The Development and Application of Peptide Reactivity Assays for Skin Sensitization Risk Assessment

G. Frank Gerberick, Leslie Foertsch, John Troutman, Petra Kern and Jean-Pierre Lepoittevin

The Procter & Gamble Company
The University of Strasbourg

Presented by: Darrell R. Boverhof
The Dow Chemical Company

January 20, 2011
Predictive Tests for Sensitization

Induction

- Oxidation
- Penetration
- Metabolism/
  - Protein binding
- DC activation

Elicitation

- GP Tests, HRIPT
- LLNA

1° T reaction
The correlation of skin protein reactivity and skin sensitization is well established and has been known for many years.

(Landsteiner and Jacobs, 1936; Dupuis and Benezra, 1982; Lepoittevin et al, 1998) Leads to stable association with proteins, in order that an immunogenic complex is created; this requires that the chemical is inherently protein reactive, or can be transformed in a protein reactive species within the skin.
Readout for Direct Peptide Reactivity Assay (DPRA): Peptide Depletion

Test chemical dissolved in acetonitrile.
Test chemical incubated with peptide (10:1 or 50:1) for 24 hours.
Peptide depletion monitored by HPLC at 220 nm.

![HPLC Graphs]

- **Un-reacted Peptide**
- **Test Chemical**
- **Reaction Mixture**

Minutes

0.00 0.20 0.40 0.60

AU

0.00 0.20 0.40 0.60
Development and Optimization of the DPRA

doi:10.1093/toxsci/kfh213
Advance Access publication July 14, 2004

Development of a Peptide Reactivity Assay for Screening Contact Allergens

G. Frank Gerberick,*1 Jeff D. Vassallo,* Ruth E. Bailey,* Joel G. Chaney,* Steve W. Morrall,* and Jean-Pierre Lepoittevin†

*The Procter & Gamble Company, Miami Valley Laboratories, Cincinnati, Ohio 45253-8707, and †Université Louis Pasteur, Laboratoire de Dermatochimie, UMR 7123, Strasbourg, France

Received April 26, 2004; accepted June 22, 2004

Development and Optimization of the DPRA

- Objective: Determine if chemical reactivity toward nucleophilic amino acids correlates with sensitization potential
  - Examined reactivity of 38 different chemicals with varying degrees of sensitization potency:
    • 11 non sensitizers
    • 7 weak sensitizers
    • 11 moderate sensitizers
    • 5 strong sensitizers
    • 4 extreme sensitizers
  - Evaluated reactivity toward glutathione, or 3 synthetic peptides (cysteine, lysine, histidine)
  - After the chemical:peptide incubation, samples analyzed by HPLC-UV for peptide depletion.
  - Also evaluated parameters such as kinetics and peptide:chemical concentration ratios

Development and Optimization of the DPRA

• Results:
  – Significant correlation was identified between sensitization potency and peptide depletion to glutathione and cysteine and lysine peptides
  – Provided initial evidence for utility of assessing peptide reactivity for assessment of sensitization potential

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Glutathione</th>
<th>Lysine</th>
<th>Cysteine</th>
<th>Histidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>55.6%</td>
<td>53.8%</td>
<td>80.8%</td>
<td>11.5%</td>
</tr>
<tr>
<td>Specificity</td>
<td>90.9%</td>
<td>100.0%</td>
<td>90.9%</td>
<td>100.0%</td>
</tr>
<tr>
<td>Accuracy</td>
<td>65.8%</td>
<td>66.7%</td>
<td>83.8%</td>
<td>36.1%</td>
</tr>
</tbody>
</table>

Development and Optimization of the DPRA

Quantification of Chemical Peptide Reactivity for Screening Contact Allergens: A Classification Tree Model Approach

G. Frank Gerberick,† Jeffrey D. Vassallo,* Leslie M. Foertsch,* Brad B. Price,* Joel G. Chaney,* and Jean-Pierre Lepoittevin†

*The Procter & Gamble Company, Miami Valley Innovation Center, Cincinnati, Ohio 45252; †Laboratoire de Dermatochimie, UMR 7123, Université Louis Pasteur, Strasbourg, France

Received February 6, 2007; accepted March 13, 2007

Development and Optimization of the DPRA

- Test chemical set expanded to 82 (all with existing LLNA data; 38 original plus 44 new)
- 3 Nucleophiles/Peptides: Glutathione, Cysteine and Lysine
- Use two ratios of peptide: test chemical (1:10 and 1:50)
- Reaction time set to 24 hours
- Monitored peptide depletion by HPLC-UV

Results based on Cys 1:10 and Lys 1:50 (n=81)

<table>
<thead>
<tr>
<th>Chemical Classification</th>
<th>Non-Sensitizer</th>
<th>Sensitizer</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Sensitizer</td>
<td>26</td>
<td>3</td>
<td>29</td>
</tr>
<tr>
<td>Sensitizer</td>
<td>6</td>
<td>46</td>
<td>52</td>
</tr>
<tr>
<td>total</td>
<td>32</td>
<td>49</td>
<td>81</td>
</tr>
</tbody>
</table>

**Predicted Classification (based on classification tree model)**

- **Sensitivity**: 88% (46/52)
- **Specificity**: 90% (26/29)
- **Accuracy**: 89% ((26+46)/81)
Use of Classification Tree Approach for Analysis of GSH, Cys and Lys Data

• A form of binary recursive partitioning
• Used when observations need to be assigned to a category based on a number of predictor variables:
  – non-sensitizer, weak, moderate, strong
• Used peptide depletion data and LLNA potency data to generate models
Prediction Model for predicting potency based on Cys 1:10 and Lys 1:50 (n=81)

NS/W/M/S-based on LLNA

Total Sample (29 / 15 / 20 / 17)

Avg Score < 22.62%

Test (29 / 11 / 3 / 0)

Avg Score < 6.376%

Minimal Reactivity (26 / 5 / 1 / 0)

Avg Score > 6.376%

Low Reactivity (3 / 6 / 2 / 0)

Avg Score > 22.62%

Test (0 / 4 / 17 / 17)

Avg Score < 42.47%

Moderate Reactivity (0 / 1 / 6 / 3)

Avg Score > 42.47%

High Reactivity (0 / 3 / 11 / 14)
Additional Analysis of Chemicals in the DPRA

• 76 new test chemicals analyzed with Cysteine and Lysine since the prediction model was developed

• Total compounds tested to date = 157
  – 38 Extreme/Strong
  – 43 Moderate
  – 38 Weak
  – 38 Non-sensitizers

• Accuracy = 85%
Inter-laboratory Studies to evaluate Direct Peptide Reactivity Assay

• We have completed 2 Inter-laboratory studies to evaluate the transferability of the DPRA.
• Scientists from Kao, L’Oreal and Givaudan visited P&G for “hands on” training
• Ring Trial 1 consisted of 15 chemicals with very good results
• Ring Trial 2 consisted of 28 chemicals
• The chemicals of Ring Trial 2 proved to be a bit more challenging but provided us with an opportunity to improve the SOP
• The 2 successful inter-laboratory studies encouraged us to move forward with ECVAM for validation of the assay.
ECVAM Pre-validation of DPRA

- Test Submission to ECVAM – February, 2009
- DPRA SOP finalized – December, 2009
- Participating labs for pre-validation study identified – January, 2010
- Training and Transfer plan approved – February 2010
- ECVAM Pre-validation
  - Phase A, Stage I: SOP training- March 31, 2010
  - Phase A, Stage II: SOP transfer- June 30, 2010
  - Phase B, Stage I: 9 chemicals- July 31, 2010
  - Phase B, Stage II: 15 chemicals- September 15, 2010
  - Data analysis (ECVAM biostatistician)- March 31, 2011
  - Final Pre-validation Report- May 31, 2011
Limitation of the DPRA is that it cannot readily measure the reactivity of pro-hapten chemical sensitizers. Pro-haptens are chemical sensitizers that are not directly reactive and must first be bio-activated in vivo to become reactive.
**Objective:** Develop a modified version of the DPRA to incorporate an activation step for identifying pro-hapten chemical sensitizers.

**Principle of the Assay**

- **Test Chemical**: Oxidant
- **Fenton Chemistry**: $\text{Fe}^{2+} + \text{H}_2\text{O}_2$
- **Blocked by desferoxamine**
- **Auto-oxidation**
- **Peroxidase(O)**
- **Peroxidase(R)**
- **Reactive Metabolite (electrophilic hapten)**
- **Readout**: Non-adducted peptide monomer measured by LC/MS/MS
- **Peptide chemical ADDUCT**: Not quantified but can be observed using MALDI
- **Peptide Dimer**: Monitored but not quantified vs monomer
- **reversed by DTT**
Optimization of Assay Conditions with Cysteine Peptide

- Peroxide concentration
- Peroxidase concentration
- Incubation time

- **Test Chemicals:**
  - 2-Aminophenol
  - Eugenol
  - 1,4-Phenylenediamine
  - 2-Methoxy-4-methylphenol
  - 3-Methylcatechol
Reactivity Screen with Cysteine under optimized Conditions

- (HRP/P (Direct)
+ HRP/P

Non-sensitizers

Pro-haptens

[Graph showing reactivity screen results for various compounds]
Reactivity Screen with Cysteine under optimized Conditions

Investigation of Peptide Reactivity of Pro-hapten Skin Sensitizers Using a Peroxidase-Peroxide Oxidation System

G. Frank Gerberick,*† John A. Troutman,* Leslie M. Foertsch,* Jeffrey D. Vassallo,* Mike Quijano,† Roy L. M. Dobson,† Carsten Goebel,‡ and Jean-Pierre Lepoittevin§

*Central Product Safety, Miami Valley Innovation Center, The Procter & Gamble Company, Cincinnati, Ohio 45253; †Analytical Global Capability Organization, Mason Business Center, The Procter & Gamble Company, Cincinnati, Ohio 45040; ‡Central Product Safety, Darmstadt Innovation Center, The Procter & Gamble Service GmbH, 64283 Darmstadt, Germany; and §University of Strasbourg, Laboratoire de Dermatochimie, UMR 7177 Strasbourg, France

Received June 25, 2009; accepted August 4, 2009

- **Peptide Reactivity Summary:**
- Depletion was generally < 10% for non-sensitizers with or without HRP
- Prohapten sensitizers showed minimal to no peptide depletion in the absence of HRP/P
- Addition of HRP/P resulted in statistically significant increases in peptide depletion for all pro-haptens
Current Process being considered for RA

Characterize peptide depletion as a function of test chemical concentration with cysteine and lysine ± HRP/P

Quantify reactivity by estimating the concentration of test chemical that depletes the peptide by 50% (RC50) within 24 hrs

Correlate with additional endpoints using an integrated testing strategy for predicting skin sensitization potential

<table>
<thead>
<tr>
<th>Peptide</th>
<th>RC50 (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteine</td>
<td>0.0166, 0.0101</td>
</tr>
<tr>
<td>Lysine</td>
<td>4.68, 2.94</td>
</tr>
</tbody>
</table>
### Preliminary Results with Cysteine and Lysine + HRP/P with Dose-Response

<table>
<thead>
<tr>
<th>Test Chemical</th>
<th>Conc range examined (mM)</th>
<th>Cysteine Peptide</th>
<th>Lysine Peptide</th>
<th>Lowest RC50 Observed (mM)</th>
<th>Corresponding Nucleophile</th>
<th>LLNA Potency Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>0.003-30</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>minimal reactivity (&lt;10%)</td>
</tr>
<tr>
<td>Hexane</td>
<td>0.003-30</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>minimal reactivity (&lt;10%)</td>
</tr>
<tr>
<td>1-Butanol</td>
<td>0.003-30</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>minimal reactivity (&lt;10%)</td>
</tr>
<tr>
<td>(+/-) Lactic acid</td>
<td>0.003-30</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>minimal reactivity (&lt;10%)</td>
</tr>
<tr>
<td>Methyl salicylate</td>
<td>0.003-30</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>minimal reactivity (&lt;10%)</td>
</tr>
<tr>
<td>Geraniol</td>
<td>0.003-30</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>minimal reactivity (&lt;10%)</td>
</tr>
<tr>
<td>3,4-Dihydrocoumarin</td>
<td>0.003-30</td>
<td>NC</td>
<td>NC</td>
<td>4.2</td>
<td>NC</td>
<td>Lysine (Direct)</td>
</tr>
<tr>
<td>2-Phenylnitroaldehyde</td>
<td>0.003-30</td>
<td>NC</td>
<td>NR</td>
<td>85.1</td>
<td>85.1</td>
<td>Lysine + HRP/P</td>
</tr>
<tr>
<td>Cinnamic alcohol</td>
<td>0.003-30</td>
<td>NC</td>
<td>37</td>
<td>NC</td>
<td>37</td>
<td>Cysteine (Direct)</td>
</tr>
<tr>
<td>Isopropylol</td>
<td>0.003-30</td>
<td>33.2</td>
<td>NC</td>
<td>NC</td>
<td>33.2</td>
<td>Cysteine (Direct)</td>
</tr>
<tr>
<td>Phenylacetaldehyde</td>
<td>0.003-30</td>
<td>NC</td>
<td>28.4</td>
<td>NC</td>
<td>28.4</td>
<td>Cysteine (+ HRP/P)</td>
</tr>
<tr>
<td>Hydroxyacetone</td>
<td>0.003-30</td>
<td>NC</td>
<td>NC</td>
<td>17.2</td>
<td>17.2</td>
<td>Lysine (Direct)</td>
</tr>
<tr>
<td>2,3-Butanediol</td>
<td>0.003-30</td>
<td>11.7</td>
<td>NC</td>
<td>NR</td>
<td>11.7</td>
<td>Cysteine (Direct)</td>
</tr>
<tr>
<td>1,2-Dibromo-2,4-dicyanobutane</td>
<td>0.003-30</td>
<td>11.0</td>
<td>13.7</td>
<td>NC</td>
<td>11.0</td>
<td>Cysteine (Direct)</td>
</tr>
<tr>
<td>Aniline</td>
<td>0.003-30</td>
<td>NC</td>
<td>10.6</td>
<td>NC</td>
<td>10.6</td>
<td>Cysteine (+ HRP/P)</td>
</tr>
<tr>
<td>Cinnamaldehyde</td>
<td>0.003-10</td>
<td>5.1</td>
<td>17</td>
<td>NC</td>
<td>5.1</td>
<td>Cysteine (Direct)</td>
</tr>
<tr>
<td>Cinnamaldehyde</td>
<td>0.003-30</td>
<td>4.3</td>
<td>16</td>
<td>NC</td>
<td>4.3</td>
<td>Cysteine (Direct)</td>
</tr>
<tr>
<td>Glyoxal</td>
<td>0.003-10</td>
<td>0.887</td>
<td>6.80</td>
<td>14.4</td>
<td>17.9</td>
<td>Cysteine (Direct)</td>
</tr>
<tr>
<td>Glutaraldehyde</td>
<td>0.003-30</td>
<td>0.753</td>
<td>9.26</td>
<td>3.74</td>
<td>3.15</td>
<td>Cysteine (Direct)</td>
</tr>
<tr>
<td>1-Chloro-2,4-dinitrobenzene</td>
<td>0.003-30</td>
<td>0.41</td>
<td>31.2</td>
<td>NC</td>
<td>0.41</td>
<td>Cysteine (Direct)</td>
</tr>
<tr>
<td>Diethyl maleate</td>
<td>0.003-30</td>
<td>0.409</td>
<td>NC</td>
<td>NC</td>
<td>0.409</td>
<td>Cysteine (Direct)</td>
</tr>
<tr>
<td>Hydroquinone</td>
<td>0.003-30</td>
<td>3.45</td>
<td>0.307</td>
<td>2.64</td>
<td>0.613</td>
<td>Cysteine (+ HRP/P)</td>
</tr>
<tr>
<td>p-Benzquinone</td>
<td>0.003-30</td>
<td>0.282</td>
<td>0.578</td>
<td>2.5</td>
<td>0.282</td>
<td>Cysteine (Direct)</td>
</tr>
<tr>
<td>1-Naphthol</td>
<td>0.003-30</td>
<td>NR</td>
<td>0.187</td>
<td>22.5</td>
<td>0.187</td>
<td>Cysteine (+ HRP/P)</td>
</tr>
<tr>
<td>4-Amino-o-toluidine</td>
<td>0.0001-1.0</td>
<td>0.137</td>
<td>0.420</td>
<td>NC</td>
<td>0.137</td>
<td>Cysteine (Direct)</td>
</tr>
<tr>
<td>Eugenol</td>
<td>0.0001-1.0</td>
<td>NC</td>
<td>0.0813</td>
<td>NC</td>
<td>0.0813</td>
<td>Cysteine (+ HRP/P)</td>
</tr>
<tr>
<td>Isoeugenol</td>
<td>0.0001-1.0</td>
<td>0.0676</td>
<td>0.0475</td>
<td>NC</td>
<td>0.0475</td>
<td>Cysteine (+ HRP/P)</td>
</tr>
<tr>
<td>1,4-Phenylenediamine</td>
<td>0.0003-2.0</td>
<td>0.0394</td>
<td>0.0165</td>
<td>NC</td>
<td>0.0165</td>
<td>Cysteine (+ HRP/P)</td>
</tr>
<tr>
<td>3-Methoxyestradiol</td>
<td>0.003-30</td>
<td>0.0186</td>
<td>0.0101</td>
<td>4.68</td>
<td>2.94</td>
<td>Cysteine (+ HRP/P)</td>
</tr>
<tr>
<td>3-Methyloestradiol</td>
<td>0.003-30</td>
<td>0.0168</td>
<td>0.00948</td>
<td>6.89</td>
<td>3.24</td>
<td>Cysteine (+ HRP/P)</td>
</tr>
</tbody>
</table>

*RC50 values were estimated using RExcel which fits a two-parameter log-logistic model to peptide reactivity data, and graphs the raw data and fitted curves.
NC = not calculated (peptide depletion did not exceed 10% across concentration range)
NR = not reported (peptide depletion did not exceed 10% for the two highest concentration tested, or depletion was low (< 20%) and did not increase with an increase in test chemical concentration)
ND = not determined (not tested to date)

Rank order from low to high for the most reactive nucleophile
Trends in peptide reactivity appear to coincide well with general trends in LLNA-based potency classifications
Summary

• Gerberick et al. have made significant progress on the development of a non-animal test for the assessment of skin sensitization potential
• Results with the DPRA have shown great promise and have led to wider validation efforts
• Initial results evaluating the addition of HRP/P to the assay system show promise for the identification of pro-haptens
• Initial RD50 potency assessment approach also looks promising