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Poster Session

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1

Optimization of the Local Lymph Node Assay: Bromodeoxyuridine Detected by Flow Cytometry in BALB/c Mice for Hypersensitivity Evaluation

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The objective of this study was to optimize the local lymph node assay (LLNA): bromodeoxyuridine (BrdU) detected by flow cytometry (LLNA:BrdU-FC) for hypersensitivity evaluation by comprehensively examining several essential parameters, including route of BrdU administration (intraperitoneal [i.p.] vs. intravenous [i.v.]), BrDU dose-response (2.5, 5, 10 mg/mouse), and BrdU time-course response (6, 24 or 48 hrs prior to study termination). The number and percentage of BrdU+B220+ and BrdU+B220- cells following treatment with the potent sensitizer 2,4-dinitrofluorobenzene (DNFB) were determined to further establish whether the assay primarily measured a T-cell response. In the time-course study, a moderate contact sensitizer, hexyl cinnamic aldehyde (HCA), was also evaluated. Female BALB/c mice were treated with vehicle (4:1 acetone:olive oil [AOO]), DNFB, or HCA for 3 days and rested for 2 days before BrdU treatment. BrdU administration via i.p. injection produced a greater number and percentage of BrdU+ cells and a larger stimulation index (SI; 27 vs. 7 following DNFB treatment) than when administered i.v. Accordingly, the i.p. route was used in subsequent studies. In the dose-response study, results showed that mice treated with 10 mg BrdU produced the greatest SI (SI = 23) following DNFB treatment. In the time-course study, the data indicated that injection of BrdU 24 hours prior to study termination produced the highest SI for both HCA (SI = 3.7) and DNFB (SI = 16.5). In conclusion, we have optimized the LLNA:BrdU-FC to produce the greatest SI (compared to vehicle control) by administering 10 mg BrdU/mouse i.p. 24 hrs prior to study termination. Furthermore, our results have indicated that the BrdU is taken up predominately by B220- cells, suggesting that the LLNA:BrdU-FC measures primarily T-cell proliferation, a cell-mediated response.

These studies were conducted at the Virginia Commonwealth University (VCU) Immunotoxicology Laboratory under National Toxicology Program Contract No. ES 05454. All animal studies were conducted in accordance with all applicable animal care and use laws, regulations, and guidelines, under a protocol approved by the VCU Institutional Animal Care and Use Committee in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility.

2

Plasmacytoid Dendritic Cell-Based Assay as an *In Vitro* Alternative Assay for Chemical Allergenicity Screening

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Human dendritic cells have been used to evaluate the allergenicity potential of chemicals and can serve as an alternative to existing animal models utilized throughout industry to monitor products for contact sensitization. Development of such a non-animal alternative assay system for hazard assessment directly addresses REACH (Registration, Evaluation, and Authorization of Chemicals). In this study, we investigated whether CD86 expression in plasmacytoid dendritic cells (pDC) can be used to identify contact allergens. Normal, non-transformed human DC were generated from CD34+ progenitor cells and the pDC fraction (CD123+/CD11c-) was harvested using fluorescent activated cell sorter (FACS) sorting. The pDC were exposed to an expanded list of chemical allergens (n=49) or irritants (n=42). Concentrations of each chemical that resulted in >50% viability, as determined by FACS analysis of propidium iodide staining of cells, were used for analysis. Allergens were identified based on stimulation index (SI) calculated by the fold increase in CD86 expression. A material that had an SI ≥1.5 in at least 50% of the pDC donors (n=2-5 donors) was considered an allergen. For 71 of the 91 materials tested, historical mouse local lymph node assay (LLNA) and human clinical data were available. Using the *in vitro* pDC assay, CD86 expression increased ≥1.5 fold for 37 of 39 allergens but not for 26 of 32 non-allergens. Based on these results, a prediction model was developed to classify chemicals as allergens or non-allergens. The *in vitro* assay performance was sensitivity=95%, specificity=81%, and accuracy=89%; these results were slightly better than those obtained using the LLNA assay which showed sensitivity=85%, specificity=84%, and accuracy=85%. CD86 expression in pDC appears to be a sensitive and specific predictor of allergenicity. The assay is advantageous because high throughput screening of chemicals is possible, donor-to-donor variation can be monitored, the cells are of human origin, and the assay is cost-effective.

3

Evaluation of the Predictive Value of ToxCast Nrf2-ARE Data for Assessing the Sensitization Potential of Pesticides

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Assessment of the skin sensitization potential of chemicals is an important aspect of the safety evaluation process. Recent regulations, as well as responsible stewardship, have pushed the development of non-animal approaches that can effectively predict skin sensitization potential for new chemical entities. A cell-based approach that has shown recent promise is activation of the Nrf2-Keap1-ARE pathway as a cellular indication for oxidative stress and exposure to electrophilic substances. Protein reactivity is the common feature of skin sensitizers and the cellular protein Keap1 has been shown to be covalently modified by electrophiles, which in turn leads to activation of the antioxidant response element (ARE) pathway via transcription factor Nrf2. A number of publications have highlighted the predictive value of this pathway, as monitored through reporter-gene assays or gene expression, for the identification of chemicals with sensitization potential.

As part of EPA's ToxCast program, activation of the Nrf2-Keap1-ARE pathway was evaluated. To explore the utility of this pathway in the prediction of sensitization potential, our lab has compared Toxcast ARE results for 23 pesticides against previous *in vivo* sensitization results. The data indicate modest performance with high sensitivity (100%) and low specificity (~ 36%), relative to animal results (guinea pig and LLNA). Using criteria put forth by ToxCast, overall concordance is almost 70%. Overall concordance increased to 78% when the criteria for a positive response was modified to include 1.5-fold increase in activation, as published by others. The ARE data appear to have better predictive value for sensitization relative to at least one QSAR platform (DEREK), which had low sensitivity (50%) and low overall concordance (65%) for these 23 substances. A combination of multiple non-animal endpoints (e.g., QSAR, peptide reactivity assessment) should be considered as part of a predictive weight of evidence assessment of chemical sensitization potential.

4

Pyridoxylamine Reactivity Kinetics as an Amine-Based Probe for Screening Electrophilic Dermal Sensitizers

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Recently we developed a nitrobenzenethiol (NBT) assay for screening thiol-reactive haptens (1). Significant correlation was observed between the NBT reactivity constant (ka) and the local lymph node assay threshold (EC3) values. In the present study we describe an amine-based (pyridoxylamine [PDA]) kinetic probe to complement the NBT assay for identification of amine-selective haptens. Using UV-Vis spectrophotometry, rate constants for 12 chemicals (including five anhydrides, three aldehydes, two quinones, hydroxyethyl acrylate, and benzyl bromide) were determined to date, where reaction times to completion ranged from 20 min to >2 h. A greater complexity was observed for electrophilic reactions to PDA relative to NBT. Preliminary assessment suggests labile reactive intermediates are formed during PDA reaction to quinones and benzyl bromide. No reactivity was observed with the thiol-selective sensitizers (propiolactone and methane sulfonyl chloride) or nonsensitizers (such as sulfanilamide and benzocaine) in this method. The results from the PDA method highlight the utility of combining this amine-based method with the previous NBT-based model to be able to identify thiol-selective, amine-selective and nonselective electrophilic skin sensitizers. The use of PDA serves as a simple, inexpensive amine-based probe that shows promise as a preliminary screening tool for electrophilic, amine-selective skin sensitizers.

Reference

(1) Chipinda et al. 2010. Chem Res Toxicol 23:918-25.

5

Validation of a Flow Cytometry-Based Photo-Local Lymph Node Assay for the Identification and Characterization of Photo-Allergens

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The increasing concerns of pharmaceutical, chemical, consumer products and cosmetics industries to protect consumers from the photosensitizing properties of chemicals (UVA and chemical-induced allergic contact dermatitis) have driven the development of photo-allergy screening assays. Rapid, cost-effective tests that identify and characterize not only sensitizers but also photosensitizing substances are needed. Currently, the photo-Buehler guinea pig sensitization assay is used to test the photosensitization potential of chemicals. The photo-LLNA assay presented here is based on a successful alternative assay we have recently validated in our laboratory, the enhanced local lymph node assay (eLLNA) using flow cytometry. Although the LLNA has been accepted as a sensitization assay by both the Organisation for Economic Co-operation and Development and U.S. Environmental Protection Agency, the use of radioactivity limits its widespread application. Both the eLLNA and the photo-LLNA replace the use of radioactivity to detect photosensitizing chemicals via incorporation of BrdU, a thymidine analog, which eliminates the need for hazardous radioactivity. Compared to the guinea pig assay, the photo-LLNA 1) reduces the number of animals and the time needed for testing, and 2) reduces pain and distress to test animals. As does the eLLNA, the Photo-LLNA increases the discriminating power of the original assay by characterizing B and T lymphocyte subpopulations (CD3+ and B220+) and measuring “activation markers” for lymphocytes such as CD69 and I-Ak. Using these additional endpoints, we were able to correctly classify 11 out of 12 known photo-sensitizers and non-photosensitizers. Additionally, using the decision tree outlined for this assay, we were able to demonstrate that 8-MOP, which has historically been considered a photo-irritant, is in fact a photosensitizer based on lymphocyte immunophenotyping and activation status.

6

Use of an Enhanced Local Lymph Node Assay to Correctly Classify Irritants and False Positive Substances

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A major weakness of the local lymph node assay (LLNA) is that irritants can produce increases in the stimulation index (SI; SI>3) in a manner analogous to true sensitizers or allergens. Due to the short duration of the LLNA, many known human irritants are not detected as such during the course of the test (ear swelling and edema). The prototypical false positive irritant in the LLNA is sodium lauryl sulfate at concentrations above 20-25%. Herein we use an enhanced LLNA with immunophenotypic endpoints and measurements of ear thickness to characterize, identify, and correctly classify additional irritants, including benzalkonium chloride and ethylenediamine. Treatment with these irritants can result in maximum SI values from 5.4 to 13.3. Using flow cytometric analysis of surface markers on lymphocytes, including B220, CD3, I-Ak (MHC), and CD69, we are able to distinguish true sensitizers from these other anomalous test substances that increase SI by unknown mechanisms. None of the false positive irritants exhibited the hallmarks of true sensitizers, specifically increases in percent B220+, I-Ak + and cd69/iak++ cells. In contrast, irritants tended to decrease the percent of B-lymphocytes while increasing the percent of T-lymphocytes in the draining lymph node. Thus, in the event that irritancy is present or suspected as a known property of the test substance, we herein define a tier-testing approach which integrates additional endpoints including ear thickness measurements and immunophenotypic analysis. This enhanced LLNA allows a more thorough assessment and accurate classification of problematic substances.

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7

ICCVAM Evaluation and International Acceptance of the Nonradioactive LLNA: BrdU-ELISA Test Method

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ICCVAM assessed the usefulness and limitations of the LLNA: BrdU-ELISA, a nonradioactive version of the traditional local lymph node assay (LLNA) that measures the amount of BrdU incorporation into the DNA of proliferating lymphocytes as an indicator of potential allergic contact dermatitis (ACD) hazards. The assays' accuracy when compared to the traditional LLNA was assessed based on data generated with 43 substances and using several different stimulation indices (SI) as decision criteria. Optimal performance was achieved using a SI ≥ 1.6; the LLNA: BrdU-ELISA correctly identified all 32 LLNA sensitizers (0% [0/32] false negatives) and 9/11 LLNA nonsensitizers (18% [2/11] false positives). The two false positives had maximum SI between 1.6-1.9. There were 18 substances with repeat tests; results for 85% (11/13) of the LLNA sensitizers and 60% (3/5) of the LLNA nonsensitizers were concordant among the repeat LLNA: BrdU-ELISA tests. ICCVAM concluded that the accuracy and reproducibility of the LLNA: BrdU-ELISA support its use to identify potential skin sensitizers and nonsensitizers. ICCVAM recommends a SI ≥ 1.6 to identify potential sensitizers since there were no false negatives relative to the LLNA. In testing situations where dose-response information is not required, or negative results are anticipated, ICCVAM recommends that the single-dose reduced LLNA: BrdU-ELISA should be considered and used, thereby reducing animal use by up to 40%. The ICCVAM-recommended protocol formed the basis of the recently adopted OECD Test Guideline 442B for the LLNA: BrdU-ELISA. Because the LLNA: BrdU-ELISA does not require radioactive reagents, more institutions can take advantage of the reduction and refinement benefits afforded by the LLNA compared to traditional guinea pig methods for ACD testing. The LLNA: BrdU-ELISA will also eliminate the environmental hazard associated with use and disposal of radioactive materials used in the traditional LLNA. ILS staff supported by NIEHS contract N01-ES-35504.

8

Assessment of Direct Peptide Reactivity as an Alternative to *In Vivo* Skin Sensitization Testing

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Direct peptide reactivity is an *in vitro* method being advanced to assess the potential of chemicals to cause skin sensitization. This method was developed because the majority of chemical allergens are electrophiles that can react with nucleophilic thiol and amino sites of proteins, a key event in the development of a sensitization response to low molecular weight chemicals. Gerberick et al. (2) utilized two different peptides containing either cysteine or lysine, and peptide binding was assessed by measuring remaining free peptide with high-performance liquid chromatography with ultraviolet detector (HPLC-UV) analysis. A modified method was developed by another research group (2) which uses a single peptide, containing both cysteine and lysine, and analyzes peptide depletion and covalent adduct formation using liquid chromatography-mass spectrometry (LC-MS) methods.

While both protocols produce relatively consistent results, some opportunities were recognized to leverage the strengths of each approach in an effort to improve the application and performance of the assay. Both approaches were evaluated in our laboratory using six sensitizers and three nonsensitizers. Incubations used both neutral and basic reaction conditions, and samples were analyzed with HPLC-UV-MS to measure peptide depletion. HPLC separation was modified to measure oxidized or dimerized peptides, adducted peptides, as well as parent peptides.

The protocol correctly identified the potential for eight out of nine reference chemicals – 5/6 sensitizers and 3/3 non-sensitizers, with the peptide developed by Natsch's group showing higher predictability. Although there were some discrepancies for three reference chemicals, these were attributed to issues with solubility and stability of test material. Hexyl cinnamaldehyde, a sensitizer with moderate potency, presented false negative results using all three peptides. Published results demonstrated inconsistent findings as well. Similarly, a trimellitic anhydride solution that showed no peptide depletion when prepared in water did so when prepared in acetone. A potential false positive result for sodium dodecyl sulfate was addressed by integrating chromatograms and mass spectrum data to distinguish retention shifts from actual peptide depletion. These modifications could be applied to improve the accuracy and reproducibility of a direct peptide reactivity assay.

References

(1) Gerberick et al. (2004) Toxicol Sci 81:332-343.
(2) Natsch and Gfeller (2008) Toxicol Sci 106:464-478.

9

A New *In Vitro* Method for the Detection of Chemical Sensitizers: Combines Peptide Binding with ARE/EpRE-Linked Gene Expression

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Chemical sensitization is a major concern for companies who are developing novel ingredients for their products. An *in vitro* method capable of replacing the current animal models would be of considerable value. The purpose of this study was establish a relationship between reactive chemicals and their expression of genes controlled by the antioxidant response/electrophilic response element (ARE/EpRE) by adding additional endpoints and building a larger validation set. The *in vitro* approach described here encompasses cell viability, direct and indirect chemical reactivity, and ARE/EpRE-mediated gene expression combined with a gated logic algorithm that identifies sensitizers, their potency category, and provides an estimated murine local lymph node assay (LLNA) EC3 value (i.e., estimated concentration of a substance expected to produce a stimulation index of 3). This model is being developed using human keratinocyte (HaCaT) cells and three-dimensional human skin models. HaCaT cells were seeded into 96-well culture plates at an initial density of 10,000 cells/well. Cells were allowed to equilibrate for 48 hr prior to beginning the experiment. Six exposure concentrations, ranging from 0.01 to 2500 μM were used for each chemical tested. Following a 24 hr exposure period the cells were harvested for analysis. The expression of 11 genes linked to the ARE/EpRE promoter was evaluated by RT-PCR. Ninety-six chemicals classified as nonsensitizers or as weak, moderate, strong or extreme sensitizers were evaluated in this model. Each chemical was evaluated for viability (MTT assay), glutathione binding and induced gene expression. A gated algorithm was developed to process the data and provide a toxicity index which was compared to LLNA EC3 values of known reference compounds using exponential regression analysis (R2 = 0.90). To challenge this model, two blinded studies were conducted each with 20 compounds. The sensitivity was determined to be 78% and the specificity was 96%. These data indicate that this *in vitro* sensitization model can identify and categorize skin sensitizers and may be a viable alternative to animal testing.

10

The Integration of Local Lymph Node Assay Data into Quantitative Risk Assessments for Dermal Sensitization to Fragrance Ingredients

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When conducting a quantitative risk assessment for the induction of dermal sensitization, both local lymph node assay (LLNA) and historical human data add to the overall weight of evidence approach used to determine potency. Contemporary human studies are not conducted to determine hazard; rather they confirm the lack of dermal sensitization at an exposure level identified as a no-effect level (NOEL) in an animal model. The LLNA is the most commonly employed model for the assessment of fragrance materials. In addition to identifying dermal sensitization hazards, the LLNA provides a quantitative measure of relative skin sensitizing potency. These potency estimates are based on interpolation of the dose response data, yielding an estimated concentration (EC3) required to elicit a positive response. In the present study, the EC3 values of 57 fragrance materials were compared to those derived from human NOELs for induction determined by historical human data from repeated insult patch tests (HRIPT) and/or maximization tests (HMAX). The human NOELs and EC3 values were converted to their dose per unit area ($\mu g/cm^2$) equivalents to allow for direct comparison. A good correlation existed between the EC3 values and the human NOELs for induction. The EC3 values were observed to predict, and in some cases underpredict, the human NOELs for approximately ~80% of the materials tested. The results from this analysis demonstrate the utility of incorporating the EC3 value into the quantitative risk assessment approach. However, the lack of correlation for several materials highlights the importance of conducting a confirmatory HRIPT.

This study relies on data generated by the authors or gathered from the literature. All data generated by the authors utilizing animals or humans was conducted in accordance with all applicable laws, regulations and guidelines. The studies were approved by either an institutional animal care and use committee or institutional review board as appropriate.

11

ICCVAM Evaluation and International Acceptance of the Nonradioactive LLNA: DA Test Method

J. Matheson,[1] A. Jacobs,[2] P. Brown,[2] R. Ward,[3] E. Margosches,[3] E. Salicru,[4] D. Allen,[4] F. Stack,[4] W. Stokes[5]

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ICCVAM evaluated the validity of the nonradioactive LLNA: DA, which measures increases in ATP content as an indicator of lymphocyte proliferation, to identify substances that may cause allergic contact dermatitis (ACD). The assay's accuracy of multiple stimulation index (SI) decision criteria was assessed for 44 substances with traditional murine local lymph node assay (LLNA) reference data. Optimal performance was achieved using a SI ≥ 1.8; the LLNA: DA correctly identified all 32 LLNA sensitizers (0% [0/32] false negatives), and 9/12 LLNA nonsensitizers (25% [3/12] false positives). The three false positives had maximum SI between 1.8-2.5. For the assessment of reproducibility, there were 14 substances with multiple tests. Results for 8/10 LLNA sensitizers (80%) and 3/4 LLNA nonsensitizers (75%) were concordant among 3-18 tests. ICCVAM concluded that the accuracy and reproducibility of the LLNA: DA support its use to identify potential skin sensitizers and nonsensitizers. ICCVAM recommends a SI ≥ 1.8 to identify potential sensitizers based on no false negatives relative to the traditional LLNA. Still, the LLNA: DA has the potential for false positives when SI values are between 1.8-2.5. The LLNA: DA might not be appropriate for testing substances that affect ATP levels (e.g., ATP inhibitors) or those that affect the accurate measurement of intracellular ATP (e.g., ATP-degrading enzymes, extracellular ATP in the lymph node). The ICCVAM-recommended protocol formed the basis of Test Guideline 442A for the LLNA: DA adopted by the Organisation for Economic Co-operation and Development. ICCVAM recommendations have been forwarded to Federal agencies for their consideration of regulatory acceptance. Since the LLNA: DA does not require radioactive reagents, regulatory acceptance will allow more institutions to take advantage of the reduction and refinement benefits afforded by the LLNA compared to traditional guinea pig methods for ACD testing. The LLNA: DA will also eliminate the environmental hazard associated with use and disposal of radioactive materials used in the traditional LLNA. ILS staff supported by NIEHS contract N01-ES-35504.

12

ICCVAM Evaluation and International Acceptance of the LLNA for Determining the Allergic Contact Dermatitis Potential of Pesticide Formulations and Other Products

J. Matheson,[1] A. Jacobs,[2] M. Wind,[1] J. Chen,[3] M. Hashim,[3] M. Lewis,[3] E. Margosches,[3] D. McCall,[3] T. McMahon,[3] J. Redden,[3] R. Ward,[3] T. Burns,[4] D. Allen,[4] W. Stokes[5]

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ICCVAM has updated its 1999 validation report on the murine local lymph node assay (LLNA) based on a recent evaluation of the usefulness and limitations of the LLNA for assessing the allergic contact dermatitis (ACD) potential of pesticide formulations. This review was initiated because the original report did not include an LLNA analysis for these types of substances, and there were growing regulatory concerns that the LLNA might not identify sensitizing pesticide formulations. LLNA data for 104 formulations were included in the evaluation, most of which are water-soluble and were therefore tested in an aqueous vehicle containing 1% Pluronic L92. Of the pesticide formulations for which LLNA and guinea pig (GP) data were available (n = 23), the LLNA classified 52% (12/23) as sensitizers, while GP tests classified only 13% (3/23) as sensitizers. All three of the pesticide formulations identified as sensitizers in the GP test were also identified as sensitizers in the LLNA; there were no instances of underprediction by the LLNA. Thus, there appears a greater likelihood of obtaining a positive result in the LLNA than in a GP test. These studies also provide data for aqueous solutions that emphasize the need for careful selection of an appropriate vehicle that maintains test substance contact with the skin (e.g., 1% Pluronic L92 in water) to achieve adequate exposure when testing such substances. Based on these data, ICCVAM agreed with an international peer review panel that the LLNA could be used for testing pesticide formulations, and products in aqueous vehicles, unless there are physicochemical properties that may interfere with the ability of the LLNA to detect the sensitizing potential of a substance. ICCVAM recommendations were forwarded to Federal agencies for regulatory acceptance consideration. Adoption of these recommendations should expand the use of the LLNA for skin sensitization testing, thereby reducing and refining animal use for this purpose. ILS staff supported by NIEHS contract N01-ES-35504.

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ICCVAM Evaluation and International Acceptance of LLNA Performance Standards

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ICCVAM develops performance standards to facilitate the efficient validation of modified versions of adequately validated alternative test methods. ICCVAM recently developed murine local lymph node assay (LLNA) performance standards based on the ICCVAM-recommended protocol. The protocol was revised to reduce the minimum number of mice per dose group from five to four (with additional direction on reducing the number of positive control animals), and to provide guidance for determining the appropriate highest test dose. The performance standards include essential test method (ETM) components, a minimum list of reference substances, and standards for accuracy and reliability. ETM components are the structural, functional, and procedural elements of a validated test method that must be included in a modified method in order for it to be evaluated using the established performance standards. LLNA ETM components include topical application of the test substance to the ears of mice, measurement of lymphocyte proliferation in the auricular lymph nodes, and use of the maximum concentration that does not result in systemic toxicity or excessive local irritation. The list of 18 required reference substances includes 13 sensitizers and 5 nonsensitizers. The accuracy and reliability standards are based on the performance of the LLNA. LLNA performance standards were included in the updated 2010 Organisation for Economic Co-operation and Development Test Guideline 429 based on ICCVAM's evaluation. These LLNA performance standards will facilitate rapid and efficient validation of modified LLNA protocols. New versions of the LLNA that provide improved performance or other advantages are expected to result in broader use of the LLNA, which will further reduce and refine animal use for allergic contact dermatitis assessments while ensuring human safety. ILS staff supported by NIEHS contract N01-ES-35504.

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Transferability, Reproducibility, and Predictivity of the Novel KeratinoSens *In Vitro* Skin Sensitization Assay: Results of a Ring-Study in Five Laboratories

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Determination of skin sensitization potential is a critical toxicological endpoint in the development and evaluation of ingredients used in fragrance, cosmetic and personal care products. With the deadline to replace animal testing for skin sensitization in the European Union quickly approaching, alternative tests are urgently needed to replace animal tests to evaluate the skin sensitization potential of novel chemicals. Several approaches, including *in vitro, in silico* and *in chemico* evaluation tools have been developed to achieve this goal. The KeratinoSens assay is a cell-based reporter gene assay that can be used to screen substances with a full dose-response assessment. It is based on a stable transgenic keratinocyte cell line. The induction of a luciferase gene under the control of the antioxidant response element derived from the human AKR1C2 gene is determined. Here we report on the results of a ring-study with five laboratories performing the KeratinoSens assay on a set of 28 test substances. The assay was found to be easily transferable to all laboratories. Overall both the qualitative (sensitizer/non-sensitizer categorization) and the quantitative (concentration for significant gene induction) results were reproducible between the different laboratories. An analysis of the transferability, the within- and between-laboratory reproducibility, and the predictivity is presented.

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Validation of a 3D Skin Model for Cosmetic, Chemical and Medical Device Phototoxicity Testing (EPARS)

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We have enhanced our validated *in vitro* phototoxicity test using human skin models by exploring inflammatory mediator and gene expression endpoints. The enhanced phototoxicity assay in reconstituted skin (EPARS) is based upon a 3D skin model that closely parallels human skin morphology. Major advantages of this test system are that test substances can be applied topically, avoiding the problems of (1) difficulty in solubilizing test materials, and (2) indirect application of test materials to cell monolayers via culture media. In addition, the tissues are composed of differentiated layers of primary human keratinocytes, a more relevant model than mouse tumor fibroblasts. Phototoxic effects are determined by measuring the viability of ultraviolet (UV)-irradiated vs. nonirradiated exposed tissues. In order to increase the sensitivity and specificity of the test, we have measured the release of cytokines into the culture media via ELISA. The release of the inflammatory factor PGE2 was shown to be an early predictor of the toxic effects demonstrated in the viability assay. When compared to human phototoxicity test results and the 3T3 NRU PT validation test material set, EPARS had 100% accuracy, sensitivity and specificity. Microarray analysis of gene expression showed that chlorpromazine treatment with UVA irradiation caused changes in gene expression over time that were not observed without UVA irradiation. These genes include those for keratins, collagens and fibronectins. EPARS is an accurate and sensitive test for detecting phototoxic substances at doses representative of those that cause actual human skin reactions. Thus, EPARS is a highly predictive phototoxicity assay, with endpoints of inflammatory mediator and gene expression that allow for investigation into the mechanisms of photosensitivity in a wide variety of consumer products.

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In Vitro Phototoxicity Test Methods Compared: 3T3 NRU PT vs. Phototoxicity Assay In Reconstituted Skin

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Currently, only the 3T3 Neutral Red Uptake Phototoxicity Test (3T3-NRU PT) has been approved as a non-animal phototoxicity test by governmental regulatory agencies. However, the 3T3-NRU PT has serious drawbacks as a model for human skin, specifically: it uses a monolayer cell culture consisting of mouse tumor cells, it is overly sensitive resulting in many false-positives, and test substances must be soluble in tissue culture media. Thus, we have developed a more relevant *in vitro* system based on three-dimensional, differentiated human keratinocyte cultures, which can accommodate a wide range of vehicles and allow direct topical application of test substances, a "Phototoxicity Assay in Reconstructed Skin". We present a side-by-side comparison between the 3T3-NRU PT and the PARS test systems using the same solar simulated light source and the eight reference standard chemicals used for validation of the 3T3-NRU PT. The PARS test correctly predicts with 100% accuracy the phototoxic potential of all reference test substances. The concentrations of test agents needed to induce cytotoxicity in reconstituted skin, when compared to the neutral red assay in 3T3 fibroblasts, is one to two orders of magnitude higher, reflecting the thickness and complexity of a three-dimensional tissue structure. This better approximates the exposure levels of chemicals needed to induce phototoxic effect in animal tests and actual human skin. In addition, the most important practical advantage gained over the 3T3 NRU PT is that test substances can be applied topically, overcoming both pH and solubility problems encountered when dosing via the culture media.

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Chemistry-Based Risk Assessment For Skin Sensitization: Quantitative Mechanistic Modelling

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Skin sensitization is a complex multistep biological process. However, by focussing on the key step which is dependent on the nature of the (putative) sensitizing chemical, predictive models based on chemical properties can be derived. The key step is covalent reaction of the sensitizer or its metabolite, with skin protein nucleophiles, and this has been found to depend on the nature of the reaction (the reaction mechanistic applicability domain), electrophilic reactivity and hydrophobicity. Based on this concept the approach to skin sensitization prediction is:

1) Assign the chemical to be predicted to its reaction mechanistic applicability domain. This can often be done by expert knowledge; if not it can be determined by chemical experimentation.
2) Obtain reactivity data and hydrophobicity data for the chemical. Often this can be done *in silico*, but if not it can be done *in chemico*, e.g. by experimental measurement of rate constants with model nucleophiles.
3) By comparison of the reactivity and hydrophobicity parameters with those of known sensitizers and nonsensitizers in the same domain, sensitization potency can be predicted by mechanism-based QSAR models or by read-across.

Examples illustrating this approach will be given. The approach offers the potential to replace the animal testing laboratory by the chemical laboratory.

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ICCVAM Evaluation and International Acceptance of the Reduced LLNA: An Alternative Test Method Using Fewer Animals to Assess the Allergic Contact Dermatitis Potential of Chemicals and Products

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Based on recommendations by ICCVAM in 1999, U.S. regulatory agencies that require the submission of skin sensitization data accepted the murine local lymph node assay (LLNA), with identified limitations, as an alternative to guinea pig tests for assessing allergic contact dermatitis (ACD). In January 2007, the U.S. Consumer Product Safety Commission (CPSC) nominated several activities related to the LLNA for evaluation by NICEATM and ICCVAM. One of the nominated activities was an assessment of the usefulness and limitations of the reduced LLNA (rLLNA). In the rLLNA, each substance is tested at only one dose level (the high dose), whereas in the LLNA, a minimum of three dose levels is tested. NICEATM and ICCVAM conducted a retrospective review of LLNA data that included 457 unique substances from 471 LLNA studies. The rLLNA's ability to correctly identify potential skin sensitizers was compared to LLNA results. The rLLNA has an accuracy of 99% (465/471), a false positive rate of 0% (0/153), and a false negative rate of 2% (6/318) when compared to the LLNA. ICCVAM concluded that the rLLNA is sufficiently accurate to distinguish between skin sensitizers and nonsensitizers. Therefore, ICCVAM recommends that the rLLNA test method should be routinely used for determining the ACD potential of chemicals and products. U.S. regulatory agencies agreed to use the rLLNA when applicable. The inclusion of an optional rLLNA in the update of Organisation for Economic Co-operation and Development Test Guideline 429 provides international acceptance. Use of the rLLNA can reduce animal use for ACD testing by 40% while continuing to support the protection of human health. ILS staff supported by NIEHS contract N01-ES-35504.