New Models in the Validation Pipeline for Allergic Contact Dermatitis Testing: DPRA, h-CLAT, and MUSST

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ECVAM Phase III Pre-validation Study

- Three methods:
  - Direct Peptide Reactivity Assay (DPRA, Procter & Gamble)
    - Uses HPLC to monitor a chemical's potential to deplete a nucleophile-containing synthetic peptide
  - Myeloid U937 Skin Sensitization Test (MUSST, L'Oréal)
    - Uses flow cytometry to monitor induction of a protein marker on the surface of a human monocytic cell line following exposure to chemical
  - Human Cell Line Activation Test (h-CLAT, Kao and Shiseido)
    - Uses flow cytometry to monitor induction of two protein markers on the surface of a human monocytic leukemia cell line following exposure to chemical

ECVAM Study – Validation Management Team (VMT) Composition

- Validation Management Group
  - David Basketter – Chair
  - Silvia Casati – Co-chair
  - Alexandre Angers – ECVAM representative
  - Thomas Cole – Chair of Chemical Selection Group
  - André Kleensang – ECVAM biostatistician
  - Anna Compagnoni – alternate ECVAM biostatistician
  - Pierre Aeby – Industry representative
  - Sebastian Hoffmann – External expert
  - Jon Richmond – External expert
- Lead laboratory Representatives
  - G. Frank Gerberick – Procter & Gamble
  - Jean Marc Ovigne – L'Oréal
  - Takao Ashikaga – Shiseido
  - Hitoshi Sakaguchi – Kao Corporation
- Liaisons
  - JaCVAM (Hajime Kojima; alternate Yasuo Ohno)
  - NICEATM (William S. Stokes; alternate Eleni Salicru)
  - ICCVAM (Joanna M. Matheson; alternate Abigail Jacobs)

ECVAM Study – Objective and Goals

- Objective:
  - Evaluate the DPRA, MUSST, and h-CLAT in view of their future incorporation into a testing strategy for fully replacing current regulatory animal tests
- Primary Goal:
  - Assess the transferability and reliability (within- and between-laboratory reproducibility) of each of the three test methods when challenged with a set of coded chemicals
- Secondary Goals:
  - Preliminary assessment of the test methods' ability to discriminate between skin sensitizing and nonsensitizing chemicals
  - Preliminary assessment of the test methods' ability to categorize skin sensitizing chemicals into the GHS sub-categories 1A (strong sensitizer) and 1B (other than strong sensitizer)

ECVAM Study – Experimental Design

- Two sequential phases:
  - Phase A: Training of participating laboratories and test method transferability
    - Stage I: Lead laboratories issue SOP and training study plan for training personnel from other testing sites
    - Stage II: Trained personnel will transfer test method to their own laboratories
  - Phase B: Formal evaluation of test method reproducibility
    - 24 coded chemicals (15 tested three times at each site)
    - Stage I: Test methods evaluated with preliminary set of 9 coded chemicals tested once (1 experiment)
    - Stage II: Test methods evaluated with additional set of 15 coded chemicals tested 3 times (3 independent experiments)
  - After each phase, reports submitted to VMT for review

Overview of DPRA

- Protein binding is a key step in induction of skin sensitization
- Cysteine and lysine containing peptides are mixed with test chemical at 1:10 or 1:50 ratio respectively
  - Reactivity is expressed as % peptide depletion measured by HPLC-UV after 24 hours incubation with test chemical
- Data analyzed using classification tree (recursive partitioning) methodology to rank reactivity as minimal, low, moderate or high
  - Minimal reactivity = nonsensitizers
  - Low, moderate or high reactivity = sensitizers
- Prediction model developed with 81 chemicals
  - Accuracy = 89%
  - Sensitivity = 88%
  - Specificity = 90%
- 157 chemicals tested to date
  - Accuracy = 85%

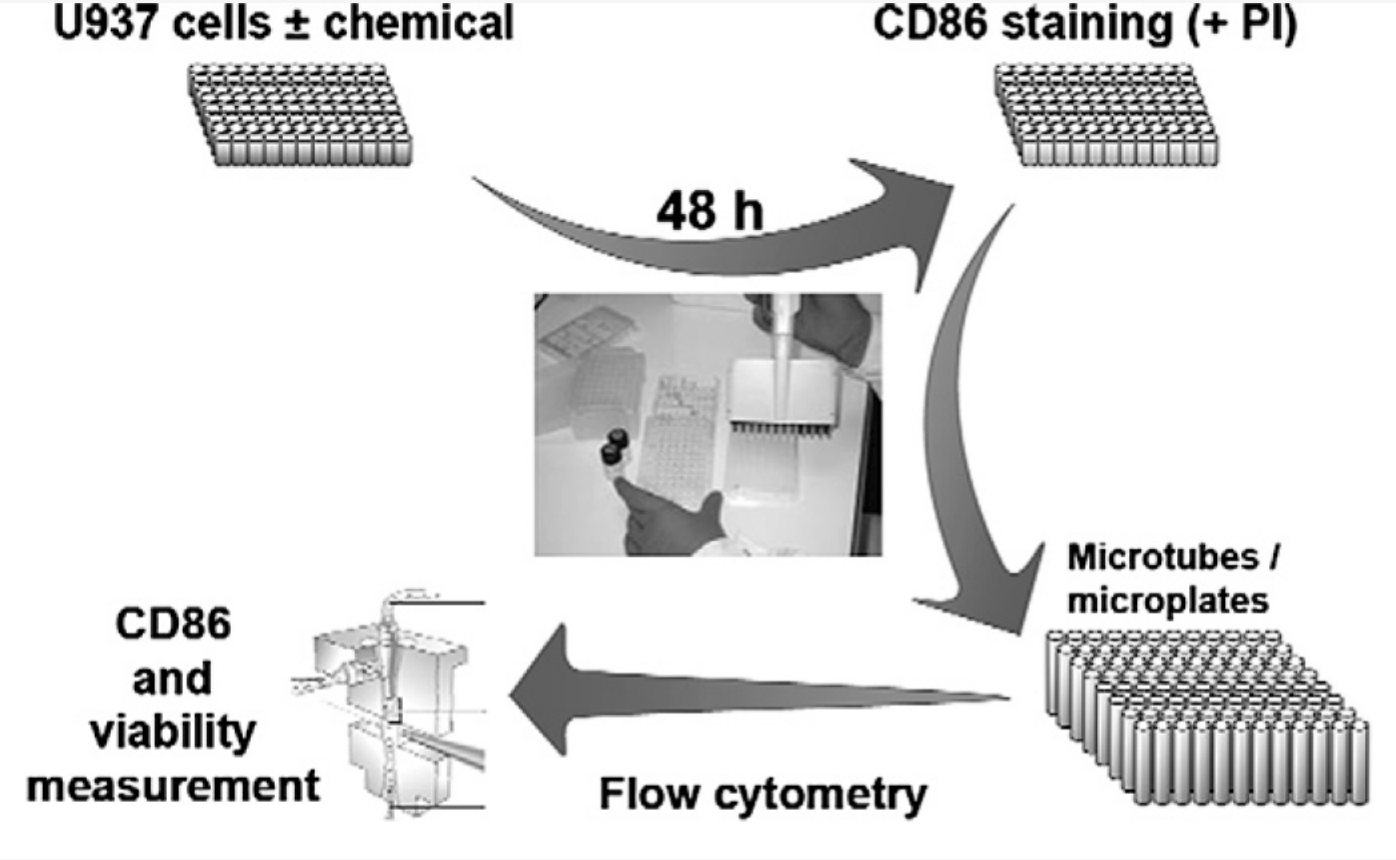
Gerberick et al. 2007. Tox Sci 97:417-427

Overview of MUSST

- Dendritic cell activation is a key event in development of skin sensitization
- Based on flow cytometry assessment of CD86 (a co-stimulatory protein) upregulation in human monocytic U937 cells[1] after exposure to sensitizing chemicals
  - U937 are dendritic-like cells and serve as a surrogate for evaluation of CD86 dendritic cell expression
- A chemical is considered sensitizing if it induces a dose-dependant increase of CD86 expression at non-toxic doses
- The stimulation index calculated represents a ratio of the % of CD86+ cells in treated vs. control cells
  - The threshold for positive is set at SI = 150
- Data presented at the 6th World Congress showed comparable results from three independent laboratories with three sensitizers (MCI/MI, hydroquinone and ethylene diamine) and two irritants (SDS and lactic acid) indicating a transferable protocol

Sundstrom and Nilsson. 1976. Int J Cancer 17:565-577

Schematic View of the MUSST Protocol



Aeby P et al. 2010. Toxicol In Vitro 24(6):1465-1473.

Overview of h-CLAT

- Dendritic cell activation is a key event in development of skin sensitization
- Based on flow cytometry assessment of CD86 (co-stimulatory protein) and CD54 (adhesion protein) upregulation in human monocytic leukemia THP-1 cells after exposure to sensitizing chemicals
  - THP-1 are dendritic-like cells and serve as a surrogate for evaluation of CD86 and CD54 dendritic cell expression
- Experiments are conducted on three different days
  - THP-1 cells are cultured with test chemicals for 24 hr using 8 doses based on the dose that afforded 75% cell viability (CV75)
- A chemical is considered a potential skin sensitizer if ≥2 runs at any dose exceed the positive criteria
  - CD86 RFI ≥ 150% and/or CD54 RFI ≥ 200%

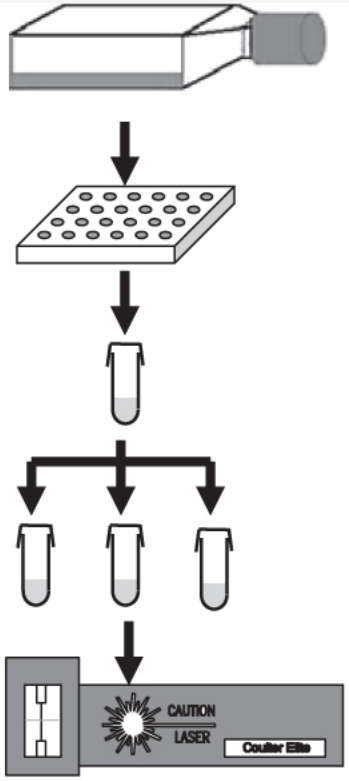
$$\text{RFI} = \frac{\text{MFI of chemical treated cells - MFI of chemical treated isotype control cells}}{\text{MFI of vehicle control cells - MFI of vehicle isotype control cells}} \times 100$$

Abbreviations :

RFI = relative flourescence intensity

MFI = geometric mean fluorescence intensity

Schematic view of the h-CLAT protocol



Pre-culture THP-1 cells for 48-72 hours

Plate (1x10^6 cells/well) in 24-well plate, treat with test chemical for 24 hours

Harvest cells, wash and block FcR (0.01% Globulins) for 15 min.

Divide cells into 3 aliquots, stain with FITC-conjugated monoclonal antibodies (isotype control, CD86, CD54) for 30 min.

Analyze by flow cytometry - mean fluorescence intensity of CD86 and CD54, cell viability by propidium iodide exclusion.

From Aeby P et al. 2010. Toxicol In Vitro 24(6):1465-1473.

- More on h-CLAT from Dr. Hitoshi Sakaguchi, Ph.D., Kao Corporation

Other Nonanimal ACD Test Methods

Undergoing Evaluation/Prevalidation

- KeratinoSens assay
  - Undergoing ring trials in preparation for ECVAM review
  - Test method submission and ECVAM assessment ongoing as of October 2010
  - Based on the Nrf2-Keap1-ARE regulatory pathway
    - Innate cellular response
    - Induced by most sensitizers[1]
  - Uses a novel cell line based on the human HaCaT keratinocyte cell line[2] containing a reporter construct with a single copy of the ARE-element of the human AKR1C2 gene
  - Have tested:
    - List of reference chemicals published by ECVAM
    - List of harmonized ICCVAM performance standards chemicals
    - 67 additional chemicals derived from the ICCVAM database
  - Amenable to high-throughput format

[1]Natsch et al. 2010. Toxicol Sci 113:284-292.
[2]Boukamp et al.1988. J Cell Biol 106:761-771.

Summary

- DPRA, h-CLAT, and MUSST undergoing prevalidation at ECVAM in view of their future incorporation into a testing strategy for fully replacing current regulatory animal tests
- KeratinoSens assay to undergo evaluation by ECVAM
- Numerous assays from research and development designed to evaluate mechanisms of skin sensitization induction
  - SenCeeTox (epidermal Inflammation)
  - VITOSENS® (dendritic cell response)
  - Keratinocyte cultures
  - Keratinocyte and dendritic cell co-culture systems
  - T-cell proliferation assays

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