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The regulatory use of the Local Lymph Node Assay for the notification of new chemicals in Europe

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ABSTRACT

The regulatory use of the Local Lymph Node Assay (LLNA) for new chemicals registration was monitored by screening the New Chemicals Database (NCD), which was managed by the former European Chemicals Bureau (ECB) at the European Commission Joint Research Centre (JRC). The NCD centralised information for chemicals notified after 1981, where toxicological information has been generated predominantly according to approved test methods. The database was searched to extract notifications for which the information for skin sensitisation labelling was based on results derived with the LLNA. The details of these records were extracted and pooled, and evaluated with regard to the extent of use of the LLNA over time, as well as for analysing the information retrieved on critical aspects of the procedure e.g. strain and amount of animals used, lymph node processing, solvent and doses selected, stimulation indices, and for assessing their level of compliance to the OECD Test Guideline 429. In addition the accuracy of the reduced LLNA when applied to new chemicals was investigated.

1. Introduction

The assessment of the skin sensitising potential of chemicals, particularly for new chemicals for which read-across information is limited, currently relies on the use of animals. Besides the conventional guinea pig tests, such as the Buhler Test (Buehler, 1965) and the Magnusson Kligman Guinea-pig Maximisation Test (Magnusson and Kligman, 1970), the mouse Local Lymph Node Assay (LLNA[1]) represents a refinement/reduction method. It measures the induction of sensitisation as a function of lymphocyte proliferation in the lymph nodes draining the site of topical application of the test substance. Chemicals are classified as contact allergens if they elicit, at one or more test concentrations, a threefold or greater increase in draining lymph node cells proliferation compared with concurrent vehicle controls (a stimulation index (SI) of 3 or more). Compared to the guinea pig tests, it eliminates the need of eliciting the immune response with a challenge dose and thus reduces animal discomfort and stress. It also generally requires fewer animals to be sacrificed for testing the chemicals (for complete details, see Organisation for Economic Cooperation and Development (OECD) Test Guideline (TG) 429 (2010)).

Following its development and optimisation, the LLNA was subjected to an extensive evaluation and was adopted in 1992 as a screening test in OECD TG 406, implying that positive responses could be considered whereas negative responses would require a confirmation in guinea pig tests (OECD, 1992).

Subsequently to the peer review conducted by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM; NIH, 1999) and endorsed by the European Centre for the Validation of Alternative Methods (ECVAM) Scientific Advisory Committee (Balls and Hellsten, 2000), in 2002 the LLNA was adopted as a stand alone method by the OECD as TG 429, which was reviewed and updated in 2010 (OECD, 2010).

Besides the issues of animal welfare described above, the LLNA has additional advantages over guinea pigs methods, such as its recognised capacity to provide predictive identification of the relative potency of skin sensitising chemicals (Loveless et al., 2010; Basketter et al., 2005; van Loveren et al., 2008). This is achieved by estimating, by linear inter- or extrapolation of the SIs for each tested dose, an EC3 value. The EC3 value describes the concentration of chemical necessary to elicit a SI of 3. The greater the potency of a skin sensitising chemical, the lower the EC3 value will be. Additionally, since it does not rely on visual observation of an allergic response, the LLNA is better suited for the testing of coloured chemicals (see, for example, Ahuja et al., 2010).

Recently a reduced version of the LLNA (reduced LLNA) which makes use of the highest dose group only to discriminate between sensitising and non-sensitising chemicals has been proposed and adopted in the revised TG 429 (OECD, 2010). The reduced LLNA

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[1] *Abbreviations used*: LLNA, Local Lymph Node Assay; rLLNA, reduced Local Lymph Node Assay; TG, Test Guideline; NCD, New Chemicals Database; NC, not classified; SI, stimulation index; HCA, hexyl cinnamic aldehyde.

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further reduces the number of animals needed with respect to the standard test and can be used in those situations where potency information is not needed.

The objective of the current study was to monitor the regulatory use of the LLNA for new chemicals registration. For this, we screened the New Chemicals Database (NCD) which was managed by the former European Chemicals Bureau (ECB) at the European Commission Joint Research Centre (JRC). The mission of the ECB included the coordination of scientific and technical work of the European Union notification scheme and risk assessment for new chemical substances (Directive 67/548/EEC including Annexes VII and VIII, Directive 93/67/EEC) notified after 1981. This scheme was revoked on the 1st of June 2008, and was replaced by the Regulation (EC) No 1907/2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH, OJ L396, 30.12.2006), which is currently managed by the European Chemicals Agency (ECHA) in Helsinki.

The database was searched to extract notifications for which the information for skin sensitisation labelling was based on results derived with the LLNA. The details of these records were compiled in order to evaluate the extent of use of the LLNA over time, and to analyse information on the procedure used in terms of strain and amount of animals used, solvent and doses selected, SIs, and other critical aspects discussed in OECD TG 429. Additionally information on labelling was used to evaluate the accuracy of the reduced LLNA when applied to new chemicals.

2. Methods

2.1. Database mining and data extraction

At the time of this evaluation there were 5288 individual substances registered in the NCD[2], from which 3792 reported having been tested for skin sensitisation hazard assessment. The Structured Notification Interchange Format (SNIF) of the notification scheme did not provide a layout tailored for LLNA results, section 4.1.70 (“skin sensitisation”) being designed for Buehler or GPMT tests. Participants were instructed to insert the results in free text either in section 4.1.70 or in section 4.7.0 (“additional toxicology tests”), using fields like “Comments” to include relevant additional information. Therefore, the 3792 notifications were further investigated with respect to the *in vivo* test used using free-text search of the terms “Local Lymph Node Assay”, “LLNA”, “406”, “429” and “mouse”.

From this search, notifications were found between 1995 and 2008 for which skin sensitisation labelling (NC or R43) was based on results generated with the LLNA. When multiple identical notifications were found for the same chemical, from different countries, only the leader file notification was kept resulting in a total number of 680 notifications. Eight of the notifications did not report the conclusion from the test. For four of these entries, SIs were reported, and we classified these chemicals according to the instructions of OECD TG 429. The other four entries were eliminated from our database. Similarly, 3 notifications reported multiple and conflicting LLNA results, with no indication of what the final conclusion was for these chemicals, and these entries were also removed. The final database then contained 673 notifications.

Each of the 673 notification was then examined individually, and information extracted and copied in an Excel worksheet to allow convenient manipulations and analyses. The information extracted consisted of:

1. Year of notification.
2. Member state from which the notification was filed.
3. The conclusion (Not Classified (NC) or Sensitiser (Xi)).
4. Strain used.
5. Animals in control group(s).
6. Animals in test group(s).
7. Vehicle used.
8. Concentrations used.
9. Stimulation indices obtained.
10. EC3 value, if applicable.
11. Method used.
12. Positive control used.
13. Positive control concentration(s).
14. Positive control stimulation index/indices.
15. Positive control EC3 value.
16. Number of animals in the positive control group(s).
17. Vehicle used for the positive control.

Not all the notifications contained all this information, in particular for points 4–17. When information was missing, the corresponding cells were left blank for this entry.

Since the information stored in the NCD is confidential, the identities/chemical structures of the substances were not extracted nor included in the present analyses and discussions. Any consideration regarding the applicability domain of the LLNA for new chemicals was therefore beyond the scope of this exercise. Additionally, taking into account the chemical classes in our analyses would have been difficult, since the NCD contains chemicals which frequently present complex multi-functional molecular structures (Eskes et al., 2007).

2.2. EC3 values

EC3 values which were not reported by the submitters were calculated when feasible, following the instructions from Gerberick et al. (2007).

Briefly, the EC3 value was calculated by linear interpolation if the dose data contained a point below (coordinates (a,b)) and a point above (coordinates (c,d)) the SI value of 3, with the formula

$$EC3 = c + \left[\frac{(3-d)}{(b-d)}\right](a-c)$$

When the dose response did not contain data points with SI values below 3, the two doses closest to the SI value of 3 were used to evaluate EC3 by log linear extrapolation, with the formula

$$EC3 = 2^{\left(\log 2(c)+\frac{(3-d)}{(b-d)}*(\log 2(a)-\log 2(c))\right)}$$

3. Results and discussion

3.1. Information found in the notifications

Of the 3792 notifications including skin sensitisation hazard assessment in the NCD of the European Chemicals Bureau in 2008, only 680 contained LLNA results. This is explained by the common utilisation of the guinea pigs test methods, in particular in the period before the LLNA was developed and included as a stand-alone method in the OECD guideline programme.

The information we have selected to extract from the notifications is described in the Methods section. Very few of the reports contained all this information; in those cases, the missing fields were left blank. Seven notifications, from which a final classification could not be derived, were eliminated from the database for the rest of the analyses.

One would assume that the minimum information required to properly analyse the results of a LLNA would include the vehicle

[2] http://ecb.jrc.ec.europa.eu/new-chemicals/