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Evaluating the sensitization potential of surfactants: Integrating data from the local lymph node assay, guinea pig maximization test, and *in vitro* methods in a weight-of-evidence approach

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ABSTRACT

An integral part of hazard and safety assessments is the estimation of a chemical's potential to cause skin sensitization. Currently, only animal tests (OECD 406 and 429) are accepted in a regulatory context. Non-animal test methods are being developed and formally validated. In order to gain more insight into the responses induced by eight exemplary surfactants, a battery of *in vivo* and *in vitro* tests were conducted using the same batch of chemicals. In general, the surfactants were negative in the GPMT, KeratinoSens and hCLAT assays and none formed covalent adducts with test peptides. In contrast, all but one was positive in the LLNA. Most were rated as being irritants by the EpiSkin assay with the additional endpoint, IL1-alpha. The weight of evidence based on this comprehensive testing indicates that, with one exception, they are non-sensitizing skin irritants, confirming that the LLNA tends to overestimate the sensitization potential of surfactants. As results obtained from LLNAs are considered as the gold standard for the development of new nonanimal alternative test methods, results such as these highlight the necessity to carefully evaluate the applicability domains of test methods in order to develop reliable nonanimal alternative testing strategies for sensitization testing.

1. Introduction

An integral part of hazard and safety assessments for consumer and occupational health is the estimation of a chemical's potential to cause allergic contact dermatitis. Currently animal tests are typically used in a regulatory context to assess a chemical's potential to induce skin sensitization. Both the murine local lymph node assay (LLNA; OECD 429) and guinea pig based tests (GPTs, OECD 406; guinea pig maximization test (GPMT) or Buehler tests) are test methods accepted by the regulatory bodies to assess this endpoint. As a 3R method, the LLNA has become the preferred method for sensitization testing in the European Union (EU) and increasingly in other countries. Within the EU, the new chemicals legislation on the registration, evaluation, authorization and restriction of chemicals (REACH) requires the submission of information on human health effects of chemicals. With few exceptions, all substances registered in accordance with REACH will require skin sensitization data. Within the framework of REACH, the local lymph node assay (OECD, 2010) is the preferred method for generating data on skin sensitizing potential. Use of other methods, including the traditionally used guinea pig tests (OECD, 1992) may only be performed under exceptional circumstances when sufficient scientific justification warrants their use.

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Following the validation of the LLNA, the observation was made that the LLNA often overestimates the sensitization potential for some substances, e.g. surfactants, fatty acids, fatty alcohols and siloxanes (Basketter et al., 2009a; Garcia et al., 2010; Kreiling et al., 2008; Penninks, 2006). Indeed, the classic example of a substance eliciting false positive responses in the LLNA is the surfactant sodium lauryl sulfate (SLS). SLS was one of the substances included in the set of chemicals used in the validation of the LLNA (Dean et al., 2001). The existing data in humans and guinea pigs indicate that this surfactant is irritating but not a sensitizer in these two species. However the LLNA identified it as a sensitizer, subsequently leading to the understanding that this was a true false positive in the assay (Basketter et al., 2009b; Dean et al., 2001). Clearly no biological assay is perfect, and in the majority of cases the LLNA appears to be accurately predictive of whether a chemical can trigger an induction of the immune system indicating a potential for being a sensitizer (Dean et al., 2001). However, if this assay is to become the only permitted assay for the future assessment of sensitizing potentials, as is by in large stipulated by REACH, it is important to establish if there are chemical classes that are incompatible with the assay as, in general, only one animal test may be conducted per endpoint due to animal welfare considerations. Based on the increased awareness within the scientific community, the recently revised version of the OECD No. 429 guideline (adopted on July 22, 2010) has taken certain aspects of applicability into account and now reads "... Despite the advantages of the LLNA over TG 406, it should be recognized that there are certain limitations that may necessitate the use of TG 406 (e.g. false negative findings in the LLNA with certain metals, false positive findings with certain skin irritants [such as some surfactant type chemicals], or solubility of the test substance). In addition, test substance classes or substances containing functional groups shown to act as potential confounders may necessitate the use of guinea pig tests (i.e. TG 406)..." (OECD, 2010).

The increasing deliberation on the ethics of animal testing has manifested itself in a regulatory context in REACH but even more so in the European Cosmetics Directive. REACH calls for alternative test methods to be used wherever possible. The Cosmetics Directive foresees a progressive phasing out of animal tests for the purpose of safety assessments of cosmetics. Marketing and testing bans apply as alternative methods are validated and adopted through EU legislation with the goal of phasing out animal tests for cosmetics by 2013. This has motivated the development of a number of alternative non-animal test methods. However, few have gone beyond intralaboratory validation, even less have been formally validated according to the ECVAM validation procedure and only a small number have achieved regulatory acceptance at this time (http://www.ecvam.jrc.ec.europa.eu/).

With the current innovation in the field of non-animal test methods, a number of *in vitro* assays have been developed to specifically assess skin sensitizing potentials, the accuracy of which are usually assessed using the LLNA as the gold standard. This is due to the ability of the assay to yield objective measurements via scintillation counting and to give information on dose responses with which an evaluation of potency is possible. Currently, the direct peptide reactivity assay (DPRA), the human Cell Line Activation Test (hCLAT) and the Myeloid U937 Skin Sensitization Test (MUSST) methods are in the prevalidation phase at ECVAM and the KeratinoSens assay will be submitted in the near future. The advantage of many of these *in vitro* assays is that the endpoints measured are linked to key stages in the mechanism leading to a skin sensitizing response. Following skin penetration, protein reactivity and the triggering of specific signaling pathways, e.g. via interleukins (Wang et al., 1999) are involved in the activation of the Langerhans cells (LC) of the skin which in turn are essential for triggering the proliferation of antigen specific T-cells. Whereas the animal tests include all these steps, the *in vitro* tests can only assess specific stages in the sensitization process. Peptide reactivity assays (Gerberick et al., 2008; Natsch and Gfeller, 2008) have been developed to assess whether the chemical can interact with synthetic peptides to mimic the formation of hapten/skin protein complexes necessary for T-cell recognition of the allergen. The keratinocytes of the epidermis are essential for generation of "danger signals", such as interleukins IL-18, IL-1β and IL-1α, in response to irritants and/or sensitizers which are required for the activation of antigen presenting cells such as the LC of the skin. One assay measuring keratinocyte activation is the KeratinoSens assay (Emter et al., 2010) which was developed following the observation that sensitizers appear to trigger the Keap1-Nrf2-ARE regulatory pathway (Natsch, 2010; Natsch et al., 2010) leading to induction of genes under the control of the Antioxidant Response Element (ARE). The activation of the antigen presenting cells themselves can be assessed by using the hCLAT or MUSST assays, both of which assess the expression of specific cell surface markers of antigen presenting cells as a measure of cell activation. A certain level of skin irritation appears to facilitate skin sensitization reactions, but on the other hand skin irritation may also be a confounding factor in animal tests and in human patch tests. The skin irritation potential can now be assessed *in vitro* with the validated Episkin™ irritation assay with reduction of cell viability in a 3-D skin model as read-out. As an option, the secretion of the danger signal interleukin-1α (IL-1α) as a measure of irritation can be used as an additional endpoint in this test. The use of an array of *in vitro* assays allows a more 'mechanistic' analysis of the various stages in the sensitizing response and generates a comprehensive dataset which can feed into a 'weight of evidence' approach. The determination of whether or not a substance is a potential human sensitizer can therefore be made more objectively.

In the current study, a comparative testing program was conducted. As the sensitization potentials of surfactants are often overestimated in the LLNA (Garcia et al., 2010; Mehling et al., 2008); the first part of the testing program was necessary to identify if the two animal assays gave the same predictions. The standard GPMT and LLNA were used to assess the sensitization potential of eight exemplary surfactants including five commercially available surfactants of high purity. In addition to the standard LLNA endpoints, additional parameters, including ear thickness and flow cytometry to measure the number of lymph node cells carrying the B220 marker, were included (Gerberick et al., 2002). In the second part of the testing program, the surfactants were tested in *in vitro* sensitization assays, namely the peptide reactivity assay, KeratinoSens assay and the hCLAT assay. To address irritancy, the often discussed confounding factor leading to overestimations of sensitization potentials in the LLNA, the Episkin™ assay with concurrent IL-1α quantitation was also included into the test program.

2. Materials and methods

2.1. Test materials

The surfactants were selected to reflect nonionic and anionic surfactant types. Selection was also based e.g. on chain length (C12–C16) and degree of ethoxylation (EO2–EO6). Five of the chemicals were obtained from Sigma Aldrich and were of analytical grade purity (>97%): sodium lauryl sulfate (SLS; No. 71729); tetraethylene glycol monotetradecyl ether (C14EO4; No. 86697), hexaethylene glycol monododecyl ether (C12EO6; No. 52044); *n*-heptyl β-d-thioglucopyranoside (Nr: H3264; thioglucopyranoside); and 1-nonane sulfonic acid sodium salt (nonane sulfonate; No. 74318). Due to the limited quantities commercially available in