Review

The murine local lymph node assay: Regulatory and potency considerations under REACH

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Abstract

From June 2007, new chemicals legislation on the registration, evaluation, authorisation and restriction of chemicals (REACH) will come into force across the European Union. This will require the submission of data on human health effects of chemicals, including chemical safety assessments which will require measurements of potency. For skin sensitization hazard identification, REACH states that the first-choice *in vivo* assay is the local lymph node assay (LLNA). This test has also been the UK competent authority's preferred test for skin sensitization since 2002, and has now replaced guinea pig tests in dossiers submitted to it under the Notification of New Substances Regulations. Advantages of the LLNA over guinea pig tests include improvements in animal welfare, a more scientific approach to hazard identification, and the inclusion of a dose–response element in the endpoint, which enables an estimation of potency. However, notifiers to the UK competent authority have sometimes been reluctant to use the assay because of concerns over false-positive reactions. Across Europe, these concerns have been heightened in the lead-up to the introduction of REACH, since the use of *in vivo* alternatives to the LLNA will require scientific justification. This review will address some of these concerns from a regulatory perspective.
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Contents

1. Background

Since 2002, the murine local lymph node assay (LLNA) (Kimber et al., 1994), as performed in accordance with OECD test guideline 429 (OECD, 2002), has been the UK competent authority's (CA) preferred test for skin sensitization. Extensive validation (Kimber et al., 1994, 1998; Kimber and Basketter, 1992) has shown that this assay is a suitable substitute for guinea pig tests (GPT) for skin sensitization, the most commonly used of which, the Guinea Pig Maximization Test (GPMT) and the Buehler Test, are described in OECD test guideline 406 (OECD, 1992). Importantly, it also has many advantages over GPT in terms of both scientific progress and animal welfare (Gerberick et al., 2000; National Institutes of Health, 1999; Sailstad, 2002). The assay provides information on a substance's ability to induce sensitization, unlike the GPT, which measure responses to the elicitation phase, and, importantly, delivers quantifiable data that enable dose response assessment. Although animals are not replaced, the numbers used are usually reduced (although a modified OECD guideline allowed half the original number of animals to be used for the GPMT) and the procedures are substantially refined. Since responses to the induction phase are measured, the LLNA does not require the elicitation of challenge-induced dermal hypersensitivity reactions, thus reducing animal discomfort. Additionally, as no adjuvant is required, the severity of the procedure and thus animal distress are reduced compared with the GPMT. The use of mice rather than guinea pigs, and the shorter experimental time involved, also greatly reduce the costs associated with testing for skin sensitization potential. For all these

reasons, in 2002 the UK CA advised its notifiers of new substances that the LLNA was now accepted as a stand-alone test, and indeed was the preferred test for skin sensitization.

However, despite its obvious advantages, reservations about the suitability of the LLNA in certain circumstances have emerged and recently it has become apparent that notifiers of new substances are reverting to the use of GPT for the testing of particular substances. Specifically, there have been concerns that some vehicles may augment or dampen lymph node cell (LNC) proliferative responses; that the assay has not been validated for the testing of formulations, including emulsions, suspensions and mixtures; and that some irritant substances give false positive responses. These concerns have become heightened with the imminent implementation of the new European chemicals legislation on the registration, evaluation, authorisation and restriction of chemicals (REACH), which specifies that the LLNA must be used for new *in vivo* testing for skin sensitization hazards: only under 'exceptional circumstances' should another *in vivo* method be used, and only when this can be scientifically justified (EC, 2006). Once REACH comes into force, the LLNA will be much more widely used throughout Europe than it currently is. REACH also requires that a chemical safety assessment be undertaken which includes dose (concentration)–response (effect) relationships for human health hazards for chemicals marketed in quantities of ten tonnes or more per annum. In view of these points, this review will address some of the concerns mentioned above and, in addition, examine the evidence for the LLNA's ability to provide information on dose–response relationships, and thus on the skin sensitization potency, of substances.

2. Vehicle effects in the LLNA

Many factors influence the induction of skin sensitization, and some of these, such as the efficiency of Langerhans' cell migration from the skin and the ability of the chemical to gain access to the viable epidermis, are likely to be influenced by the vehicle or formulation in which an allergenic chemical is applied. Since the LLNA uses only topical application of test substance, the choice of vehicle is particularly important (Kimber and Basketter, 1992). OECD test guideline 429 provides guidance on the concentration of substance and in choosing a vehicle in which to administer it (OECD, 2002), with five vehicles being recommended, although others may be used with scientific justification. The preferred vehicle is acetone/olive oil (4:1, v/v; AOO), whilst one of the less favoured but still recommended vehicles is propylene glycol (PG). However, some studies have indicated that these vehicles may themselves affect LNC proliferation and thus give misleading results. It has been assumed that the most important effect of vehicle or formulation matrices is to alter the skin penetration and effectiveness with which a chemical allergen gains access to an intact epidermis (Basketter et al., 2001a). However, there may be a variety of other ways in which a delivery matrix affects the development of skin sensitization (reviewed by Basketter et al., 2001a).

2.1. Olive oil

AOO is the vehicle most commonly used in LLNA submissions for the notification of new substances to the UK CA (Cockshott et al., 2006). Olive oil forms an occlusive layer on the skin, thus preventing epidermal water loss with a consequent increase in the penetration of water-soluble compounds. However, it has been reported that olive oil may itself cause contact allergy (Malmkvist Padoan et al., 1990). Additionally, Montelius et al. (1996) reported highly variable results with olive oil in the LLNA; 100% olive oil gave SI values that ranged from 16.4 to 22.7, dependent on the supplier, whilst two out of three AOO mixtures resulted in a stimulation index (SI) greater than 3 (the default cut-off value between a positive and a negative response when radioactive material is used to measure cell proliferation), with, in one case, dpm from nodes of individual animals ranging from 213 to 1160. It was therefore suggested that AOO was not a suitable vehicle for the LLNA, and that the results also had implications for the use of olive oil in other predictive methods and in patch testing. Dimethyl formamide (DMF) was advocated as a more reliable alternative.

However, despite these results and the report of contact allergy to olive oil (Malmkvist Padoan et al., 1990), clinical experience indicates that it is not a contact allergen. AOO was also found to increase the responses to formalin: formalin diluted in water had no effect on LNC proliferation, formalin applied neat was a weak contact sensitizer (SI of 4.0), whereas formalin 25% in AOO was a severe sensitizer (SI 17.8) (Edwards et al., 1994). In another study, two independent laboratories evaluated the skin sensitization potential of 1,4-dihydroquinone in seven different vehicles (Lea et al., 1999). Although AOO did not lead to the highest SI values of the substance in the tested vehicles (it was comparable to acetone and methyl ethyl ketone, MEK), there was some variability in the responses: the dpm/node values for the allergen-activated LNC were generally equivalent between the two laboratories, except for 1% 1,4-dihydroquinone in AOO, in which case the result from one laboratory was almost twice that from the other laboratory. AOO was also found to give similar levels of LNC proliferation to other tested vehicles (dimethyl sulphoxide (DMSO), MEK, DMF, ethanol/distilled water 90:10) in LLNA experiments in which four chemical allergens were compared, although there was not always a clear dose response (Wright et al., 2001). However, in some cases the vehicle had a marked effect on the LNC proliferative responses and thus the apparent sensitizing potency (Wright et al., 2001; Table 1).

Basketter and Kimber (1996), after reporting their findings with fresh and aged (for 6 months) olive oil, concluded that AOO was a suitable vehicle for use in the LLNA. The aged olive oil generally resulted in more LNC proliferation, and with both oils there was a dose response, with 100% olive oil giving SI values of marginally greater than 3. However, a mixture of 4:1 acetone/olive oil resulted in a negative result; moreover, comparison of historical data from untreated controls and from acetone/olive oil controls showed only a slightly higher proliferation in the latter. A later study, in which oxazolone was applied in one of five vehicles (acetone, ethanol, DMF, DMSO or AOO), measured the effect of the vehicle on allergic contact sensitization (ACS) and proliferative responses in LNC together with specific antibody production to oxazolone (van't Erve et al., 1998). Oxazolone in all five vehicles resulted in sensitization, as determined by mouse ear swelling, but there were differences in the magnitude of the response, with DMSO and AOO causing the most vigorous reactions and DMF the least. In previously sensitized animals, challenge with the substance in ethanol led to significantly higher LNC cell

Table 1
Examples of the effect vehicles may have on LLNA EC3 values and the categorization of chemicals as sensitizers

Chemical	Vehicle[a]	EC3 value (%)	Category[b]
Isoeugenol[c]	AOO	1.0	Strong
	MEK	1.0	Strong
	DMF	1.4	Strong
	PG	2.5	Moderate
	DMSO	0.9	Strong
	EtOH/ddw (90:10)	1.8	Strong
	EtOH/ddw (50:50)	4.9	Moderate
Cinnamic aldehyde[c]	AOO	1.7	Strong
	MEK	1.1	Strong
	DMF	0.5	Strong
	PG	1.4	Strong
	DMSO	0.9	Strong
	EtOH/ddw (90:10)	1.6	Strong
	EtOH/ddw (50:50)	1.2	Strong
3-Dimethylpropylamine[c]	AOO	2.2	Moderate
	MEK	1.8	Strong
	DMF	1.7	Strong
	PG	> 10	Moderate
	DMSO	3.2	Moderate
	EtOH/ddw (90:10)	4.1	Moderate
	EtOH/ddw (50:50)	7.1	Moderate
1,4-Dihydroquinone[d]	Acetone	0.08	Extreme
	MEK	0.09	Extreme
	AOO	0.15	Extreme
	DMF	0.21	Strong
	DMSO	0.35	Strong
MCI/MI[e,f]			
	AOO	0.0049	Extreme
	MEK	0.0068	Extreme
	DMF	0.0075	Extreme
	DMSO	0.0075	Extreme
	Acetone	0.0076	Extreme
	PG	0.048	Extreme
Potassium dichromate[g]	1% L92	0.17	Extreme
	DMSO	0.05	Extreme
	DMF	0.0327	Extreme
Nickel sulphate[g]	1% L92	2.5	Moderate
	DMSO	4.8	Moderate
	DMF	>5.0	Moderate

[a] AOO, acetone olive oil (4:1); MEK, methyl ethyl ketone; DMF, dimethyl formamide; PG, propylene glycol; DMSO, dimethyl sulphoxide; EtOH, ethanol; ddw, double distilled water.

[b] According to the scheme of Basketter et al. (2005a) (Table 2).

[c] Wright et al. (2001).

[d] Lea et al. (1999).

[e] MCI/MI, methylchloroisothiazolinone/methylisothiazolinone.

[f] Warbrick et al. (1999a).

[g] Ryan et al. (2002).

numbers and proliferative responses than when applied in DMSO, but there was little difference between the other groups. Ratios of IgG2a/IgG1 were variable and depended on the vehicle used, but were the highest after sensitization with oxazolone in AOO, indicative of the production of Th1 cytokines, which are associated with ACS. Although not directly comparable to the LLNA because the challenge rather than induction phase responses were measured, this confirmed that vehicles affect ACS, not just the induction phase as measured

by LLNA, although LNC proliferation does not always predict the magnitude of ear swelling responses. Emphasizing the importance of vehicle selection in testing for skin sensitization potential, particularly in the case of weak sensitizers, it was found that ethylene diamine gave positive LLNA responses when applied in AOO but not when applied in 3:1 acetone/water (Kimber et al., 1998). This material has some potential to cause ACS, but this is probably augmented by its being applied topically in medicaments to inflamed skin. Conversely, when the weak sensitizer benzocaine, another ingredient of topical medicines, was applied in a variety of vehicles (including AOO) in a LLNA, there was no dose response and application in none of the vehicles elicited a positive response (Warbrick et al., 2000).

A recent report has demonstrated that background levels of LNC turnover obtained with AOO significantly increased over time in one laboratory (Betts et al., 2007a). AOO was the only vehicle investigated in this way, so it is not known if a similar phenomenon would occur with other vehicles. Possible propounded explanations were differences in variables such as the spontaneous levels of lymphocyte turnover, the source or purity of the olive oil, or changes in the operator. However, it was noted that the SI values obtained with a known contact sensitizer were comparable over time, indicating that changes in background lymph node values were mirrored by changes in the vigour of responses to skin sensitizers. It was suggested that laboratories conducting LLNAs should monitor changes in the thymidine incorporation values obtained from vehicle-treated control animals and be vigilant for any that fall outside the laboratory's normal range.

Therefore, it is apparent that some variability in proliferative responses to AOO has been reported. Possibly, the use of olive oil as a vehicle could lead to a positive assay for substances that would otherwise achieve a borderline SI value, although it has not led to an increase in the number of positive notifications to the UK CA, despite being the most commonly used vehicle (Cockshott et al., 2006). As such, there would currently appear to be little justification for discouraging the use of AOO. However, the assay of individual lymph nodes would allow identification of outliers so that the median could be used instead of the mean, or outliers could be excluded; further consideration may need to be given to this question.

2.2. Propylene glycol

Propylene glycol is also an OECD-approved vehicle (OECD, 2002), but there have been concerns that it may dampen proliferative responses to sensitizers. Some of the variables that may affect the extent to which sensitization occurs were investigated when the contact allergen 2,4-dinitrochlorobenzene (DNCB) was tested in a LLNA applied in either acetone or PG (Heylings et al., 1996). DNCB provoked a significantly more vigorous LNC proliferative response when administered in acetone, although there was no variation in dendritic cell accumulation associated with application in the two different vehicles. Percutaneous absorption of DNCB across mouse skin was significantly greater from 2 h onwards when administered in acetone, but over a 24-h period the cumulative absorption was similar with both vehicles. It was concluded that alterations in the initial percutaneous absorption and/or disposition might be important in the subsequent development of sensitization.

Warbrick et al. (1999a) investigated the contact sensitizing activity of a known contact allergen, methyl-chloroisothiazolinone/methylisothiazolinone (MCI/MI) in the LLNA when applied in the OECD guideline 429-approved vehicles and in acetone. Application of MCI/MI in DMF, acetone and MEK gave the highest proliferative responses, whereas MCI/MI applied in propylene glycol (PG) gave much weaker responses, with an SI of greater than 3 (4.7) attained only at the highest concentration. Thus, at the highest concentration MCI/MI gave a positive response in all the tested vehicles, but with different EC3 values (the concentration of test substance estimated to cause a threefold increase in LNC proliferative activity) (Table 1). The EC3 values in the other vehicles were very similar.

In another study, the skin sensitization potential of a weak skin sensitizer, 1,4–dihydroquinone, was evaluated in seven different vehicles (Lea et al., 1999). In five of the seven vehicles tested there was a dose-related LNC proliferative response (Table 1), but in acetone/physiological saline (1:1) and PG, the substance was negative at 1%, although in further testing it gave a positive response at a concentration of 2.5% or greater in both vehicles. When four known contact allergens administered in seven vehicles were tested in the LLNA, the chemicals administered in PG tended to result in lower LNC proliferation than in most of the other vehicles (Wright et al., 2001). However, this was not universally true: responses to cinnamic aldehyde in PG were greater than when AOO was used (Table 1). The application of 3-dimethylpropylamine up to 10% in PG did not give a positive SI value, and it was not possible to increase the concentration because of poor solubility, indicating that, in any case, PG would not be the best

vehicle for application of this chemical for the purposes of hazard identification testing.

2.3. The effects of other vehicles

The use of dimethyl sulphoxide (DMSO) as a vehicle may increase the sensitivity of the LLNA when metal salts, including nickel and copper salts, which are generally negative in this test, are applied to the skin (Ikarashi et al., 1992; Ikarashi et al., 1993c; Ryan et al., 2002). DMSO applied as a vehicle control has tended to give higher proliferative responses than the other tested vehicles (Kimber et al., 1995, 1998; Lea et al., 1999), which is consistent with its being an irritant (see later section on false-positive responses to irritants). As with skin sensitization potential, the irritancy properties of a chemical may be influenced by the vehicle in which it is applied (Loveless et al., 1996; Vohr and Ahr, 2005). For example, polyethylene glycol and DMF enhanced the irritancy of cinnamic aldehyde (Vohr and Ahr, 2005). SLS when applied in ethanol gave a much lower SI value than when applied in DMF (Loveless et al., 1996).

Because of the higher proliferative responses observed with DMSO, it has been suggested that it may not be a suitable vehicle for weak allergens, since high vehicle responses result in reduced SI values (Ikarashi et al., 1993b). However, this contradicts the finding of greater responses to metal salts when applied in this vehicle. DMSO stimulates cell-mediated immunity but with a reduction in antigen-specific antibody production (van't Erve et al., 1998). Toxicity (as determined by the presence of oedema and erythema) was noted when dibromodicyanobutane (International Nomenclature Cosmetic Ingredient name methyldibromoglutaronitrile) was administered in DMSO but not in other vehicles, leading to the suggestion that animals should be carefully observed to detect local toxicity (Wright et al., 2001). DMF is a penetration enhancer and so can also increase the sensitivity of the LLNA to weak allergens (Robinson and Cruze, 1996), although, as for other vehicles, its effects may be unpredictable. However, although some vehicles improve skin penetration and availability of a substance, this is not always the sole explanation for differences in responses (van't Erve et al., 1998).

Since wholly aqueous vehicles should be avoided (OECD, 2002), Ryan et al. (2002) attempted to identify an alternative vehicle for water-soluble substances. Pluronic L92 (L92), a non-ionic surfactant, was chosen based on its skin wetting characteristics, low acute toxicity and low irritation potential. Concentrations of L92 up to 50% did not induce positive responses in the LLNA.

Proliferative responses to dinitrobenzene sulphonic acid and formaldehyde formulated in 1% L92, water, DMSO or DMF were compared, with the finding that both chemicals were positive in all vehicles, but that the relative potency varied (potency ranking DMF ≥ DMSO > 1% L92 > water). It was recommended that, if a test substance is soluble in DMSO or DMF, they are the preferred vehicles because of their ability to increase epidermal permeability and hence increase bioavailability by enhanced penetration of the test substance. However, if higher concentrations can be achieved in aqueous vehicles, the recommendation was that L92 is preferable to water alone. Additionally, if occupational or consumer exposure will be in a water-based matrix, it was suggested that 1% L92 provides a suitable vehicle for the assessment of sensitization risk.

More recently, ethanol and diethyl phthalate (DEP), individually and in two different combinations, have been investigated as alternative vehicles for the application of fragrance materials (Lalko et al., 2004). These vehicles were selected because of their representation of the matrix in which human skin exposure to fragrance materials is likely to occur. The tested materials all gave positive responses in the four tested vehicles, although the EC3 values varied depending on both the vehicle and the tested material in an unpredictable manner. A comparison of a 1:3 mixture of ethanol:DEP with AOO indicated that both vehicles resulted in comparable background turnover of draining LNC and of LLNA responses to hexyl cinnamic aldehyde (Betts et al., 2007a). The authors concluded that 1:3 ethanol:DEP is a suitable alternative vehicle for use in the LLNA.

2.4. Vehicle effects in non-murine systems

It is important to recognise that vehicle effects on hypersensitivity responses elicited after application to the skin are not specific to the LLNA; they have been recognised in humans for a long time (Kligman, 1966; Marzulli and Maibach, 1975) and, moreover, vehicle effects have been implicated in erroneous or variable results from human patch tests, especially false-negatives (Danneman et al., 1983; Liden and Boman, 1988). Vehicle effects have also been recognized in guinea pigs (Andersen et al., 1985; Liden and Boman, 1988; Magnusson and Kligman, 1970), including contradictory responses to chlorocresol in AOO and PG (with chlorocresol in AOO being more sensitizing than the same concentration in PG), even though chlorocresol absorption from the two preparations was equivalent (Andersen et al., 1985). In a test of the antihistamine triprolidine in a vehicle of 0.5% oleic acid in PG, the

Buehler test underestimated the skin sensitization that subsequently occurred in clinical tests (Robinson et al., 1991).

Guinea pig methods, with their complex methodology and qualitative end-points, are not well suited for the prediction of such vehicle effects, whereas the LLNA, with its objective and quantitative output, is more suitable (Basketter et al., 2001a). Interestingly, two mineral oils that had been used in GPMT tested positive in a LLNA (Edwards et al., 1994), raising the possibility that their use in the GPMT may have masked responses to weak sensitizers. It has been reported that responses in GPT appear to be polarized to the extremes, with chemicals tending to be classified as non-sensitizers or strong sensitizers, with far fewer substances in the weak and moderate categories (Basketter et al., 2001a). However, at present the published data on hazard identification in traditional GPT is more limited than that in the LLNA. Notwithstanding, a review of 244 chemicals with sensitizing activity in humans and animal tests (including but not limited to the GPMT, Buehler assay and LLNA) demonstrated that a positive animal test is a reliable indicator for a contact allergenic potential in humans (Schlede et al., 2003). Additionally, there is generally a close concordance in hazard identification in the GPT and the LLNA (Basketter and Scholes, 1992; Edwards et al., 1994; Kimber et al., 1990b; Yamano et al., 2005).

2.5. The effects of vehicles on potency estimation

Wright et al. (2001) examined the usefulness of the LLNA for the evaluation of reliable skin sensitizing activity as a first step in the risk assessment process. One question addressed was if the effect of the vehicle on a particular chemical allergen can be predicted. In the cases of dimethylaminopropylamine, isoeugenol, and cinnamic aldehyde administered in AOO, MEK, DMSO and DMF, the effects were consistent, with the lowest EC3 values; similarly, PG and ethanol/water both gave higher EC3 values with all three chemicals. However, application of isoeugenol and cinnamic aldehyde in DMF and DMSO gave contradictory results: both had the same EC3 value in DMSO, but in DMF cinnamic aldehyde was more potent than isoeugenol. Therefore, it may be difficult to generalise about the effects of vehicles on sensitization potency. Other studies have also shown that the choice of vehicle may affect the EC3 value (Edwards et al., 1994; Lea et al., 1999; Warbrick et al., 1999a). However, it is generally found that for a particular sensitizing chemical, a range of EC3 values may be obtained which, nevertheless, would generally lead to the substance being categorized in the same class

Table 2
Proposed categories for skin sensitizers based on LLNA EC3 values

Category	LLNA EC3 value (%)	
	ECETOC (2003) and Kimber et al. (2003)	Basketter et al. (2005a)
Extreme	<0.1	≤0.2
Strong	≥0.1 to <1	>0.2 to ≤2
Moderate	≥1 to <10	>2
Weak	≥10 to ≤100	–

of human sensitizer (see Section 3.2 and Table 2; Ryan et al., 2002; Warbrick et al., 1999a; Wright et al., 2001). In a further study, 1,4-dihydroquinone would have been categorized as either an extreme or a strong sensitizer, depending on the vehicle (Lea et al., 1999). Table 1 provides some examples of how the application vehicle may affect the EC3 value and thus the sensitization category into which a chemical would be placed. In cases where different animal test results would lead to different categorization, it has been suggested that the higher potency category should apply (Basketter et al., 2005a).

2.6. The testing of formulations

Guinea pig and mouse predictive test methods for the identification of chemicals with the potential to cause ACS are usually conducted with discrete chemicals. However, since topical exposure to chemicals more usually occurs in the context of mixtures and formulations, it would be appropriate to test chemicals in the formulations in which exposure to them would be most likely to occur. Additionally, the formulation of a product will be a critical factor in the risk assessment process. Unfortunately, to date, the testing of formulations has not been validated in any assay method (Basketter et al., 2005a), including GPT, with the testing of finished products and formulations usually being confined to humans. A particular problem is the testing of aqueous formulations, since wholly aqueous vehicles are not suitable for testing in the LLNA (OECD, 2002). Another problem arises in the testing of pesticides, which are often complex mixtures that are not compatible with the traditional vehicles advocated for use in the LLNA. Alternative vehicles such as L92 may provide at least a partial solution to both these problems (Ryan et al., 2002; Woolhiser et al., 2007).

Components of the formulation may, as is the case with vehicles, alter the active substance's bioavailability and apparent sensitization potential (Heylings et al., 1996; Warbrick et al., 1999a); for example, the inclusion of sodium lauryl sulphate (SLS) may enhance skin penetration, leading to more accurate patch tests for weak

allergens (Seidenari et al., 1996). Irritation to a transdermal formulation of an antihistamine was mainly caused by the 0.5% oleic acid in PG vehicle (Robinson et al., 1991), which, as discussed later, may result in increased LNC proliferative responses. Therefore, the formulation of a chemical can affect responses measured by the LLNA. However, it is difficult to determine if the influences of a formulation are an artefactual effect of the assay system or a true reflection of the altered skin sensitization potential of the chemical in a particular matrix. This is an important research area to be pursued. If it is a true reflection of altered skin sensitization potential, the LLNA could be established as a valuable means of detecting such alterations, leading to more accurate hazard identifications and risk assessments. Assays in the mouse can help to define the mechanisms through which a formulation may influence sensitization. Such mechanisms may include enhanced acquisition of antigen by Langerhans' cells (Dearman et al., 1996), increased migration of dendritic cells into draining lymph nodes (Cumberbatch et al., 1993) and altered skin penetration (Dearman et al., 1996; Heylings et al., 1996). Components of a formulation may also have different effects on the efficiency of sensitization dependent upon the nature of the inducing allergen (Dearman et al., 1996), or may influence the chemistry and thus the toxicity of the active substance (Calvin, 1992). Although the mechanisms involved in the alteration of sensitization responses are not required to be known for regulatory purposes, information on them would increase understanding and the development of tests for formulations. Additionally, a lack of understanding of the mechanisms involved and their predictability should indicate a need to undertake hazard identification testing with the end-use formulation, where possible.

Therefore, vehicles can affect not only LNC proliferation, as measured by the LLNA, but also ACS (Andersen et al., 1985; Kligman, 1966; Liden and Boman, 1988; Magnusson and Kligman, 1970; Marzulli and Maibach, 1975; van't Erve et al., 1998), and affect both cellular and humoral responses (van't Erve et al., 1998). However, sensitization to not all substances is affected by the vehicle (Marzulli and Maibach, 1975). Results with different vehicles emphasize the need to conduct, where possible, positive control assays with the same vehicle as is used with the test substance. As far as is possible, the vehicle used in contact sensitization assays should be related to the end-use formulation. However, currently, no animal predictive test system has been validated for the testing of formulations. In cases where the vehicle has an impact on the degree of skin sensitization, it is tending to modulate the potency of the allergen, so that

hazard identification is usually unaffected; a European Centre for Ecotoxicology and Toxicology (ECETOC) task force considered that vehicle-related effects have little influence on hazard identification (ECETOC, 2003), which has also been the experience in a UK regulatory setting (Cockshott et al., 2006). Although the relative potency of a chemical, as measured by the EC3, can be altered by application in different vehicles, this often would not result in categorization into a different potency group. Considering these points, the influence of vehicle on LLNA results has probably been over-estimated in the past and, in reality, is of relatively minor importance, provided that factors such as the solubility and toxicity of the test material in the chosen vehicle are taken into account.

3. Estimation of sensitization potency with the LLNA

Under REACH there will be a duty on registrants to produce chemical safety assessments, which will include dose–response relationships and derived no-effect levels (DNELs) for human health hazards, for chemicals marketed in quantities of ten or more tonnes per annum. The use of the LLNA in the assessment of sensitization potency of substances is based on the end-point of the assay being related directly to the efficiency of skin sensitization. Elicitation thresholds (which are indicators of both the induction and elicitation phases of sensitization), as determined by GPT, tend to correlate poorly with induction potency, and there is often a large variation in elicitation thresholds between individuals that depends on numerous factors. For example, elicitation thresholds are generally lower than induction thresholds and may occur at lower doses on repeated exposure, so that the protection of individuals who are already sensitized becomes increasingly difficult (Felter et al., 2003). Therefore, a European Union Expert Group on Sensitization considered that 'it would be inappropriate to define elicitation thresholds as a function of skin sensitizing potency' and that the LLNA was better suited than GPT to categorize the potency of skin sensitizers (Basketter et al., 2005a). Based on the proposal that relative skin sensitization potency is best described as a function of the concentration of chemical necessary for the acquisition of sensitization, relative potency is determined in the LLNA by the derivation of an EC3 value. The design of GPT, which requires irritant concentrations of the test substance to be used for the induction phase, also limits the ability of these tests to provide potency information (ECETOC, 2000; Kimber et al., 2001). Although it has been possible to derive EC3 values for tests conducted in

guinea pigs (Andersen et al., 1995), based on the intra-cutaneous induction concentration that sensitized 50% of the animals (see also Arts et al., 2006 for a summary of methods to determine dose–response relationships from GPT), the objective and quantitative nature of the LLNA's end-point, its focus on induction of sensitization only and the incorporation of a dose–response assessment make it far more suitable for the estimation of relative potency.

3.1. The EC3 value

The EC3 value is mathematically derived by linear interpolation (Basketter et al., 1999b) and is the concentration estimated to induce an SI of 3. The EC3 value can be used as an objective measure of relative potency when comparing two or more chemicals. Thus, the dose–response data generated by the LLNA make this test more informative than GPT for the identification of sensitization potency and enable more accurate risk assessments. It should be emphasised that the EC3 value is not a measure of absolute potency.

Importantly for risk assessment purposes, the EC3 value is highly reproducible within and between laboratories (Loveless et al., 1996; Warbrick et al., 1999b) and is also stable over time (Dearman et al., 1998). An international collaborative trial of seven chemicals in five independent laboratories demonstrated the ability of the LLNA consistently to predict sensitization potential and potency (Loveless et al., 1996). This was confirmed in a further evaluation of test performance in two independent laboratories over a 4-month period. Derived EC3 values for the potent contact allergen paraphenylene-diamine were highly consistent, with calculated values of between 0.06% and 0.09% from one laboratory and between 0.09% and 0.20% from the other (Warbrick et al., 1999b). Dearman et al. (1998) tested a skin sensitizer of moderate potency (hexyl cinnamic aldehyde) in five separate experiments in one laboratory over ten months. The EC3 values for all five experiments ranged from 4.01% to 9.63%. Taking this investigation a stage further, EC3 values ranging from 7.0% to 12.2% for hexyl cinnamic aldehyde, differences in relative potencies that were considered to be trivial, were determined from three laboratories over 8 years (Dearman et al., 2001). These reproducibilities are in contrast to results from GPT, with which reproducibility between laboratories is more difficult to achieve, not least because of the subjective nature of the end-point.

The inherent biological variation in the EC3 value for a chemical, owing to heterogeneities in the test system, has been investigated for isoeugenol (Basketter and Cadby, 2004). In this study, 29 individual EC3 values for isoeugenol ranged from 0.5 to 2.6%, with a mean value of 1.2% (standard deviation 0.6%), which supported the view that 'the biological variation associated with the estimation of EC3 values means that any particular EC3 value can be halved or doubled'. Indeed, it has been suggested that small differences in EC3 values are probably biologically insignificant (Kimber et al., 2002). The current view is that classification schemes should be based on no less than 10-fold differences in EC3 values (ECETOC, 2003; Kimber et al., 2002). To circumvent these biological variations, an alternative method of classifying chemicals has been proposed (Takeyoshi et al., 2005), in which chemicals are tested concurrently with three reference contact allergens in different human contact allergen classes and categorized in accordance with their comparative SI values.

3.2. The relevance of the EC3 value to the estimation of human sensitization potency

When the relative skin sensitizing potencies of glutaraldehyde and formaldehyde were estimated in the LLNA, glutaraldehyde was found to be the more active (Hilton et al., 1998), which was consistent with clinical observations. In another study, in which the relative skin sensitizing potencies of three biocides as assessed by the LLNA were compared with data generated in human volunteers, the EC3 values agreed with the observed potencies in the volunteers (Basketter et al., 1999c). A more extensive comparison of the EC3 values of twenty chemicals (all applied in AOO) with their known sensitizing activities in humans was then conducted (Basketter et al., 2000). The chemicals had been categorised into one of five classes of sensitizing potency, based on expert clinical judgement, from class 1 (strongest allergens) to class 5 (non-sensitizing). Those chemicals considered to be strong allergens in humans had correspondingly low EC3 values (0.01–0.08%). Class 2 chemicals (moderate allergens) had EC3 values in the region of 1%, whilst the values for those in class 3 (weak allergens) were around 10%. Those chemicals in classes 4 and 5, which would, from a regulatory view, not be classified as skin sensitizers, had little activity in the LLNA and could not be assigned an EC3 value. It was concluded that the potency ranking of the chemicals in the LLNA correlated well with their ability to cause skin sensitization in humans. In contrast, although the GPMT adequately detected sensitization hazard, it failed to rank chemicals correctly according to their sensitization potency in humans (Basketter and Chamberlain, 1995).

In a progression from the study by Basketter et al. (2000), quantitative data, determined from human non-diagnostic repeat patch test studies, together with clinical judgement, were used to assign no effect levels to more than 21 chemicals, which were then compared with LLNA EC3 values (Gerberick et al., 2001b; Griem et al., 2003). A good concordance existed between the human no effect levels and the EC3 values across all potency categorizations, which was particularly strong for the non-sensitizers (Gerberick et al., 2001b). It was noted that the use of murine data is conservative, because rodents tend to show a higher skin penetration for chemicals than humans (Griem et al., 2003). A comparison of the EC3 values of 46 contact sensitizers with the lowest doses per unit skin area that resulted in positive human repeat insult patch tests or human maximization tests showed a significant positive correlation and led to a suggested four potency groups based on EC3 values (Schneider and Akkan, 2004). Despite the merit of the EC3 in determining sensitization potency, it was noted that it 'is not an absolute value which can be extrapolated directly to humans', but rather that it 'enables a comparison of the allergenic potency of one potential sensitizer with that of another' (Gerberick et al., 2001b). Further evidence in support of this statement and the correlation between the EC3 value and contact allergenic potency in humans was provided in an evaluation of the derived EC3 values of ten aldehydes with varying degrees of allergenicity in man (Basketter et al., 2001b). The EC3 values predicted accurately the class of skin sensitizer to which nine of the ten aldehydes were assigned. The exception was vanillin, an extremely weak allergen, which had an EC3 of >50%.

An ECETOC task force and an EU expert group have recommended that contact allergens should be categorized based on their LLNA EC3 values as extreme, strong, moderate or weak (Basketter et al., 2005a; ECETOC, 2003; Kimber et al., 2003; Table 2). It was also acknowledged that the guideline GPT have limited possibilities for potency evaluation, but that ranking of allergenic potency could sometimes be derived, albeit, often, with a high degree of uncertainty (Basketter et al., 2005a). Based on the EU expert group categorization system, the potency of eleven chemicals for which LLNA and human data existed were reviewed; in all cases, the categorization based on LLNA EC3 values agreed with that based on human data and with most, but not all, of the findings of GPT (Basketter et al., 2005a). An extensive review has provided a database of 211 chemicals, encompassing the chemical and biological diversity of known chemical allergens, that have been categorized as extreme, strong, moderate, weak or non-sensitizers,

based on their EC3 values (Gerberick et al., 2005). Currently, an OECD expert group is considering the introduction of a system to classify sensitizers as 'weak' or 'strong', based on published schemes (Basketter et al., 2005a; Kimber et al., 2003), into the Globally Harmonized System of classification and labelling of chemicals (GHS).

Although a low potency generally indicates that a chemical will have less impact on human health than one with a high potency, other factors also need to be considered. For example, there is a high incidence of human sensitization to nickel salts (Kligman and Basketter, 1995), despite their being intrinsically very weak skin sensitizers; this incidence appears to be attributable to the extensive human exposure to these chemicals (Dearman et al., 1999; Kimber and Basketter, 1997). Another example of this phenomenon is methyl methacrylate, which is a weak sensitizer, with an EC3 value of 60–90%, but has been associated with increasing prevalences of dermal sensitization in those exposed to plastic materials (Betts et al., 2006).

3.3. The use of the LLNA in risk assessment

As has been discussed earlier in this review, the vehicle in which a chemical is applied can alter the EC3 value obtained in the LLNA. Further research into the effects of the vehicle on potency classification would be informative; notwithstanding, risk assessments should preferably consider the vehicle in which the substance was tested and, where possible, this should be in a formulation that is close to that in which human exposure is likely to occur. In practice, to lend refinement to the risk assessment process, it has been suggested that the EC3 value should be considered as specific to both the chemical sensitizer and the vehicle in which it was tested (Gerberick et al., 2001b). Where this is not possible, a sensitization uncertainty factor to account for the different product matrix may need to be applied during the risk assessment (Felter et al., 2003).

Risk assessment is generally viewed as a four-step process: hazard identification, dose–response assessment, exposure assessment, and risk characterization. The LLNA is able to provide information for the first and second steps of the risk assessment process. Although EC3 values do not provide an absolute measure of skin sensitizing activity that can be directly extrapolated to thresholds for human exposure, they do provide a basis for comparison against other contact allergens of known potency in humans and for an estimation of relative risk (Kimber et al., 2002; Kimber and Basketter, 1997). Potency is defined as a function of the amount of chem-

ical required for the acquisition of skin sensitization (Kimber et al., 2002). A critical exposure determinant for the development, and thus the evaluation, of skin sensitization risk is the dose per unit area of skin exposed (Robinson et al., 2000) rather than the total amount of chemical to which a subject is exposed. If GPT are considered in this context, only the Buehler method allows for precise calculation of the dose per unit area. The LLNA uses open application of the test substance, but the standardized application to the dorsum of the ear (an area calculated to be approximately $1\,cm^2$ (Kimber et al., 1994)) allows for the calculation of the dose per unit area. Additionally, exposure is via the relevant route. The use of adjuvant and intradermal injections in the GPMT causes difficulty in interpreting data in terms of direct extrapolation for skin sensitization in humans (Basketter et al., 1997). In contrast, the LLNA EC3 values of 26 chemicals had a linear relationship with their thresholds for the induction of sensitization derived from human repeated insult patch tests (Basketter et al., 2005b). The reproducibility of EC3 values is an additional, important feature for the assessment of risk.

Although human potency data are valuable for risk assessment, for many chemicals such data are not available. In these cases, it has been argued that LLNA EC3 values provide absolute potency information that is applicable to quantitative risk assessment (Griem et al., 2003). Based solely on LLNA data, two kinds of safe skin area dose levels have been derived: an 'acceptable non-sensitizing area dose' to protect non-allergic individuals against skin sensitization; and an 'acceptable non-eliciting area dose' to protect already sensitized individuals against elicitation of ACS. When applied to the sensitizers MCI/MI, cinnamic aldehyde and nickel, these approaches were found to give good agreement with clinical experience and experimental data (Griem et al., 2003).

Test systems have usually been considered to provide a means to assess a chemical's potency in relation to other chemicals tested in the same system, since the extrapolation of potency and threshold values from animals to humans is complicated by many factors, including interspecies variations and the system employed (including application frequency, occlusion, contact area) (Boukhman and Maibach, 2001). For example, in the LLNA, the exposure duration for the induction of sensitization is only three days. However, in real life, occupational and consumer exposure often lasts for months or years, involving both the induction and elicitation phases. To address this issue, mice were repeatedly exposed (at 7-day intervals for two months) to concentrations of sensitizers that did not induce an SI of ≥ 3 in the LLNA, to determine if, with prolonged exposures, they surpassed this threshold (van Och et al., 2003). The LNC proliferations measured at 60 days were no different from those achieved following a 3-day LLNA, which may indicate that no effect levels can be established for skin sensitization with the LLNA (Arts et al., 2006). This supposition is supported by the linear relationship of LLNA EC3 values of 26 chemicals with their thresholds for the induction of sensitization derived from human repeated insult patch tests (Basketter et al., 2005b). Additionally, clear thresholds for induction concentrations below the EC3 have been demonstrated (Scott et al., 2002), consistent with the increasing evidence for the existence of induction thresholds in humans (Boukhman and Maibach, 2001). However, the complexity of the induction–elicitation responses, with the degree to which skin sensitization has developed influencing the dose of chemical required to elicit a reaction (Scott et al., 2002), may mean that it will be necessary to consider separate dose–response relationships for sensitization and elicitation when establishing minimum exposure levels for chemicals that cause ACS.

Data are often not robust enough to identify a no-observed effect level (NOEL) with a high degree of precision. Therefore, for the purposes of quantitative risk assessment, an arbitrary classification scheme to assign default NOEL values based on LLNA EC3 values converted to dose per unit area of skin and/or human data has been developed (Gerberick et al., 2001a). Felter et al. (2003) described an approach for converting an EC3 value to a no-observed adverse effect level (NOAEL) which, for the case study chemical, hydroxycitronellal, was close to the NOAEL and low-observed adverse effect levels (LOAEL) determined from human tests. The NOAEL was then used to assign the chemical into one of the six categories proposed previously (Gerberick et al., 2001a), each with a default NOAEL. A 'sensitization reference dose' was calculated as the default NOAEL divided by a composite sensitization uncertainty factor, which could then be used to establish an acceptable safety level or to calculate a margin-of-safety compared to an actual human exposure. Demonstrations of the correlation of the LLNA EC3 value with NOAELs in non-diagnostic human patch tests (Basketter et al., 2005b; Gerberick et al., 2001b, 2003) indicated that this approach can be taken to identify non-diagnostic human repeat patch test NOELs without resorting to human testing.

A notable case of false-negative responses in the LLNA (Basketter et al., 1994; Basketter and Scholes, 1992) and other predictive tests (reviewed in Kimber et al., 1994) is that of metal salts, although it has been

possible to achieve positive, albeit weak and variable, responses to nickel sulphate in standard LLNA and MEST (Kimber et al., 1990a). The sensitivity of the LLNA to weak allergens such as nickel salts has been enhanced by modifications to the guideline protocol, such as abrasion of the dorsal ear surface prior to application of the test chemical (Ikarashi et al., 1992) or the use of two application phases, an intradermal injection in Freund's complete adjuvant followed by topical application (Ikarashi et al., 1993a). However, these amendments negate many of the advantages that the guideline LLNA protocol has over previous tests for contact sensitization. Nickel is widely used in jewellery, spectacles and buttons and allergic reactions are common, occurring in as many as 10% of the normal exposed population (Kligman and Basketter, 1995). Probably, nickel is in reality a weak contact allergen, with the high prevalence of nickel allergy in humans occurring as a result of extensive exposure rather than the inherent allergenic potential of the metal (Dearman et al., 1999; Kimber and Basketter, 1997). Interestingly, recent work has raised the possibility that the negative results in murine predictive tests may be the result of tolerance to nickel, induced by frequent oral exposure to low levels of nickel ions in the cage environment (Draeger et al., 2004).

4. False-positive reactions to irritants

For some time it has been recognised that irritants can give positive responses in the LLNA (Basketter et al., 1994; Loveless et al., 1996; Montelius et al., 1998, 1994), and concerns have recently been heightened by an apparent belief amongst notifiers to the UK CA that the assay results in an unacceptable level of false-positive responses (P. Evans, personal communication). Most of the information on LNC proliferative responses to irritants has been generated with the anionic surfactant SLS.

4.1. Sodium lauryl sulphate

For a long time it has been recognised that co-administration of allergen with the skin irritant SLS, resulting in mild inflammation at the exposure site, can increase the incidence of sensitization (Kligman, 1966). Notably, the OECD guideline for the GPMT (OECD, 1992) requires the topical application of SLS before the topical induction phase to achieve maximum sensitivity, in cases where the test chemical is not itself irritant. A modified LLNA that included pre-treatment with SLS has also been described, which enabled the detection of the sensitizing potential of 10 weak human contact allergens of low molecular weight (van Och et al., 2000).

Table 3
Examples of stimulation indices (SI) obtained when sodium lauryl sulphate has been tested in the LLNA

Concentration tested (%)	SI	Reference
25	4.2	Basketter et al. (1994)
25	5.3	Basketter et al. (1998)
25	7.6	Montelius et al. (1994)
20	3.5 to 8.6	Loveless et al. (1996)
10	Positive	Cumberbatch et al. (1993)
40	Negative	Kimber and Weisenberger (1989)
40	1.71	Ikarashi et al. (1993c)
25	1.13	Ikarashi et al., 1993a
20	1.49	Ikarashi et al., 1992

Although there have been isolated reports of sensitization to SLS in humans, (Foussereau et al., 1974; Prater et al., 1978), it is considered not to be a sensitizer. Despite this, there is a body of evidence to show that SLS produces adequate LNC proliferation for it to be identified by the LLNA as a potential sensitizer, albeit, often, with SI values that are only marginally above the classification limit (Table 3) (Basketter et al., 1994, 1998; Basketter and Kimber, 1996; Cumberbatch et al., 1993; Loveless et al., 1996; Montelius et al., 1994). An immunohistochemical assessment of T-cell proliferation, in a modified LLNA, detected a weak proliferation following exposure to SLS (Boussiquet-Leroux et al., 1995). However, the responses to SLS are not consistent between studies, even when conducted in the same laboratories (Table 3). For example, when two allergens were applied in combination with 10% SLS, the proliferative responses were additive in one case and synergistic in the second (Montelius et al., 1994). In another study, SLS failed to induce measurable LNC proliferation even when applied at a concentration that provoked local inflammation of the ear (Kimber and Weisenberger, 1989). It was also negative in the hands of other investigators (Ikarashi et al., 1993a,c), despite the mice's ears having been abraded before application to increase sensitivity (Ikarashi et al., 1992).

The reason for the positive responses to SLS has not been fully elucidated, although it is assumed that the dermal trauma caused by slight irritation will enable an easier access of allergenic chemical into and through the skin. One proposed explanation is that SLS stimulates cytokine (TNF-α) production in the epidermis, which leads to Langerhans' cell migration and the arrival of active dendritic cells in draining lymph nodes, with a subsequent proliferative activity (Cumberbatch et al., 1993; Loveless et al., 1996). These dendritic cells may

carry 'environmental antigens' (Basketter et al., 1996; Kimber and Basketter, 1992); the standard, radioactive LLNA protocol does not allow differentiation of antigen-specific immune responses from non-specific inflammatory reactions. Also, in practice, there is not a rigid division between skin irritants and sensitizers, and most contact allergens will have some ability to produce cutaneous inflammation (Dearman et al., 1999); for example, some materials used in weapons maintenance were shown to be both irritants and sensitizers (Arfsten et al., 2006).

4.2. LLNA responses to other irritants

Because SLS has the ability to cause the epidermal migration of Langerhans' cells (Cumberbatch et al., 1993), it has been proposed that this irritant is a special case, particularly since the majority of non-sensitizing skin irritants are negative in the LLNA (Dearman et al., 1999). For example, dibutyl phthalate, which induces very slight skin irritation, failed to elicit any proliferative activity when applied alone (Dearman et al., 1996). A modified assay was negative for the contact urticant benzoic acid and also for propylene glycol, even though the latter caused local inflammation when applied to abraded skin (Gerberick et al., 1992). Several studies have found methyl salicylate to be negative in the LLNA (Basketter and Scholes, 1992; Gerberick et al., 1992; Kimber et al., 1991, 1995, 1998). A safety assessment of the toxicological properties of a panel of salicylates also concluded that methyl salicylate was negative in the LLNA, whereas the response to salicylic acid varied according to the vehicle: it was positive when applied in acetone but negative in AOO (Cosmetic Ingredient Review Expert Panel, 2003). Two essential oils that were positive for irritancy at high concentrations gave negative results in the LLNA when tested at up to 50% (Craig et al., 2004).

However, positive responses to non-sensitizing irritants other than SLS have occasionally been reported and are often at odds with other studies. Positive responses to SLS, chloroform/methanol, Triton-X 100, oxalic acid, methyl salicylate and nonanoic acid have been documented (Montelius et al., 1998, 1994). In contrast, other studies found methyl salicylate to be negative (Basketter and Scholes, 1992; Gerberick et al., 1992), which was corroborated during inter-laboratory trials (Kimber et al., 1991, 1995, 1998); however, it has been suggested that the discrepancy in the findings of different investigators is due to differences in the concentrations of irritants administered (Montelius et al., 1998). A weak T-cell proliferation with salicylic acid has been detected

(Boussiquet-Leroux et al., 1995), and a later inter-laboratory study identified a false-positive, albeit weak (SI 3.3), response to isopropyl myristate applied at high concentrations (≥50%) (Ryan et al., 2000). Previously, three petrochemicals with irritant properties but that were not contact sensitizers in humans had been reported to be positive in the LLNA (Edwards et al., 1994); however, of these, two also gave positive responses in the GPMT.

The issue of false positives in both the GPMT and the LLNA has been addressed by Basketter et al. (1998). Whilst it was acknowledged that SLS and the structurally similar anionic surfactant C_{12-13} β-branched primary alcohol sulphate were weakly positive, and the response to another substance was equivocal, the majority of tested irritants (11 of 14) was negative. Included amongst these was the cationic surfactant benzalkonium chloride, which had previously tested positive in a modified assay (Gerberick et al., 1992). Another irritant, salicylic acid, was also positive in the latter study. However, the situation with these two chemicals is confused, since they have been reported as rare sensitizers (DeGroot et al., 1984; Rudzki and Kloslowska, 1976; Schallreuter et al., 1986). As discussed earlier, the irritant properties of a chemical may be influenced by the vehicle in which it is applied.

4.3. Responses to irritants in other animal and human tests

The LLNA measures non-specific proliferation, which has sometimes led to the assumption that it may give a higher incidence of false-positive responses than other predictive tests. However, irritant substances may also produce false-positive responses in the GPMT and other GPT which, clinically and histologically, are difficult to distinguish from mild allergic reactions (ECETOC, 2000; Kligman and Basketter, 1995; Maurer et al., 1991). Because of this, it can be difficult to demonstrate weak sensitizing properties of irritant chemicals in standard GPT (Botham et al., 1991). Re-challenge has been advocated as way to differentiate allergic from irritant reactions in GPT (ECETOC, 2000), although this adds to the complexity and animal welfare issues of the tests. The sometimes conflicting results with irritants seen in the LLNA also occur in GPT: benzalkonium chloride was negative in a GPMT (Gad et al., 1986) but has been reported to be a rare sensitizer, including in guinea pigs (Schallreuter et al., 1986). When responses to petrochemicals in different test systems were compared, two out of three test materials with irritant potential but not known to be human sensitizers were positive in

the GPMT (Edwards et al., 1994). SLS has also been associated with false-positive skin reactions in humans (Kligman and Epstein, 1975). In humans as well as guinea pigs, it can be difficult to distinguish allergic reactions from irritant reactions by morphology, particularly when the reactions are weak (Wahlberg, 1996).

4.4. Potential methods to differentiate irritancy from sensitization

The original selection of an SI value of 3 as being indicative of skin sensitization potential was made empirically. Statistical analysis of a large LLNA dataset for which GPT and/or human data were also available indicated that an SI of 3.4–3.6 would give a more accurate indication of hazard, but, in the view of the authors, it was preferable to err on the side of caution to ensure better health protection (Basketter et al., 1999a). However, it should be recognised that this SI value of 3 is not an inviolate figure that must be rigorously applied in all circumstances: interpretation of the test results should incorporate the consideration of other factors, such as dose–response relationships and normal biological responses (Dearman et al., 1999). For example, a weakly positive response to a high concentration of a known irritant would lead to the suspicion of a false positive (Basketter et al., 1998). According to the criteria suggested by Basketter et al. (1998) for identifying false-positive reactions in the LLNA, positive reactions with SLS would be suspect on account of the substance's significant irritancy, the absence of a structural alert for skin sensitization, and its giving a low dose response only at high concentrations.

Examination of differential immune responses to sensitizers and non-sensitizing irritants can be used to confirm such conclusions. For example, measurement of antigen expression on Langerhans' cells enabled the differentiation of skin sensitizers and an irritant concentration of SLS (Aiba and Katz, 1990; Cumberbatch et al., 1992). A combination analysis of the phenotype of draining LNC and the cell number per node improved the identification of irritants and allergens (de Silva et al., 1993; Sikorski et al., 1996). This approach was further refined by calculating the ratio of the B cell activation marker B220[+] population in treated versus vehicle draining lymph node samples, which enabled the differentiation of allergens from a panel of irritants that included SLS, benzalkonium chloride, methyl salicylate and salicylic acid (Gerberick et al., 2002). The B220+ ratio has also confirmed that positive LLNA responses to the fragrance material high purity d-limonene are owing to irritation rather than sensitization (Lalko and Api,

2007). A revision of the model used to predict such effects has recently been proposed (Betts et al., 2007b). Analysis of T cell activation/memory markers in the draining lymph nodes has also been used (Gerberick et al., 1997). Differential expression of Langerhans' cell antigens and epidermal cytokine mRNA have also been investigated (reviewed by Kimber, 1996). Currently, such proposed investigations to differentiate irritancy from sensitization, with their increased complexity and expense compared with the standard LLNA, are useful as research tools rather than for routine hazard identification for regulatory purposes.

Modifications of the standard LLNA have also enabled a better differentiation of sensitizers and irritants. For example, a 'long' protocol (animals were pre-exposed to the test substance applied under an occluded patch for 2 days prior to auricular application) did not show T-cell proliferation with SLS or salicylic acid, whereas the standard 'short' protocol did demonstrate weak proliferation with both these irritants (Boussiquet-Leroux et al., 1995). Another variant of the LLNA was able to accurately predict the sensitization potential of a weak sensitizer and SLS (Ikarashi et al., 1993a); unfortunately, the need for an intradermal application phase negated some of the advantages of the standard LLNA. The inclusion of a topical challenge phase (tier II of the LLNA) in equivocal cases has been suggested to enable the separation of irritants from sensitizers (Ulrich et al., 2001). An alternative proposed approach to determine relative sensitizing and irritant potential has been to combine measurements of LNC proliferation and acute, non-specific inflammatory responses (Ehling et al., 2005a,b; Homey et al., 1998; Vohr and Ahr, 2005). For example, the 'Integrated Model for the Differentiation of Skin reactions' (IMDS) involves the measurement of both LNC proliferation (cell counts and lymph node weights) and acute, non-specific inflammatory responses by ear thickness or ear weight (Homey et al., 1998; Vohr et al., 2000). Measurements of ear thickness have recently been combined with immunophenotypic end-points to correctly characterize a panel of irritants that included benzalkonium chloride and ethylenediamine (Reeder et al., 2007). Since OECD guideline 429 permits alternative end-points to the incorporation of radioactivity, registrants would be able to provide evidence of this sort or that suggested by Basketter et al. (1998) to support claims for chemicals to be irritants rather than sensitizers. To date, no such data have been submitted to the UK CA. The increased complexity and cost of obtaining measurements of the additional end points mentioned above would probably not be justifiable routinely, but could be valuable in cases

where the registrant strongly suspects or has good evidence that a substance is irritant but not a sensitizer, or in cases of a borderline SI value. As previously noted, registrants and regulators should recognise that the SI value of 3 is not a set-in-stone figure.

To summarise, the potential for false-positive reactions to irritants in the LLNA has been extensively investigated and characterised, so that it should usually be relatively straightforward to identify suspect results. In contrast, false-positive responses in the GPMT are poorly characterized, and it is pertinent that this test was developed to have optimal sensitivity, with no consideration given to its specificity (Basketter et al., 1998). Despite the likelihood that an SI of 3 as the threshold in the LLNA may be slightly precautionary (Basketter et al., 1999a), the predictive accuracy of this test has been shown to be equivalent to GPT in terms of identifying significantly sensitizing chemicals in humans (Gerberick et al., 2000). However, it should be remembered that many chemicals possess both irritant and sensitizing properties.

5. Animal welfare considerations

It is an important aspect of testing to identify human health hazards under REACH that animal testing should be minimised, and that, where new *in vivo* tests are necessary, animal welfare should be a prime concern. For skin sensitisation testing, REACH specifies that the 'LLNA is the first-choice method for *in vivo* testing. Only in exceptional circumstances should another test be used. Justification for the use of another test shall be provided' (EC, 2006). GPT are not specifically mentioned or excluded, but their use in preference to the LLNA will require scientific justification. GPT have major disadvantages in terms of animal welfare: they are prolonged studies over at least 3 weeks; sensitization may be elicited, causing discomfort to the animals; the concentration of test substance used for each induction exposure should cause mild-to-moderate skin irritation; and large numbers of animals are used (a minimum of ten or twenty in the treatment group for the GPMT and Buehler method, respectively) (OECD, 1992). Additionally, the GPMT (the GPT historically favoured by EU regulators) requires intradermal injections of the test substance in Freund's complete adjuvant (FCA) during the induction phase, which increases the severity of the procedure and thus animal distress. The combination of adjuvant, high intradermal induction concentration of test material, and occlusive dressing can lead to severe local effects. A test report submitted to the UK CA under the Notification of New Substances Regulations

(NONS) for a GPMT stated that 'test sites showed ulceration, necrosis and scabbing, which is a typical reaction of FCA. The effect became more severe after topical application of the test material.' It is also not unusual for control animals to exhibit adverse reactions. When conducting the Buehler assay, bandaging should be very tight to ensure occlusion and adequate levels of sensitivity. In terms of animal welfare, these tests should be viewed as outdated and causing unacceptable levels of suffering.

The use of the LLNA is a vast improvement in terms of refinement of testing procedures (minimal animal handling, no use of adjuvant, no elicitation phase and topical application only, so less discomfort and distress; assays conducted over six days) and, generally, a reduction in the numbers of animals used (four to five per group), especially when compared with the Buehler assay. Because of these improvements, in 2002 the UK CA adopted the LLNA as the preferred method for skin sensitization testing under NONS, informing notifiers that, from then, alternative tests would require full justification on a case-by-case basis. Shortly afterwards, this view was endorsed by the Home Office, which licenses animal testing in the UK, when it issued a statement that the LLNA was the method of first choice and that a scientific, case-by-case justification would be required for other, more aggressive tests. As a result, no new GPT have been conducted in the UK on industrial chemicals since October 2002.

6. Conclusions

The GPMT and other GPT were not a 'gold standard' against which to compare the LLNA. The LLNA is a relatively recent introduction to the battery of regulatory tests used to classify substances' hazardous potentials, and as such it is to be expected that there may yet be much to be learned about its capabilities and, also, its limitations. However, it offers significant advantages over the tests that preceded it, and, importantly for regulatory purposes, it has been more extensively investigated and validated than GPT. The potential problems that have been identified and covered in this review are not specific to the LLNA but rather are an inherent difficulty of using artificial animal tests, and they apply also to GPT. An advantage of the LLNA is that it is far less artificial than, for example, the GPMT. Issues that have been seen as a disadvantage, such as the potential for variability in EC3 values with different vehicles, can be turned to an advantage, for example in the production of more refined and accurate risk assessments. UK regulatory experience has indicated that the LLNA is not either over- or under-

predictive for skin sensitization hazard compared with the GPMT (Cockshott et al., 2006). Many modifications to the OECD-guideline test have been published, but for routine regulatory use the guideline method has proven to be robust and easy; in any case, the guideline does allow for some modifications such as the use of alternative vehicles and end-points. Although *in vitro* tests (reviewed by Kimber, 1996) and (quantitative) structure activity relationship approaches for skin sensitization hazard identification have been under development for some time, these are currently not suitable as stand-alone methods for regulatory purposes, and it is likely that the LLNA will remain the method of choice for some time to come.

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