Regulatory Toxicology and Pharmacology 52 (2008) 290–298

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

Local lymph node assay (LLNA): Comparison of different protocols by testing skin-sensitizing epoxy resin system components

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ARTICLE INFO

Article history:
Received 13 May 2008
Available online 13 September 2008

Keywords:
Local lymph node assay variants
Quantification of lymph node stimulation
Vehicle effect
Potency ranking of sensitizers
Epoxy resin system components

ABSTRACT

Thirteen epoxy resin system components were tested in the LLNA with regard to their sensitizing potency. Lymph node stimulation was quantified not only by measuring the incorporation of [^{3}H]-thymidine into the ear lymph nodes but also the counts of cells recovered from these organs. Equivalent figures were obtained with both endpoints used for the evaluation of lymph node cell proliferation if the reference stimulation indices were adjusted. When dissolved in acetone, all test substances showed skin-sensitizing potential, mainly on the boundary between "strong" and "moderate" according to common potency evaluation schemes. Replacing acetone with acetone/olive oil (4:1) as a vehicle for four selected test items, resulted in considerably lower estimated concentrations for sensitization induction. The challenges in comparing the results obtained by different LLNA variations are discussed.

1. Introduction

The principle of the murine local lymph node assay (LLNA), which detects the sensitizing properties of test substances by measuring lymphocyte proliferation in the auricular lymph nodes, was published in 1989 (Kimber and Weisenberger, 1989). A report on the first collaborative validation study was released in 1991 (Basketter et al., 1991). In these initial studies the stimulation of the lymph nodes, i.e. cell proliferation, was measured by [^{3}H]-thymidine incorporation. In 1999 the LLNA was accepted by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM, 1999; Dean et al., 2001) and in Europe by the European Centre for the Validation of Alternative Methods (ECVAM; Balls and Hellsten, 2000) as a valid alternative to guinea pig assays, although the need for further modifications was also noted.

The method had already been incorporated into Skin Sensitisation Guideline No. 406 of the OECD (1992) and by the EPA in 1998 (OPPTS 870.2600, Skin Sensitization). However, it took another 10 years until the LLNA was accepted as a stand-alone test by the OECD Test Guideline 429 (OECD, 2002), and a revised EPA guideline was published in 2003 (OPPTS 870.2600, Skin Sensitization: EPA 712-C-03-197, March 2003).

So far, some of the recommendations contained in the two guidelines or the ICCVAM report have been implemented rather inconsistently by different researchers, but have become increasingly prominent as a result of growing experience (Basketter et al., 1994; Loveless et al., 1996; Montelius et al., 1998).

One of these points is the concern about a possible confounding impact of irritant properties of test materials or other non-specific activation of immune competent cells which may cause non-specific cell proliferation in the draining lymph node and thus – in the worst case – lead to false positive or "unexpected" results (Montelius et al., 1994, 1998; Basketter et al., 1994, 1998; Loveless et al., 1996; Vohr et al., 2000; Vohr and Ahr, 2005; Ulrich et al., 2001a; Kreiling et al., 2008). Limitations of all three tests available in regulatory toxicology for predicting sensitizing properties of compounds, i.e. the guinea-pig maximization test, the occluded patch test of Buehler and the LLNA, were reviewed by Basketter and Kimber (2007) in large part.

A number of new endpoints and modifications of the LLNA have been suggested as a means of discriminating effectively between the irritant and sensitizing properties of test items (Homey et al., 1998; Ulrich et al., 2001a; Suda et al., 2001; Takeyoshi et al., 2001; Yamashita et al., 2005; Gerberick et al., 1996, 2002). In this context, several authors have introduced a flow cytometric evaluation of activated cells in the ear draining lymph nodes (Takeyoshi et al., 2001; Yamashita et al., 2005; Gerberick et al., 1996, 2002).

Another important matter is the demand for the avoidance of aqueous formulations. They should not be used in the LLNA because they drip off the ears immediately after application. OECD Guideline 429 recommends, in order of preference, acetone/olive oil (4:1 v/v, "AOO"), dimethyl formamide, methyl ethyl ketone,

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doi:10.1016/j.yrtph.2008.08.018

propyleneglycol and dimethyl sulfoxide as suitable vehicles, adding that "others may be used if sufficient scientific rationale is provided". An IPCS/WHO workshop concluded that the LLNA had never been validated for the use of aqueous formulations or mixtures, and the recommendation was to have the existing data re-evaluated in this respect by ICCVAM (van Loveren et al., 2008).

Last but not least, the standard Local Lymph Node Assay has been challenged for the *in vivo* use of radioactive labeling of the draining lymph nodes. Many laboratories wanted to circumvent this cost-intensive method, which may also show high individual variations of [^{3}H]-thymidine incorporation into the lymph node cells. On the other hand modifications of the LLNA described by different authors, which avoid the use of radioactive *in vivo* labeling, have been criticized by others as being not sensitive enough (Gerberick et al., 1992; van Och et al., 2000; Piccotti et al., 2006). However, it is obvious that for endpoints other than thymidine incorporation, their individual specific cut-off value or threshold (SI or EC value) has also to be determined based on the variance and the maximum stimulation index achievable with the method used (Vohr et al., 2000; Suda et al., 2001; Ehling et al., 2005a,b; Sakaguchi et al., in press).

Due to their importance in occupational settings, the Institute for Occupational Safety and Health of the German Social Accident Insurance (BGIA) decided to investigate a set of epoxy resin system components by means of LLNA under GLP conditions, with the aim of ranking these substances according to their sensitizing potency. Epoxy resins are used for a variety of purposes, e.g. in the construction, metalworking, turbine, automotive and paint industries. Although the hardened, finished polymers are considered to be virtually inert, the handling of uncured resin system components is causing a growing number of cases of allergic contact eczema (Geier et al., 2003, 2004; Tavakoli, 2003). Epoxy resin systems are made up of an epoxy resin and a (mostly aminic) curing agent or hardener. In order to process epoxy resins of high viscosity, so-called reactive thinners are added, such as monoglycidyl ethers of phenols or glycidyl ethers based on mono- or difunctional aliphatic or cycloaliphatic alcohols. Unfortunately, the resins themselves as well as reactive thinners and curing components have a sensitizing effect. Hence, there is a need to substitute highly sensitizing epoxy resin system components with substances with lower sensitizing potency (Kalberlah, 2007).

The European project "EPOXYCODE" (Terwoert and Spee, 2005) has suggested a ranking based predominantly on physico-chemical data and qualitative hazard categories. This approach necessarily neglects some important physiological principles. Studies of exposed workers have revealed that different components show different incidences of allergic contact dermatitis (e.g. Geier et al., 2004). However, they are not able to determine whether these differences are based on the varying sensitizing potency of the components or are affected by exposure.

BGIA has urged the performing laboratories to employ different LLNA protocol variations when testing epoxy resin system components. Here we are presenting the main results of this project with a focus on the comparison of radioactive vs. non-radioactive methods. But we will also address the matter of the incorporation of skin irritation in the evaluation of the data. In addition, the influence of the vehicle on the results has been studied for selected test compounds.

2. Material and methods

2.1. Animals

CBA/CaOlaHsd female mice were used throughout all studies described herein. Six to ten weeks old animals were purchased from Harlan Winkelmann GmbH, Borchen, Germany. The individually housed animals were identified by cage cards.

The animals were housed in Makrolon type I cages in fully air-conditioned rooms in which a central air-conditioning system ensured a temperature in the range of 20–24 °C and a relative humidity in the range of 30–70%. The illumination period was as follows: 12 h light (6.00 a.m.–6.00 p.m.) and 12 h darkness (6.00 p.m.–6.00 a.m.). Tap water and diet was provided *ad libitum*. The experiments were conducted in accordance with German animal welfare legislation in an AAALAC certified laboratory.

2.2. Chemicals

Acetone *pro analysi* from Merck or Riedel de Haen, Germany was used as vehicle. Methyl-[^{3}H]–thymidine TRA 120 was purchased from GE Healthcare, Germany. The tested epoxy resin system components (Table 1) were provided by the purchasers in the same quality as in ready-to-use preparations.

In order to facilitate concentration selection, a pretest was carried out with each chemical using 3 mice and a standard concentration of 5%. Ear and lymph node weights were measured as indicators of ear skin irritation. Based on the results of the pretests the high concentration for each chemical was selected and two lower concentrations were tested spaced by a factor of 3 each. If an indication of ear skin irritation was observed, lower concentrations were used for the main studies.

2.3. Protocol

Each component was tested in a separate study, which was carried out in accordance with OECD Guideline 429 under GLP conditions. In addition to the endpoints described in this guideline, lymph node cell counts as well as ear weight and thickness were determined. These latter parameters served as indicators for acute ear reactions which could lead to non-specific activation of draining lymph node cells (Homey et al., 1998; Vohr et al., 2000; Ulrich et al., 2001a,b).

For each study, groups of 6 female CBA/Ca mice were treated with different concentrations of the test substances in acetone or with acetone alone (vehicle control). Because single animal evaluation was performed, six animals per test group were used, in order to ensure at least 5 valid results per test group to achieve sufficient statistical power. In additional studies acetone/olive oil (4:1) was used in order to compare the findings to those obtained with acetone. The studies used 3 test groups and 1 control group, each. Twenty five microliter per ear of the respective test substance preparation was applied to the dorsum of both ears for three consecutive days. The control group was treated solely with 25 μL per ear of the vehicle. The correctness of the concentrations and the stability of the test substance preparations were routinely checked by GC analyses in acetonitrile/deionized water mixtures (1:1 v/v).

Three days after the last application, the mice were injected intravenously (i.v.) with 20 μCi of [^{3}H]-thymidine in 250 μL of sterile saline into a tail vein. About 5 h after the [^{3}H]-thymidine injection, the mice were sacrificed and the auricular lymph nodes on both sides were removed and the weight of each animal's pooled lymph nodes was determined. Lymph node response was evaluated by measuring the cellular content and [^{3}H]-thymidine incorporation into lymph node cells (indicators of cell proliferation). To this end, single cell suspensions were prepared from the pooled lymph nodes of each animal as soon as possible after dissection by carefully passing the lymph nodes through an iron mesh (mesh size 200 μm) into 6 mL of phosphate-buffered physiological saline. For determination of cell counts, an aliquot of each suspension was further diluted with Casy®ton in a ratio 1:500. The cell count was