterion of SI ≥ 1.6 was used to identify sensitizers vs. nonsensitizers, compared to the traditional LLNA, accuracy was 95% (41/43), with a false positive rate of 18% (2/11) and a false negative rate of 0% (0/32). The two false positive substances, hexane (SI = 1.76 and 1.89) and lactic acid (SI = 1.80, 1.89, and 2.53), produced SI values between 1.6 and 1.9 in the LLNA: BrdU-ELISA. 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 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: BrdU-ELISA results when borderline positive results (e.g., SI values between 1.6 and 1.9) are produced to confirm that such results are not false positive. Both of the LLNA nonsensitizers with positive results in the LLNA: BrdU-ELISA, lactic acid and hexane, had minimal peptide reactivity. No unique characteristics were identified that could be used as rationale for excluding any particular types of substances from testing in the LLNA: BrdU-ELISA.

An evaluation to determine the robustness of the optimum SI ≥ 1.6 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 criteria or on the resulting number of false positives or false negatives (**Appendix C, Annex VII**).

Figure 3-1 shows that SI values for the LLNA: BrdU-ELISA are generally lower than those for the traditional LLNA at comparable 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: BrdU-ELISA results. However, the accuracy analyses reported in the BRD are based on individual test results and not on a geometric mean. The SI values for **Figure 3-1** are provided in **Table 3-3**.

Table 3-2 Performance of the LLNA: BrdU-ELISA for 43 Substances in Predicting Skin Sensitizing Potential Using Alternative Decision Criteria to Identify Sensitizers

Alternate Criterion	Accuracy		Sensitivity		Specificity		False Positive Rate		False Negative Rate		Positive Predictivity		Negative Predictivity	
	%	(No.[1])	%	(No.[1])	%	(No.[1])	%	(No.[1])	%	(No.[1])	%	(No.[1])	%	(No.[1])
Statistics[2]	91	(39/43)	97	(31/32)	73	(8/11)	27	(3/11)	3	(1/32)	91	(31/34)	89	(8/9)
≥95% CI[3]	88	(38/43)	100	(32/32)	54	(6/11)	46	(5/11)	0	(0/32)	86	(32/37)	100	(6/6)
≥2 SD[4]	91	(39/43)	100	(32/32)	64	(7/11)	36	(4/11)	0	(0/32)	89	(32/36)	100	(7/7)
≥3 SD[5]	91	(39/43)	91	(29/32)	91	(10/11)	9	(1/11)	9	(3/32)	97	(29/30)	77	(10/13)
SI ≥ 5.0	49	(21/43)	31	(10/32)	100	(11/11)	0	(0/11)	69	(22/32)	100	(10/10)	33	(11/33)
SI ≥ 4.5	58	(25/43)	44	(14/32)	100	(11/11)	0	(0/11)	56	(18/32)	100	(14/14)	38	(11/29)
SI ≥ 4.0	63	(27/43)	50	(16/32)	100	(11/11)	0	(0/11)	50	(16/32)	100	(16/16)	41	(11/27)
SI ≥ 3.5	74	(32/43)	66	(21/32)	100	(11/11)	0	(0/11)	34	(11/32)	100	(21/21)	50	(11/22)
SI ≥ 3.0	84	(36/43)	78	(25/32)	100	(11/11)	0	(0/11)	22	(7/32)	100	(25/25)	61	(11/18)
SI ≥ 2.5	93	(40/43)	91	(29/32)	100	(11/11)	0	(0/11)	9	(3/32)	100	(29/29)	79	(11/14)
SI ≥ 2.0	95	(41/43)	94	(30/32)	100	(11/11)	0	(0/11)	6	(2/32)	100	(30/30)	85	(11/13)
SI ≥ 1.9	95	(41/43)	94	(30/32)	100	(11/11)	0	(0/11)	6	(2/32)	100	(30/30)	85	(11/13)
SI ≥ 1.6	95	(41/43)	100	(32/32)	82	(9/11)	18	(2/11)	0	(0/32)	94	(30/32)	100	(9/9)
SI ≥ 1.5	95	(41/43)	100	(32/32)	82	(9/11)	18	(2/11)	0	(0/32)	94	(30/32)	100	(9/9)
SI ≥ 1.3	93	(40/43)	100	(32/32)	73	(8/11)	27	(3/11)	0	(0/32)	91	(32/35)	100	(8/8)

Abbreviations: CI = confidence interval; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine (BrdU); No. = number; SD = standard deviation; SI = stimulation index

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

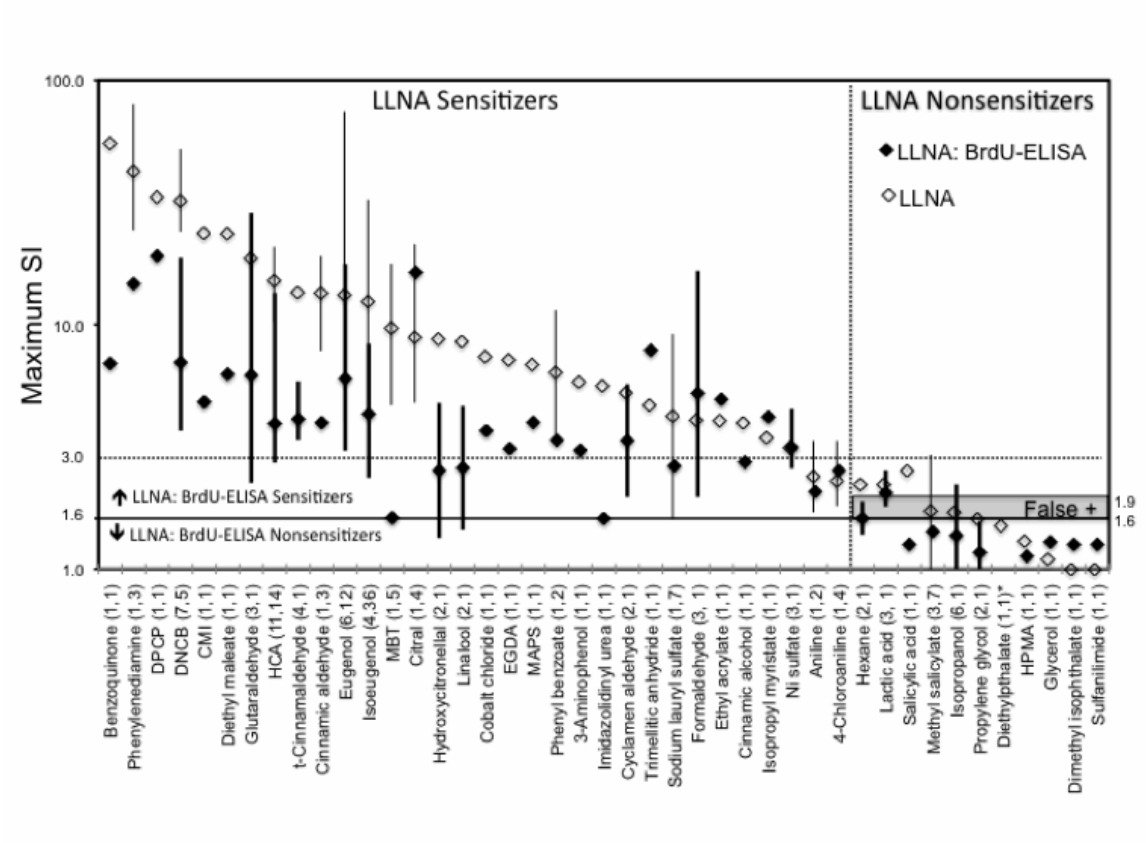
[2] Analysis of variance for difference of group means when substances were tested at multiple doses or *t*-test when substances were tested at one dose. The absorbance data were log-transformed prior to analysis of variance. Significance at $p < 0.05$ was further tested by Dunnett's test.

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

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

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

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



Abbreviations: CMI = 5-chloro-2-methyl-4-isothiazoline-3-one solution; DPCP = diphenylcyclopropanone; DNCB = 2,4-dinitrochlorobenzene; EGDA = ethylene glycol dimethacrylate; False + = false positive results in the LLNA: BrdU-ELISA (based on most prevalent result for substances with multiple tests) were in the SI range between 1.6 and 1.9; HCA = hexyl cinnamic aldehyde; HPMA = 2-hydroxypropyl methacrylate; LLNA = murine local lymph node assay; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; MAPS = 4-methyl aminophenol sulfate; MBT = 2-mercaptobenzothiazole; Ni = nickel; SI = stimulation index.

[1] LLNA: BrdU-ELISA and traditional LLNA responses at comparable test doses are shown. Symbols show the SI for substances with one test result or geometric mean maximum SI for substances with more than one test result. **Table 3-3** shows the individual values used. Bars show the range of values reported for multiple test results (heavy bars for LLNA: BrdU-ELISA and light bars for traditional LLNA). Numbers in parentheses beside the chemical names show the number of SI values for the LLNA: BrdU-ELISA and then the number of SI values for the traditional LLNA used in this figure. The number of SI values used in the figure may be different from the total number of SI values available since only comparable test doses and vehicles 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 maximum SI between 1.6 and 1.9 include hexane and lactic acid.

* The LLNA: BrdU-ELISA SI for diethyl phthalate is outside of the displayed data range and is not shown (SI < 1).

Table 3-3 Maximum SI Values of 43 Substances Evaluated in the LLNA: BrdU-ELISA Compared to the Traditional LLNA

Substance Name[1]	Test Vehicle[2]	LLNA: BrdU-ELISA Maximum SI Values[3]	Traditional LLNA Maximum SI Values
Sensitizers (LLNA: BrdU-ELISA SI ≥ 1.6 and Traditional LLNA SI ≥ 3.0)			
Benzoquinone (1,1)	AOO	6.94	52.30
1,4-Phenylenediamine (1,3)	AOO	14.70	23.30, 37.40, 75.30
Diphenylcyclopropenone (1,1)	AOO/ACE	19.10	31.70
2,4-Dinitrochlorobenzene (7,5)	AOO	3.68, 4.50, 5.29, 6.26, 6.53, 12.30, 18.80	23.00, 24.00, 26.80, 36.70, 49.60
CMI (1,1)	DMF	4.83	22.70
Diethyl maleate (1,1)	AOO	6.27	22.60
Glutaraldehyde (3,1)	ACE	2.25, 3.72, 28.60	18.00
HCA (11,14)	AOO	2.72, 2.87, 3.02, 3.27, 3.34, 3.40, 3.60, 3.64, 3.84, 5.90, 13.50	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
trans-Cinnamaldehyde (4,1)	AOO	3.37, 3.50, 4.11, 5.86	13.10
Cinnamic aldehyde (1,3)	AOO	3.97	7.60, 15.80, 18.40
Eugenol (6,12)	AOO	3.05, 3.17, 3.18, 7.09, 12.30, 17.70	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 (4,36)	AOO	2.36, 2.43, 7.20, 8.36	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
MBT (1,5)	DMF	1.62	4.60, 9.10, 9.50, 10.80, 17.10
Citral (1,4)	AOO	16.40	4.70, 6.20, 9.30, 20.50
Hydroxycitronellal (2,1)	AOO	1.34, 4.78	8.50
Linalool (2,1)	AOO	1.45, 4.65	8.30
Cobalt chloride (1,1)	DMSO	3.68	7.21
EGDA (1,1)	MEK	3.11	7.00
MAPS (1,1)	DMF	3.98	6.70
Phenyl benzoate (1,2)	DMF/AOO	3.37	3.50, 11.10
3-Aminophenol (1,1)	AOO	3.06	5.70

continued

Table 3-3 Maximum SI Values of 43 Substances Evaluated in the LLNA: BrdU-ELISA Compared to the Traditional LLNA (continued)

Substance Name[1]	Test Vehicle[2]	LLNA: BrdU-ELISA Maximum SI Values[3]	Traditional LLNA Maximum SI Values
Sensitizers (LLNA: BrdU-ELISA SI ≥ 1.6 and Traditional LLNA SI ≥ 3.0)			
Imidazolidinyl urea (1,1)	DMF	1.61	5.50
Cyclamen aldehyde (1,1)	AOO	1.97, 5.71	5.16
Trimellitic anhydride (1,1)	AOO	7.85	4.60
Sodium lauryl sulfate (1,7)	DMF	2.64	1.60, 2.60, 4.10, 5.10, 5.10, 5.40, 8.90
Formaldehyde (3, 1)	ACE	1.97, 4.40, 16.60	4.00
Ethyl acrylate (1,1)	AOO	4.95	3.98
Cinnamic alcohol (1,1)	AOO	2.74	3.90
Isopropyl myristate (1,1)	AOO	4.19	3.40
Ni sulfate (3,1)	DMSO	2.58, 2.66, 4.53	3.10
Aniline (1,2)	AOO	2.07	1.70, 3.30
4-Chloroaniline (1,4)	AOO	2.53	1.80, 1.80, 2.50, 3.30
Traditional LLNA Nonsensitizers (SI <3.0) with Borderline Positive SI Values in LLNA: BrdU-ELISA (1.6 < SI < 1.9; see bold text)			
Hexane (1,1)	AOO	1.38, **1.89**	2.20
Lactic acid (3,1)	DMSO	**1.80**, **1.89**, 2.53	2.20
Nonsensitizers (LLNA: BrdU-ELISA SI < 1.6 and Traditional LLNA SI < 3.0)			
Salicylic acid (1,1)	AOO	1.26	2.50
Methyl salicylate (3,7)	AOO	1.40, 1.44, 1.44	0.90, 1.10, 1.72, 1.90, 2.10, 2.30, 2.90
Isopropanol (6,1)	AOO	0.94, 0.98, 1.01, 1.57, 2.04, 2.22	1.70
Propylene glycol (2,1)	AOO/Water	0.87, 1.57	1.60
Diethyl phthalate (1,1)	AOO	0.88	1.50
HPMA (1,1)	AOO	1.13	1.30
Glycerol (1,1)	Water/DMF	1.29	1.10
Dimethyl isophthalate (1,1)	AOO	1.26	1.00
Sulfanilamide (1,1)	DMF	1.26	1.00

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); CMI = 5-Chloro-2-methyl-4-isothiazoline-3-one solution; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; EGDA = ethylene glycol dimethacrylate; HCA = hexyl cinnamic aldehyde; HPMA = 2-hydroxypropyl methacrylate; LLNA = murine local lymph node assay; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; MAPS = 4-methyl aminophenol sulfate; MBT = 2-mercaptobenzothiazole; Ni sulfate = nickel (II) sulfate hexahydrate; SI = stimulation index.

[1] Numbers in parentheses beside the substance names indicate the number of tests for the LLNA: BrdU-ELISA followed by the traditional LLNA, which may differ from the total number of tests available since only the most comparable test doses and vehicles were included.

[2] The vehicle used was the same in LLNA: BrdU-ELISA and traditional LLNA tests, except where indicated (e.g., vehicle used in the LLNA: BrdU-ELISA/vehicle used in the traditional LLNA).

[3] The bold text indicates SI values having potential false positive results (1.6 < SI < 1.9) for individual LLNA: BrdU-ELISA tests

3.5 Test Method Reliability (Intra- and Interlaboratory Reproducibility)

The BRD details the evaluation of intra- and interlaboratory reproducibility of the LLNA: BrdU-ELISA test method. Intralaboratory reproducibility was assessed using a concordance analysis of sensitizer/nonsensitizer results, and a coefficient of variation (CV) analysis of SI values and EC1.6 values (estimated concentration needed to produce an SI of 1.6). The qualitative analysis shows that multiple tests of 12 substances (10 LLNA sensitizers and two nonsensitizers) yielded 100% concordance for sensitizer/nonsensitizer outcomes for 83% (10/12) of the substances. The concordant results for one nonsensitizer, hexane, however, were incorrectly positive for both tests (2/2 tests had SI ≥ 1.6). In the quantitative analyses, the CVs for the SI values of 13 substance/concentration combinations that were tested up to five times each ranged from 1% to 80%. In addition, the CVs for the EC1.6 values of four substances that were tested up to five times at multiple doses ranged from 37% to 118%.

When using SI ≥ 1.6 as the threshold to distinguish sensitizers from nonsensitizers, the qualitative interlaboratory reproducibility analysis of 10 substances (seven sensitizers and three nonsensitizers) that were tested in three to seven laboratories indicated 100% agreement (3/3, 6/6, or 7/7) among the laboratories for nine substances (seven sensitizers and two nonsensitizers). However, one of the nonsensitizers, lactic acid, for which there was 100% agreement among the laboratories, was a false positive (i.e., 3/3 laboratories had SI ≥ 1.6). There was 67% (4/6) agreement among the tests for the remaining nonsensitizer. Interlaboratory CVs for the EC1.6 values of the seven sensitizers ranged from 31% to 93%.

When using SI ≥ 1.6 to classify sensitizers, the concordance analysis for the 18 substances with multiple tests indicated that the SI results for 85% (11/13) of the sensitizers (based on traditional LLNA results) were 100% concordant (i.e., all tests yielded SI ≥ 1.6) (**Table 3-4**). The SI results for the remaining two sensitizers included one test with SI < 1.6 and another test with SI > 1.6. The SI results for 60% (3/5) of the nonsensitizers were 100% concordant. All tests for two of the three nonsensitizers yielded SI < 1.6. All tests for the third nonsensitizer yielded SI values between 1.6 and 1.9, the narrow region in which false positive results occurred. The concordance for the other two nonsensitizers was 71% (5/7) for SI < 1.6 and 67% (2/3) for SI values between 1.6 and 1.9.

Table 3-4 Concordance of LLNA: BrdU-ELISA Tests across Maximum SI Categories

Substance	LLNA: BrdU-ELISA Nonsensitizers (Maximum SI ≤ 1.6[1])	LLNA: BrdU-ELISA Sensitizers (Maximum SI ≥ 1.6)		Total Tests
		1.6 < Maximum SI < 1.9[1]	Maximum SI ≥ 1.9[1]	
Sensitizers[2]				
Cyclamen aldehyde	0 (0%)	0 (0%)	0 (100%)	2
2,4-Dinitrochloro-benzene	0 (0%)	0 (0%)	9 (100%)	9
Diphenylcyclopro-penone	0 (0%)	0 (0%)	3 (100%)	3
Eugenol	0 (0%)	0 (0%)	9 (100%)	9
Formaldehyde	0 (0%)	0 (0%)	3 (100%)	3
Glutaraldehyde	0 (0%)	0 (0%)	5 (100%)	5

continued

Table 3-4 Concordance of LLNA: BrdU-ELISA Tests across Maximum SI Categories (continued)

Substance	LLNA: BrdU-ELISA Nonsensitizers (Maximum SI ≤ 1.6[1])	LLNA: BrdU-ELISA Sensitizers (Maximum SI ≥ 1.6)		Total Tests
		1.6 < Maximum SI < 1.9[1]	Maximum SI ≥ 1.9[1]	
Sensitizers[2]				
Hexyl cinnamic aldehyde	0 (0%)	0 (0%)	12 (100%)	12
Hydroxycitronellal	1 (50%)	0 (0%)	1 (50%)	2
Isoeugenol	0 (0%)	0 (0%)	3 (100%)	3
Linalool	1 (50%)	0 (0%)	1 (50%)	2
Nickel sulfate	0 (0%)	0 (0%)	3 (100%)	3
1,4-Phenylenediamine	0 (0%)	0 (0%)	2 (100%)	2
trans-Cinnamaldehyde	0 (0%)	0 (0%)	4 (100%)	4
Nonsensitizers[2]				
Hexane	0 (0%)	2 (100%)	0 (%)	2
Isopropanol	5 (71%)	0 (0%)	2 (29%)	7
Lactic acid	0 (0%)	2 (67%)	1 (33%)	3
Methyl salicylate	3 (100%)	0 (0%)	0 (0%)	3
Propylene glycol	3 (100%)	0 (0%)	0 (0%)	3

Abbreviations: LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; 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] According to traditional murine local lymph node assay results.

3.6 Animal Welfare Considerations: Reduction, Refinement, and Replacement

The LLNA: BrdU-ELISA will use the same number of animals as the updated ICCVAM-recommended traditional LLNA 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: BrdU-ELISA may lead to further reduction in use of the GP tests, which would provide for reduced animal use and increased refinement due to the avoidance of pain and distress that occur in the GP tests when substances cause ACD. Additionally, the LLNA: BrdU-ELISA test method protocol requires fewer mice per treatment group (a minimum of four animals/group) than either of the GP 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: BrdU-ELISA included two public review meetings by an independent scientific peer review panel, multiple opportunities for public comments (see **Section 1.0**), consideration of the OECD Expert Consultation on the LLNA, 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: BrdU-ELISA. This chapter summarizes the ICCVAM consideration of these reports and comments. The peer review panel reports and public comments are provided as **Appendices D** and **E**, respectively. The report of the OECD Expert Consultation on the LLNA is not publicly available.

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: BrdU-ELISA 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 LLNA Expert Consultation viewed that despite certain limitations, the LLNA: BrdU-ELISA 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. The experts reviewed LLNA: BrdU-ELISA results for 12 additional substances and four substances previously tested that were received by NICEATM after the Panel meeting. Like the Panel, OECD member country experts questioned the regulatory utility of the LLNA: BrdU-ELISA since specific guidance on how to classify substances with SI values in the range of uncertainty had not been 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 $\geq$ 1.6 provided the 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 1.6 and 1.9) as potential skin sensitizers.

ICCVAM considered the Panel report and the OECD Expert Consultation recommendations, and concluded that the single SI decision criterion of SI ≥ 1.6 to classify sensitizers would avoid false negative results as well as indeterminate results, which are not useful for regulatory purposes. Borderline results that may occur between 1.6 and 1.9 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. 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, a prescreen test should be performed in order to define the appropriate dose level to test in the LLNA: BrdU-ELISA. The Panel and the OECD Expert Consultation agreed in principle with ICCVAM that use of a reduced LLNA: BrdU-ELISA test method protocol instead of the multidose LLNA: BrdU-ELISA 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 the 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 should 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 ≥ 1.6, the OECD Expert Consultation recommended that borderline positive results (i.e., SI values between 1.6 and 1.9) 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 1.6 and 1.9 in the LLNA: BrdU-ELISA 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 the LLNA: BrdU-ELISA 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: BrdU-ELISA 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

continued

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

Table 4-1 Opportunities for Public Comment (continued)

Opportunities for Public Comments	Date	Number of Public Comments Received
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
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 draft ICCVAM 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: BrdU-ELISA.

1. One commenter indicated that several nonradioactive detection methods for the LLNA (e.g., 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: BrdU-ELISA. 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: BrdU-ELISA 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: BrdU-ELISA.

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 updated 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.

Two written comments were relevant to the LLNA: BrdU-ELISA.

1. One commenter noted that the LLNA: BrdU-ELISA was recommended for use by ICCVAM pending receipt of additional information, which the commenter supported, and using alternative decision criteria. The commenter further noted that ICCVAM qualified their acceptance and recommended a weight-of-evidence approach. The commenter indicated that while it is usually good scientific practice to evaluate any test method results in a weight-of-evidence manner, qualifications such as these challenged the recommendations and gave incentive to conduct more testing, when in reality the method evaluated had acceptable performance and should simply be recommended.

- The January 2008 draft ICCVAM recommendations for the LLNA: BrdU-ELISA indicated that the test method may be useful for identifying substances as potential skin sensitizers and nonsensitizers but recommended that more data and information were needed before final recommendations could be made. The January 2008 draft ICCVAM recommendations did not recommend using a weight-of-evidence approach to hazard classification.

2. Another commenter agreed with the January 2008 draft ICCVAM recommendation that more information and data were needed for the LLNA: BrdU-ELISA in order to conduct a meaningful assessment of the procedure's performance relative to the traditional LLNA. The commenter further agreed with the ICCVAM recommendation that it was important to have information regarding the interlaboratory performance of the assay. The commenter also had a suggestion regarding Table 6-2 of the January 2008 draft BRD. Since an alternative SI cutoff for the LLNA: BrdU-ELISA was identified (i.e., SI $\geq$ 1.3) a comparison of LLNA: BrdU-ELISA EC1.3 values to traditional LLNA EC3 values would be helpful.

- A comparison of data for the alternative SI values is included in the final ICCVAM BRD (see **Appendix C**).

Two oral comments were relevant to the LLNA: BrdU-ELISA.

1. One commenter agreed with ICCVAM that the LLNA: BrdU-ELISA and the LLNA: DA should be evaluated separately from one another because they have different treatment schedules. The tests have very little similarity, other than using CBA mice and measuring lymphocyte proliferation.
2. Another commenter explained that the rationale for selection of the CBA/JN strain of mice for the LLNA: BrdU-ELISA was that the sensitivity of the strain to p-benzoquinone was greater than that of the other two strains tested (i.e., BALB/cAnN and CD-1).

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 comments 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, National Institute of Environmental Health Sciences (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 (see **Appendix E3**).

There were no public comments specific to the LLNA: BrdU-ELISA.

Regarding the LLNA: BrdU-ELISA, one SACATM member indicated that the LLNA BrdU-ELISA had potential based on an accuracy of 83% (19/23) but a detailed protocol had not been provided and it was premature to make judgments.

The January 2008 draft ICCVAM recommendations included a statement that a sufficiently detailed protocol of the test method, including a defined and adequately justified decision criterion for distinguishing between sensitizers and nonsensitizers, was required. NICEATM subsequently obtained the detailed protocol, which was included in the revised draft BRD that was evaluated by the Panel in April 2009.

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, revised draft test method protocols, and revised draft LLNA performance standards 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: BrdU-ELISA.

1. The commenter supported the revised draft ICCVAM recommendation that the LLNA: BrdU-ELISA can be used for ACD testing with specific defined limitations in the decision criteria. That is, that substances falling within the intermediate SI 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 $\geq$ 1.6, to classify sensitizers. However, ICCVAM recommends that borderline positive results (i.e., SI values between 1.6 and 1.9) should be evaluated with other available information (e.g., dose-response information, evidence of systemic toxicity or excessive local 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.

2. The commenter further noted that the Panel recommended that the LLNA: BrdU-ELISA 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, especially since there are no available data for comparison.

- As outlined in the test method recommendations, ICCVAM considers the applicability domain for the nonradioactive LLNA methods 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: BrdU-ELISA.

One oral comment was relevant to the LLNA: BrdU-ELISA.

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: BrdU-ELISA 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 test 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: BrdU-ELISA.

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 GP test methods where radioactivity is prohibited.

The commenter also indicated that the number of animals used in the LLNA: BrdU-ELISA was eight animals per dose group and for ethical reasons the LLNA: BrdU-ELISA might be avoided.

- The commenter misunderstood the number of animals required by the LLNA: BrdU-ELISA. The ICCVAM-recommended protocol for the LLNA: BrdU-ELISA indicates that four animals per dose group are recommended.

The commenter further indicated that the justification for replacing the GP is not provided for the LLNA: BrdU-ELISA and that it should be mentioned.

- As indicated in Section 10.0 of the final ICCVAM BRD (**Appendix C**), the LLNA: BrdU-ELISA evaluates only the induction phase of skin sensitization and therefore discomfort to animals associated with the elicitation phase is eliminated. Additionally, the LLNA: BrdU-ELISA test method protocol requires fewer mice per treatment group (a minimum of four animals per group) than either of the GP tests (10-20 animals/group for the Buehler test and 5-10 animals/group for GPMT).

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 (see **Appendix E4**).

There were no public comments specific to the LLNA: BrdU-ELISA.

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

One 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.

Regarding the LLNA: BrdU-ELISA, another SACATM member indicated that the use of two SI decision criteria in the LLNA: BrdU-ELISA (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 $\geq$ 1.6 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 $\geq$ 1.6 as the decision criterion results in a false positive rate of 18% (2/11) compared to the traditional LLNA. Since the two false positive substances in the LLNA: BrdU-ELISA produced SI values between 1.6 and 1.9, 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, or other testing data) to confirm that such results in the SI range are positive.

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