Bayer HealthCare

Neepa Y. Choksi, Ph.D.

Senior Staff Toxicologist

Integrated Laboratory Systems, Inc./Contractor supporting the NTP Interagency Center for the Evaluation of Alternative Toxicological Methods
National Institute of Environmental Health Sciences

MD EC-17, P.O. BOX 12233

Research Triangle Park, NC 27709

Bayer HealthCare AG

PH-PD Toxikologie
Prof. Dr. H.-W. Vohr
D - 42096 Wuppertal
Telefon: 0202 - 36 8811
Telefax: 0202 - 36 4137
E-Mail: hans-werner.vohr@bayerhealthcare.com

6/20/07

**Concerns:**
**Data package 1, submitted to NICEATM and ICCVAM for further evaluation of the LLNA and modifications of it**

In 2001 experts of several institutes (authority, academia, industry) in Europe decided to initiate a catch-up validation of a modification of the standard - radioactive - LLNA as described before by Homey et al. and Vohr et al. [Ref. 1.1 and 1.2.]. From the very beginning the studies were supported by the VCI (Verband der Chemischen Industrie e.V. (German chemical industry federation)).

It was decided to test 3 (first round) and 9 (second round) international standards out of a list of 26 standards under full GLP compliance. The substances should be submitted blinded by an independent coordinator to the participating labs. A well-known expert from the Swiss authority Swissmedic, T. Maurer, accepted to supervise the study, to select the test substances including submission of the test items as well as to organize the data submission to an independent statistician (J. Hüsler, University of Bern, Switzerland).

It was decided to start with a pilot study using HCA as test substance to finally harmonize the protocol used by the participating labs. In addition, a new evaluation scheme was agreed on which takes the assessment of skin reaction due to irritation into account [Ref. 1.3.].

Afterwards a first round with 3 test substances and two strains of mice (BALB/c and NMRI outbred) had been carried out. The test items were not only blinded but also labeled differently for each participating lab for this first part of the study by the coordinator. An intermediate assessment of the still blinded test substances served as a milestone to continue or not, and to

select one of the mouse strains for the second round of the study. Because of extremely good correlation of the data between labs it was decided to continue with another 9 standards in a second round with BALB/c.

All 9 participating labs measured weights and cell counts of the draining lymph nodes, and for acute skin reaction ear weights (8mm punch). Ear thickness was measured in some labs in addition. One lab used radioactive labeling as well, and one lab used NMRI also with all standards.

All raw data were sent to T. Maurer who forwarded these to J. Hüsler for statistical evaluation [Ref.1.4.]. Only after the overall evaluation the codes were de-blinded by T. Maurer.

Evaluation based on cell count indices turned out to be as sensitive as the radioactive method. The cut-off concentrations (EC values) were very similar for both methods (cf. also publications of the catch-up validation).

The additional determination of acute ear (skin) reaction by ear weight/ear thickness turned out to be very useful for further assessment of the lymph node reaction, i.e. to exclude false positive results. Results of this catch-up validation have been published in peer reviewed papers [Ref 1.5. and 1.6.] and at different meetings in poster sessions.

With respect to the cut-off values (EC (Effective Concentration) values) it is obvious that each parameter (end point) requires its own specific cut-off value. This is accepted since decades for example in guinea pig assays: >= 30% positive reactions in M&K tests or >= 15% positive reactions in Bühler tests.

For the radioactive labeling the cut-off value has been fixed to that concentration of test substance that induces a 3 times increase in stimulation index, i.e. the so-called EC3 value. For cell count indices such cut-off values are much lower, for example 1.5 times increase of stimulation index. This is understandable by the facts that cell count indices have i) lower individual variances compared to 3H-Thymidine incorporation, and ii) lower maximum stimulation indices compared to radioactive labeling. For example, a strong sensitizing substance may easily induce indices about 30-50 by 3H-Thymidine incorporation but only indices about 4-5 by cell counting. However, crucial for the assessment are not impressive high stimulation indices, but reliable determination of a safe and accurate cut-off value, so the reasonable and reliable determination of the concentration of a test substance exceeding it. These concentrations exceeding the thresholds can then be compared between methods and modifications, and are indeed comparable as it has been shown by our catch-up validation! In [Ref. 1.7.] the results of EC1.5 values of all participating labs are averaged and the classification range of potency given as calculated in the different labs. Statistically significant increases were taken into account just as all stimulation indices exceeding the cut-off value. i.e. EC1.5, without being of statistical significance.

Interestingly, there was an extremely good correlation between statistically significant increases in stimulation indices and the exceeding of thresholds or cut-off values. Similar finding have already been published by Gerberick et al. in 1992 [Ref. 1.8.] as can be taken from the attached table (statistically significant indices in red):

Table 1 (modified after Gerberick et al., 1992) showing significant stimulation indices of two different endpoints, i.e. cell counting or radioactive labeling obtained with international standards.

| Compound | Cell counts | 3H.Thymidine |
|---|---|---|
| Benzalkonium chloride 0,5% | 2,70 | 9,00 |
| 1% | 4,08 | 11,10 |
| 2% | 2,93 | 7,60 |
| Benzocaine 5% | 1,39 | 1,30 |
| 10% | 0,99 | 1,00 |
| 20% | 1,12 | 1,30 |
| DCNB 0,001% | 0,94 | 0,80 |
| 0,05% | 2,06 | 10,70 |
| 0,10% | 2,83 | 21,10 |
| Ethylendiamine 1% | 1,06 | 1,10 |
| 5% | 1,07 | 1,10 |
| 10% | 1,77 | 2,20 |
| Eugenol 25% | 2,72 | 5,40 |
| 50% | 2,70 | 10,60 |
| 75% | 2,72 | 10,50 |
| Glutaraldehyde 3,1% | 2,54 | 9,80 |
| 6,20% | 4,52 | 21,40 |
| 12,50% | 5,35 | 22,90 |
| MCI/MI 50ppm | 3,04 | 8,10 |
| 500ppm | 5,68 | 27,80 |
| 1000ppm | 4,59 | 48,20 |
| Nickel cloride 2,5% | 0,98 | 1,30 |
| 5% | 1,50 | 2,60 |
| 10% | 1,96 | 6,60 |
| Oxazolone 0,0001% | 0,94 | 1,60 |
| 0,005% | 1,62 | 8,70 |
| 0,05% | 4,52 | 55,20 |
| TNCB 0,01% | 3,02 | 18,00 |
| 0,05% | 6,62 | 80,30 |
| 0,10% | 7,23 | 103,30 |

Beside all references mentioned here in the text two reports with all standards tested in one lab with BALB/c or NMRI (outbred) mice are also included in this package 1. Of course, the test substances are called in both reports A to L, but A to C were differently named in each participating lab.

The actual identity of these standards can be taken from the following Table 2:

**Round I**

| Code | Compound | Proposed classification | Reference |
|---|---|---|---|
| HCA | Hexylcinnamaldehyde | Sensitiser | Dearman 2001 |
| A | p-hydroquinone | Sensitiser | Kimber 1998 |
| B | SDS | Irritant | Basketter 1992 |
| C | 4-aminobenzoic acid | Negative | Basketter 1992 |

**Round II**

| Code | Compound | Proposed classification | Test concentrations | Reference |
|---|---|---|---|---|
| D | Xylene | Irritant | 10, 30, 100% | Kligman 1966 |
| E | Octanoic acid | Weak Irritant | 1, 3, 10% | ECETOC 1995 |
| F | MCI | Sensitiser | 0.03, 0.1, 0.3% | Botham 1991 |
| G | Mercaptobenzothiazole* | Sensitiser | 3, 10, 30% | Scholes 1992 |
| H | Isoeugenol | Sensitiser | 3, 10, 30% | Basketter 1992 |
| I | Potassium dichromate | Sensitiser | 0.3, 1, 3% | Basketter 1992 |
| K | Hydroxycitronellal | Sensitiser | 6, 20, 60% | Basketter 1992<br>Montelius 1994 |
| L | Tween 80 | Irritant | 10, 30, 100% | Magnusson 1969 |

Kind regards,

H.-W. Vohr

References:


1.1. Homey, B, von Schilling, C., Blümel, J., Schuppe, H.-C., Ruzicka, T., Ahr, H.-J., Lehmann, P. and Vohr, H.-W.
An Integrated Model for the Differentiation of Chemical-induced Allergic and Irritant Skin Reactions
Toxicol. Appl. Pharmacol. **153**, 83-94, 1998

1.2. Vohr, H.-W., Blümel, J., Blotz, A., Homey, B. and Ahr, H.J.
An intra-laboratory validation of IMDS: Discrimination Between (Photo)Allergic and (Photo)Irritant Skin Reactions in Mice.
Arch. Toxicol., 73, 501-509, 2000

1.3. Protocol of the kick-off meeting of the European catch-up validation study of the modified LLNA

1.4. First statistical evaluation of the second round (test substances D-L) of the catch-up validation by J. Hüsler, Bern, Switzerland

1.5. Ehling G, Hecht M, Heusener A, Huesler J, Gamer AO, v. Loveren, H., Maurer Th, Riecke K, Ullmann L, Ulrich P, Vandebriel R, Vohr H-W
An European Inter-Laboratory Validation of Alternative Endpoints of the Murine Loacl Lymph Node Assay. First ROUND.
Toxicology, 212, 60-68, 2005

1.6. Ehling G, Hecht M, Heusener A, Huesler J, Gamer AO, v. Loveren, H., Maurer Th, Riecke K, Ullmann L, Ulrich P, Vandebriel R, Vohr H-W
An European Inter-Laboratory Validation of Alternative Endpoints of the Murine Loacl Lymph Node Assay. 2nd ROUND.
Toxicology, 212, 69-79, 2005

1.7. Table (PP) showing the classification of the standards tested in the catch-up validation based on different end points and methods according the ECETOC Technical report No. 87, 2003.

1.8. Gerberick GF, House RV, Fletcher ER, Ryan CA
Examination of the Local Lymph Node Assay for Use in Contact Sensitization Risk Assessment
Fundamental and Applied Toxicology, 19,438-445, 1992