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Chemical applicability domain of the Local Lymph Node Assay (LLNA) for skin sensitization potency. Part 1. Underlying physical organic chemistry principles and the extent to which they are represented in the LLNA validation dataset

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1. Introduction

Skin sensitization leading to allergic contact dermatitis remains a significant human health concern and is the leading cause of occupational illness in many countries (Diepgen, 2003; Diepgen and Coenraads, 1999; McDonald, 2006).

Predictive assessment of substances for their skin sensitization potential still relies on animal testing. Although human test data are available for a number of chemicals, and are used, as well as for risk assessment purposes, to assess predictive performance of animal tests, routine testing of new chemicals on humans is not an option. The two main test animals are the guinea pig (used in the guinea pig maximization test (GPMT) and the Buehler test) and the mouse (used in the Local Lymph Node Assay (LLNA)) (Magnusson and Kligman, 1969; Buehler, 1965; Gerberick et al., 2007; OECD, 1992; OECD, 2002).

Under REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) (EC, 2006), the LLNA is stated to be the first choice method for *in vivo* testing (ECHA, 2015). In the LLNA, skin sensitization hazard is defined as a function of the ability of the test chemical to provoke immune activation (lymphocyte proliferation) in lymph nodes draining the site of topical application (Gerberick et al., 2000; Basketter et al., 1996, 2002). A substance is classified as a sensitizer if it induces a three-fold stimulation index (SI) or greater at one or more test concentrations (Kimber et al., 1994; Basketter et al., 2000, 2003). The LLNA is able also to provide a reliable measure of relative skin sensitizing potency. Potency is measured by derivation of an estimated concentration of substance, applied on three consecutive days, required to induce a three-fold SI value (EC3) as compared with concurrent vehicle controls (Basketter et al., 1999). EC3 values are usually considered to be accurate within a factor of ca. 2 (Basketter et al., 2000).

For at least the last 15 years, there has been a great deal of effort to investigate and develop non-animal approaches to evaluate skin sensitization potential and potency (Jowsey et al., 2006; Adler et al., 2011; Jaworska et al., 2013; Teubner et al., 2013; Patlewicz et al., 2014). Regulations in Europe in particular, such as REACH and the Cosmetics Regulation have provided significant momentum (EC, 2006; EC, 2009), and since the publication of the Adverse Outcome Pathway (AOP) for skin sensitization (OECD, 2012), there have been even greater efforts to anchor the development and evaluation of assays to key events within the AOP. An adverse outcome pathway (AOP) describes the causal linkage between initial molecular events and an adverse outcome at individual or population levels (Ankley et al., 2010).

The LLNA is commonly the standard against which the performance of alternative/*in vitro* test methods are judged. However, in common with all predictive test methods, the LLNA is subject to

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false positives and false negatives with an overall level of accuracy of approximately 90% (Basketter et al., 2009). At this point, it is appropriate to consider the various causes of disagreement between LLNA results and findings or experience in guinea pigs and humans.

Firstly there may be cases of genuinely false positives – an SI value > 3 is achieved in the LLNA but the biological mechanism is not sensitization. Conversely there may be cases of genuine false negatives, where for some reason the chemical is unable to sensitize mice but can sensitize guinea pigs and/or humans.

Secondly, it should be borne in mind that the boundary between sensitizers and non-sensitizers is fuzzy. There are chemicals that are completely incapable of sensitizing and there are chemicals that have enough potency to produce positive results in any test. However there are others whose ability to sensitize is marginal (e.g. due to low but not zero chemical reactivity), and these may be categorized as non-sensitizing in one assay (e.g. failing to produce an SI > 3 at any concentration in the LLNA) and as weakly sensitizing in another assay or on the basis of clinical evidence (e.g. a low but not zero incidence of reports of human sensitization). Conversely some marginal sensitizers might fail to register positives in guinea pig assays and not have any reports of human sensitization (particularly when human exposure is not extensive) yet be categorized as sensitizers in the LLNA.

Thirdly, chemicals can give positive results due to impurities they contain. If human exposure is mainly to the "commercial grade" material whereas animal testing is with higher purity "laboratory grade" material, there may be incidences of human sensitization that, in comparison with negative results in the animal assays, can be interpreted as false negatives in the latter. Conversely, a laboratory synthesized chemical can sometimes contain more of a sensitizing contaminant than when it is produced on a manufacturing scale, and a positive animal result with the former material may be misinterpreted as a false positive.

In the context of the evaluation and validation of novel test methods it would be extremely helpful to understand in which areas of chemistry the performance of the LLNA may be unreliable. In this regard it will be useful to define the chemical applicability domains of the LLNA in which false predictions are liable to occur. This is particularly critical when considering the outcomes of new *in vitro* test methods in order to be able to discriminate whether the result is reasonable or whether the outcome is impacted by uncertainty in the LLNA, which is commonly used as the basis of comparison.

In this paper we review the chemical mechanisms of skin sensitization, provide an overview of the test methodologies for the LLNA, the guinea pig assays and generation of human potency data, and present the ranges of key physico-chemical parameters for the chemicals that were used in the formal validation process ((NIH Publication No. 99-4494 1999)) to compare LLNA findings with human and guinea pig skin sensitization potency. Further papers will discuss specific areas of chemistry where LLNA data may not correlate with human and guinea pig potency.

2. Chemical mechanisms of skin sensitization

Skin sensitization is a T-cell mediated immune response. The biological mechanism of skin sensitization is summarized briefly below (Barratt et al. 1997).

The skin sensitizing chemical acts as a "hapten", i.e. binds to skin protein in the epidermis so as to make it antigenic. The antigenic protein is processed by dendritic cells in the epidermis and these dendritic cells are consequently stimulated to migrate to a lymph node where they present the antigen to naïve T-cells. As a result, T-cells with receptors able to specifically recognize the antigen are stimulated to proliferate and circulate throughout the body. These events take place during the induction stage of a sensitization test.

On subsequent exposure to the same sensitizer, or a second sensitizer cross-reactive with the first, protein binding and processing of the resulting antigenic protein by dendritic cells again occurs, after which the antigen presented by the dendritic cells is recognized by the circulating T-cells, triggering a cascade of biochemical and cellular processes which produce the clinical sensitization response. These events take place at the challenge stage of a sensitization test.

From the above it is clear that the molecular initiating event (MIE) in the AOP for the sensitization process (OECD, 2012) is the reaction of the chemical (or its abiotic/metabolic transformation product) with skin protein. Consequently, reaction chemistry underpins all mechanistic attempts to predict skin sensitization from structural and physical properties. In order to understand these approaches, some description of reactivity and its implications for skin immunology is required.

2.1. The Relative Alkylation Index model

The Relative Alkylation Index (RAI) model (Roberts and Williams, 1982) has proved to be a useful tool in analyzing sensitization data. It is based on the concept that the degree of sensitization produced at induction, and the magnitude of the sensitization response at challenge, depends on the degree of covalent binding (haptenation; alkylation) to carrier protein occurring at induction and challenge. The RAI model is a general mathematical model of what is now referred to in AOP terminology as the MIE, i.e. the step in which skin protein reacts covalently with the chemical (as such or after activation), and it forms the theoretical basis for the mechanism-based QSARs(QMMs) correlating potency with physico-chemical parameters for both guinea pig data and LLNA data.

The RAI is an index of the relative degree of carrier protein haptenation and was derived from differential equations modelling competition between the carrier haptenation reaction in a hydrophobic environment and removal of the sensitizer through partitioning into polar lymphatic fluid.

In its most general form the RAI is expressed as:

$$\mathrm{RAI} = \mathrm{logD} + \mathrm{a\ logk} + \mathrm{b\ logP} \quad (1)$$

Thus according to the RAI model the degree of haptenation increases with increasing dose D of sensitizer, with increasing reactivity (as quantified by the rate constant or relative rate constant k for the reaction of the sensitizer with a model nucleophile) and with increasing hydrophobicity (as quantified by log P, P being the octanol/water partition coefficient).

Equation (2) provides the basis for combined dose-response/structure-activity models in which the biological response BR (e.g. SI value in an LLNA test, total erythema score in a guinea pig test) varies as the dose and/or identity of the chemical vary:

$$\mathrm{Log\ BR} = \mathrm{A.RAI} + \mathrm{B} \quad (2)$$

For the more usual approach of expressing toxicity in terms of the dose required to produce a specific effect (in the case of the LLNA, an SI value of 3), equations (1) and (2) give the general quantitative mechanistic model (here using EC3, the concentration required to give an SI value of 3 in the LLNA):

$$\mathrm{pEC3} = \mathrm{a\ logk} + \mathrm{b\ logP} + \mathrm{c} \quad (3)$$

Thus the key parameters are reactivity and hydrophobicity.