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Evaluation of the performance of the reduced local lymph node assay for skin sensitization testing

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ABSTRACT

The local lymph node assay (LLNA) is the preferred method for classification of sensitizers within REACH. To reduce the number of mice for the identification of sensitizers the reduced LLNA was proposed, which uses only the high dose group of the LLNA. To evaluate the performance of this method for classification, LLNA data from REACH registrations were used and classification based on all dose groups was compared to classification based on the high dose group. We confirmed previous examinations of the reduced LLNA showing that this method is less sensitive compared to the LLNA. The reduced LLNA misclassified 3.3% of the sensitizers identified in the LLNA and misclassification occurred in all potency classes and that there was no clear association with irritant properties. It is therefore not possible to predict beforehand which substances might be misclassified. Another limitation of the reduced LLNA is that skin sensitizing potency cannot be assessed. For these reasons, it is not recommended to use the reduced LLNA as a stand-alone assay for skin sensitization testing within REACH. In the future, the reduced LLNA might be of added value in a weight of evidence approach to confirm negative results obtained with non-animal approaches.

1. Introduction

The assessment of the skin sensitization potential of chemicals is required within the REACH regulation (registration, evaluation, authorisation, and restriction of chemicals). The local lymph node assay (LLNA) is the first method of choice to identify skin sensitizers (EU, 2007). The LLNA is a murine assay that measures cell proliferation in the auricular lymph nodes draining the ear after topical application of the test substance for three consecutive days (Kimber and Weisenberger, 1989; Kimber and Dearman, 1991; Ryan et al., 2005). After extensive evaluation, this method was in 2002 adopted as an OECD test guideline (TG 429) and was revised recently (OECD, 2010). In the LLNA, three dose groups are included and substances are classified as sensitizers when the proliferation in one of these dose groups is threefold higher compared to vehicle controls (stimulation index (SI) $\geqslant 3$). For borderline results, the strength and statistical significance of the dose–response can be used for the decision-making. The dose–response information is also used to estimate the potency, which is the interpolated or extrapolated concentration at which the SI = 3 is induced. This EC3 value correlates well with human potency (Basketter et al., 2005).

From an animal welfare perspective, the LLNA is considered as a refinement and reduction method compared to the guinea pig methods in described in OECD TG 406 (guinea pig maximization test and Buehler test) (OECD, 1992). The LLNA is a refinement since the challenge dose that is used in the guinea pig methods is not required thereby reducing animal discomfort and stress. Compared to the Buehler test, the LLNA is a reduction method, as a minimum of 16 mice is recommended in the LLNA compared to a minimum of 30 guinea pigs in the Buehler test (OECD, 1992). Despite this, skin sensitization testing using the LLNA still requires a large amount of animals for REACH purposes, since this endpoint has to be assessed at the lowest tonnage threshold. It is estimated that skin sensitization testing will require 8–10% of the total number of animals needed for REACH. This adds up to approximately 384,000–553,000 mice needed for skin sensitization testing within REACH (Van der Jagt et al., 2004).

To further decrease the number of animals required for this endpoint the reduced LLNA was proposed, in which only one exposure group (the highest dose) is included, rather than three (Kimber et al., 2006; Ryan et al., 2005). The reduced LLNA was included in OECD TG 429 recently besides the traditional LLNA (OECD, 2010). An important drawback of the reduced LLNA is that it does not provide information on dose–response relationships, hence on potency. Another drawback of the reduced LLNA is that certain substances induce dermal toxicity when high exposure concentrations are used. This can lead to impairment of the immune response and reduced proliferation in the draining lymph nodes even if the substance has sensitizing activity. Substances that induce such a bell-shaped curve are misclassified in the

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reduced LLNA when the SI value in the high dose group is below three, while the SI values in the low or mid dose groups are equal to or higher than three.

A retrospective evaluation of published LLNA data showed that 1.9% of the substances with a positive LLNA result were misclassified in the reduced LLNA (Kimber et al., 2006; ICCVAM, 2009). A more recent evaluation, using the New Chemicals Database (NCD) of the EU Joint Research Centre showed that 4.9% of the substances would have been misclassified when the reduced LLNA was used instead of the LLNA (Angers-Loustau et al., 2011). To confirm these previous examinations and to assess whether the reduced LLNA can replace the LLNA for skin sensitization testing under REACH, the performance of this assay for classification was evaluated using LLNA data from registrations submitted to the European Chemicals Agency (ECHA) for REACH.

2. Methods

2.1. Data base mining

To obtain LLNA data for substances registered for REACH, the OECD eChemportal (http://www.echemportal.org) was used. This is a free public database that contains information on substance properties and direct links to collections of information prepared for government chemical review programmes at national, regional, and international levels. This portal provides access to information and classification results according to the globally harmonized system of classification and labelling of chemicals (GHS) of industrial substances, pesticides and biocides.

A search was conducted within eChemportal focused on substances for which skin sensitization data were available. The search was refined by only selecting data based on the following criteria:

- Study result type: "experimental result",
- Reliability: "1" (reliable with no restrictions)
- Type of method: "in vivo"
- Type of study: "mouse local lymph node assay (LLNA)"
- Test guideline, qualifier: "according to"
- Test guideline, guideline: "OECD guideline 429 (LLNA)".

2.2. Data extraction

The database was evaluated in September 2012 and at that time the search resulted in information of 835 substances all tested for REACH. Information of the individual substances was converted to a database in Excel. This database was screened for duplicates based on CAS number and 17 duplicates were removed. After careful screening of all available data, registrations that did not provide LLNA data for the separate dose groups were excluded as well. A total of 34 substances were discarded for lacking dose–response information. Additionally, substances that were tested in modifications of the LLNA were discarded. Twelve substances were tested in the BrdU-ELISA LLNA (OECD TG442B). The reason to exclude these modified LLNA approaches is that they use a different read-out and do not have a single cut-off value. Furthermore, the results obtained in the three dose groups were not provided either. Twenty-four substances were tested in a modification of the LLNA using cell number and ear thickness as a read-out. This method has not been accepted as an OECD guideline and substances were excluded from further analysis.

For each of the remaining 748 substances LLNA data in the registrations were analyzed carefully. For all substances classified as sensitizers, SI values were added to the Excel database. This Excel database is available as Supplementary data and offers a direct link to the eChemportal database for all substances as well. The predictive value of the reduced LLNA was assessed by comparing classification based on the high dose group to classification when all three dose groups were taken into account. In both instances, the cut-off value of SI $\geqslant 3$ was used to classify skin sensitizers.

3. Results and discussion

3.1. Classification of substances using the traditional and reduced LLNA

The performance of the LLNA was evaluated using a database that contained information of 748 unique substances that were tested according OECD guideline TG 429. Table 1 show that according to the traditional LLNA 29.8% of the substances was classified as a sensitizer. The reduced LLNA would classify 28.9% of the substances.

3.2. Performance of the reduced LLNA using different databases

The analysis of the REACH registration database showed that the accuracy and sensitivity of the reduced LLNA were slightly lower compared to the traditional LLNA, 99.1% and 96.7%, respectively (Table 2). The specificity was equal in these methods, since a substance that is negative in the LLNA will be negative in the reduced LLNA as well. Seven substances were misclassified as non-sensitizers in the reduced LLNA compared to the traditional LLNA, resulting in a false-negative rate of 3.3%. This is slightly lower than the percentage reported by Angers-Loustau et al. (2011), who found a percentage of 4.9% of misclassified substances. The studies that have done a retrospective analysis (Kimber et al., 2006; ICCVAM, 2009) report lower false-negative rates of 1.9%, which might be explained by a lower number of substances evaluated and differences in substances present in the database (Table 2).

3.3. Misclassified substances: dose–response curves and EC3 values

The misclassified substances were analyzed further, using the information from the REACH registrations. The chemical structures of the substances are shown in Fig. 1, with the exception of fluoronicomethanol. The reason for this exclusion is that there is ambiguity as to the structure of this substance, because its CAS number brings up to different compounds. For each substance a dose response curve was generated (Fig. 2). To find additional information that could explain misclassification, the registrations were further analysed with a focus on potency of the sensitizers, irritating properties (Table 3) and information on acute toxicity (weight loss, lethality or other signs of toxicity).

In the LLNA, potency is calculated by determining the concentration at which the SI value of 3 is induced, this value is called the EC3. These values are used to subcategories chemicals according to their potency. Basketter et al. (2005) proposed three categories: extreme (EC3 $\leqslant 0.2$), strong (EC3 >0.2 and $\leqslant 2$) and moderate sensitizers (EC3 >2).

The potency values, expressed as the LLNA EC3 values, are shown in Table 3. For o-anisidine and $2MeO_3$-Im4KA2 the EC3 val-

Table 1
Classification of substances based on the LLNA or the reduced LLNA.

LLNA[§]	Total	Not classified	Classified
Traditional	748	535 (71.5%)	223 (29.8%)
Reduced	748	542 (72.4%)	216 (28.9%)

[§] Substances were classified as sensitizers when the SI value was equal to or higher than 3 in one of the dose groups for the traditional LLNA or in the high dose group for the reduced LLNA. The table shows absolute and relative (in brackets) numbers of substances that were either not classified or classified as sensitizers.