Assessing the sensitization/irritation properties of micro-organisms

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  – MD Gregorio Loprieno operator exposure
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  – Dr. Maristella Rubbiani general toxicity
Alternatives to Animal Testing

• REPLACE
  – Using no animals
    • In vitro micronucleus test (-S9)

• REFINE
  – Obtaining more information and less suffering
    • Local Lympho Node Assay

• REDUCE
  – Less animals
    • Acute Toxicity Limit Test
Gregorio Loprieno

• Degree in Medicine
  – DNA damage of cosmetics
• PhD in Environmental and Food Toxicology in Milan University
  – Hg exposure through fish consumption
• Master in Hygiene and Preventive Medicine
• Now at Public Health Unity c/o ASL2 Lucca, responsible for consumer protection, pesticides, cosmetics and environment-health relationship
Animal use for irritancy/sensitization

- **Council Directive 86/609/EEC** of 24 November 1986, on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes, *foresees that* each Member State should collect, and periodically make publicly available, statistical information on the use of laboratory animals.

- According to the Fifth Report (**COM/2007/675 final**) by the European Commission (EC), published in 2002, 12.1 million animals were used in the European Union (EU) Member States; **56,900 of these animals were used for the assessment of skin sensitisation and irritancy**.
Micro-organism and irritation

• The product must be evaluated with respect to skin and eye irritation since both micro-organisms and their metabolites/toxins, and its degradation products, other ingredients in the product, by-products from the production medium and any other impurities could be possible causes of irritation.

• Respiratory irritation could also arise when liquids are handled which could give rise to aerosols which might contain cells, spores or virus particles that are spread via the air.
Testing Irritancy

• The purpose is to identify substances with skin and/or eye irritation potential (cellular toxicity).

• Information derived from irritancy tests serves to identify possible hazards to a population exposed voluntary or by accident.

• Respiratory irritancy could lead to pulmonary function impairment and chronic respiratory diseases, however it might be difficult to test for.

• Excluding corrosivity, irritancy is an acute and transient phenomena without chronic consequences.
Test Methods

• Animal methods (dermal and eye irritation) are still in use but protocols were revised for reducing numbers and doses.

• Data submitted for Annex I inclusion were developed on animal studies.

• A wide range of *in vitro* or *ex vivo* tests are published and validation programmes are focusing on a relevant test battery for chemicals classes and/or function (micro-organism ?).
Micro-organisms as potential sensitizers

• All micro-organisms should be regarded as potential sensitizers. Until new and reliable methodologies have been developed for assessing sensitisation by micro-organisms, this view should by default be reflected in classification and labelling and in recommendations for the use of personal protection equipment.

• Micro-organisms may cause a kind of allergic response in the bronchi upon repeated inhalation of larger doses. This has to be taken into account in the evaluation as a general risk.
Testing Sensitization

• The purpose is to identify substances with skin sensitization potential (immunologic type IV reaction).

• Determination of the potential to cause or elicit skin sensitization reactions is an important element in evaluating a substance’s toxicity.
  – Dermal sensitization – Contact Dermatitis
  – Respiratory sensitization – Asthma

• Skin and respiratory sensitizations are chronic diseases which leads to inability and function loss in humans.
Test Methods

• Classical methods (GPMT and Buehler) are still in use but now a new testing guidelines is available (LLNA)

• Data submitted for Annex I inclusion are only with classical methods, but often they were contradictory and not revealing the sensitization potential of the products based on micro-organism so a conservative approach must be chosen.
### An Example

<table>
<thead>
<tr>
<th>Method - Year</th>
<th>Tested material</th>
<th>GLP</th>
<th>Result</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buehler</td>
<td>Technical XXX powder</td>
<td>Yes</td>
<td>Positive 5.0%</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Negative 0.5%</td>
<td></td>
</tr>
<tr>
<td>GPMT *</td>
<td>Technical YYY granules</td>
<td>Yes</td>
<td>Negative</td>
<td>Strain not reported</td>
</tr>
<tr>
<td>Human Patch</td>
<td>Technical ZZZ liquid</td>
<td>No</td>
<td>Negative</td>
<td>Different strain Limited study</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Not acceptable</td>
<td></td>
</tr>
</tbody>
</table>

* According to EPA OOPS 870.2600

**Final evaluation : Potential human sensitizer**
(f) Screening tests. The mouse ear swelling test (MEST) (see paragraphs (i)(9), (i)(10), (i)(11), and (i)(12) of this guideline) or the local (auricular) lymph node assay (LLNA) (see paragraphs (i)(13), (i)(14), (i)(15), and (i)(16) of this guideline) in the mouse may be used as screening tests to detect moderate to strong sensitizers. If a positive result is seen in either assay, the test substance may be designated a potential sensitizer, and it may not be necessary to conduct a further test in guinea pigs. If the LLNA or MEST does not indicate sensitization, the test substance should not be designated a nonsensitizer without confirmation in an accepted test using guinea pigs.
Alternatives in Sensitization

• On 24\textsuperscript{th} April 2002, OECD adopted the Guidelines for the testing of Chemicals, number 429 "Skin Sensitization: Local Lymph Node Assay”

• On 1998 EPA included LLNA as a screening test for pesticides guidelines.

• On March 2003 LLNA reached the status of authorized test system in evaluating sensitization potential
### EPA 1998

**Screening tests**
- Local Lymph Node Assay (LLNA)
- Mouse ear swelling test (MEST)

**Authorized protocols**
- Buehler test.
- Guinea-pig maximization test (GPMT)
  - *Open epicutaneous test*
  - *Maurer optimization test*
  - *Split adjuvant technique*
  - *Freund’s complete adjuvant test*
  - *Draize sensitization test*
  - *under justification/reasoning*

### EPA 2003

**Authorized protocols**
- Local Lymph Node Assay (LLNA)
- Buehler test.
- Guinea-pig maximization test (GPMT)
A different strategy

<table>
<thead>
<tr>
<th>If</th>
<th>Then</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUEHLER POS</td>
<td>POS</td>
</tr>
<tr>
<td>GPMT NEG</td>
<td></td>
</tr>
<tr>
<td>&amp; LLNA NEG</td>
<td></td>
</tr>
<tr>
<td>BUEHLER POS</td>
<td>NEG</td>
</tr>
<tr>
<td>GPMT NEG</td>
<td></td>
</tr>
<tr>
<td>&amp; LLNA POS</td>
<td></td>
</tr>
<tr>
<td>BUEHLER POS</td>
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<td></td>
</tr>
<tr>
<td>&amp; LLNA QUE</td>
<td></td>
</tr>
</tbody>
</table>
LLNA - A different approach

• In LLNA the ability of a material to potentially elicit a delayed-type hypersensitivity response is evaluated by the mitotic proliferation of lymphocytes within the draining auricular lymph nodes.

• Measurement of the degree of cell proliferation is quantified by incorporation of $^3$H-thymidine into DNA of replicating lymph node lymphocytes.

• Interpretation is based on a statistically significant difference in the stimulation index (SI) between the test and negative control groups.
## GPMT against LLNA

<table>
<thead>
<tr>
<th></th>
<th>GPMT</th>
<th>LLNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>On-Test Time</td>
<td>28 days</td>
<td>6 days</td>
</tr>
<tr>
<td>Adjuvant</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Dose Response</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Induction Phase I</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Induction Phase II</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Challenge Phase</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Testing Endpoint</td>
<td>Subjective</td>
<td>Quantitative</td>
</tr>
</tbody>
</table>
A better choice?

- LLNA is faster and cheaper than GPMT
- Accuracy and specificity are high (≈ 80%)
- Results are quantitative and ready for potency estimation
- Results are useful for setting up a factual database using QSAR techniques
- LLNA is a step forward from *in vitro* to *in silico* sensitization
However ...

- LLNA is only able to look at the first phase of induction
- LLNA is limited by the nature of the test material
  - Adhesiveness, liquid or powder ...
  - Irritancy, pH ...
- Most appropriate for single chemical, rather than aqueous solutions, extracts, fabrics, mixtures, preparations
- The use of the LLNA for potency categorization of induction of skin sensitization needs to be validated
And also ...

- Products based on Micro organism are composed by:
  - Spores, Toxins, Proteins, fragments
  - Culture media
  - Formulants and co-formulants
  - What else?

- The possibility to use LLNA for micro organism products has to be confirmed
Conclusion

- Irritancy: at the moment is very difficult to elaborate an alternative strategy simply because there is no data reference
- Sensitization: is reasonable to promote the evaluation of LLNA for products based on micro-organism, but the methods must be proved to be useful for micro-organism products.
... So ... substantial impediments which still exist must be faced if we are to meet the sort of deadlines (March 2013) imposed by politicians in Europe for total replacement of in vivo sensitization tests

David A. Basketter 2008