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THE PERFORMANCE OF THE LOCAL LYMPH NODE ASSAY WITH CHEMICALS IDENTIFIED AS CONTACT ALLERGENS IN THE HUMAN MAXIMIZATION TEST

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Abstract—For many years, tests in the guinea pig have been the favoured option for the identification of the skin sensitization potential of chemicals. However, the mouse has been used widely in immunology research and can represent a viable alternative. A variety of murine assays have been described, including several methods based on ear swelling as an endpoint. Another option is to assess induced lymph node cell proliferation and it is this which forms the basis of the murine local lymph node assay (LLNA). The LLNA has undergone several successful interlaboratory validations and compares well with standard guinea pig assays. In the present study, the performance of the LLNA was examined with chemicals tested previously in the human maximization test (HMT). 30 chemicals, 23 of which proved positive in the HMT and seven of which were negative, have been tested. All but four of the materials found positive in the HMT also tested positive in the LLNA. Of these four, sulfanilamide and paraben esters would not classify as skin sensitizers in the guinea pig maximization test and nickel has been found to yield variable results in a number of predictive animal tests. Of the seven substances which proved negative in the HMT, six were also negative in the LLNA, the exception being sodium dodecyl sulfate. These data demonstrate that the LLNA is able to identify accurately chemicals which have the potential to cause significant allergic contact dermatitis in humans.

INTRODUCTION

The guinea pig is currently the species of choice for the identification of chemicals which have the potential to cause skin sensitization. The test methods used most commonly are the guinea pig maximization test (GPMT) (Magnusson and Kligman, 1970) and the occluded patch test described by Buehler (1965) (for reviews of the topic see Botham *et al.*, 1991; Kimber, 1992). From time to time however, the mouse has been proposed as an alternative and a range of test methods described: the mouse ear swelling test (Gad *et al.*, 1986) and variants thereof (Sailstad *et al.*, 1993), the mouse ear swelling assay (Descotes, 1990), and the non-invasive mouse ear swelling assay of Thorne *et al.* (1991a,b). However, the only murine assay which has the benefit of a substantial and successful interlaboratory validation is the local lymph node assay (LLNA) (reviewed by Kimber and Basketter, 1992). Furthermore, the LLNA has been the subject of detailed comparisons with the other main animal models (reviewed by Kimber *et al.*, 1994). Consequently, the assay is accepted as a screen for skin sensitizers by the Organisation for the Economic Cooperation and Development (OECD, 1993).

In view of the above, we have undertaken a comparison of LLNA data with results obtained previously from a prospective human assay, the human maximization test (HMT) (Kligman, 1966). Using a standard optimized method, the sensitizing potential of a range of chemicals was evaluated in humans to provide what could be regarded as a definitive dataset in terms of human hazard identification (Kligman, 1966). The results with 30 chemicals tested in the HMT have been compared with results from recent studies of the LLNA.

MATERIALS AND METHODS

Human maximization test. All the data presented in this paper are derived either from the comprehensive publication of Professor Kligman (Kligman, 1966) or work performed by him which was commissioned by the Research Institute for Fragrance Materials (Ford *et al.*, 1988; Opdyke 1974a,b, 1975, 1976 and 1978). Test details for the HMT are given in Table 1.

Local lymph node assay. Much of the data were taken from earlier work (Basketter and Scholes, 1992). New LLNA data were generated using the standard protocol defined previously (Kimber and

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Abbreviations: GPMT = guinea pig maximization test; HMT = human maximization test; ^{3}HTdR = [^{3}H]methyl thymidine; LLNA = local lymph node assay; LNC = lymph node cells; PBS = phosphate buffered saline; RIFM = Research Institute for Fragrance Materials; SDS = sodium dodecyl sulfate; TCA = trichloroacetic acid.

Basketter, 1992). Female CBA/Ca mice were used at an age of 8–12 wk. Groups of mice (n = 4) were treated by daily topical application for 3 consecutive days with 25 μl of one of three concentrations of the test chemical on the dorsum of each ear. Control mice were treated with vehicle alone in an identical manner. 5 days after the first topical application, all mice were injected iv with 250 μl phosphate buffered saline (PBS) containing 20 μCi of [^{3}H]methyl thymidine (^{3}HTdR) (Amersham, UK). The mice were killed 5 hr later and the draining auricular lymph nodes excised and pooled for each experimental group. A single cell suspension of lymph node cells (LNC) was prepared by gentle disaggregation through 200 mesh stainless-steel gauze. Pooled LNC were pelleted by centrifugation at 190 *g* for 10 min, washed twice with 10 ml PBS and resuspended in 3 ml 5% trichloroacetic acid (TCA). After incubation overnight at 4°C, the precipitate was recovered by centrifugation, resuspended in 1 ml 5% TCA and transferred to 10 ml scintillation fluid. Incorporation of ^{3}HTdR was measured by β-scintillation counting. The proliferative response of LNC was expressed as mean radioactive disintegrations per minute per lymph node (dpm/node for each experimental group and as the ratio of ^{3}HTdR incorporation into LNC of test nodes relative to control nodes [test:control (T:C) ratio]. A chemical was regarded as a sensitizer in the LLNA if at least one concentration resulted in a T:C ratio of 3 or greater and the data were not incompatible with a biological dose response (Kimber and Basketter, 1992). Details of the test concentrations and vehicles used for each chemical are given in Table 2.

Table 1. Test details for the human maximization test (HMT)

Chemical	Test concentrations (%)* Induction	Challenge
Glyoxal	10.0	2.0
Potassium dichromate	2.0	0.25
p-Phenylene diamine	10.0	0.5
Thioglycerol	50.0	5.0
Tetrachlorosalicylanilide	5.0	1.0
Mercuric chloride	2.0	0.05
Diethylenetriamine	10.0	10.0
Beryllium sulfate	5.0	1.0
Butylglycidyl ether	10.0	10.0
Chlorpromazine	25.0	10.0
Formaldehyde	5.0	1.0
Penicillin G	25.0	10.0
Nickel sulfate	10.0	2.5
Cinnamic aldehyde	8.0	2.0
Cobaltous sulfate	25.0	2.5
2-Mercaptobenzothiazole	25.0	10.0
Citral	8.0	8.0
Aniline	20.0	10.0
Neomycin	25.0	10.0
Sulfanilamide	25.0	10.0
Benzocaine	25.0	10.0
Hydroxycitronellal	10.0	10.0
Methyl/propyl paraben	25.0	10.0
p-Aminobenzoic acid	25.0	10.0
Geraniol	6.0	6.0
Methyl salicylate	8.0	8.0
6-Methyl coumarin	4.0	4.0
Resorcinol	15.0	5.0
Salicyclic acid	20.0	10.0
Sodium dodecyl sulfate	10.0	1.0

*Precise details of test conditions, vehicles etc. are contained in the original publications.

Table 2. Test details for the local lymph node assay

Chemical	Test concentrations (%)*	Vehicle
Glyoxal	5.0/10.0/25.0	DMF
Potassium dichromate	0.1/0.25/0.5	DMSO
p-Phenylene diamine	2.5/5.0/10.0	AOO
Thioglycerol	10.0/25.0/50.0	DMF
Tetrachlorosalicylanilide	0.25/0.5/1.0	Acetone
Mercuric chloride	5.0/10.0	AOO
Diethylenetriamine	5.0/10.0	AOO
Beryllium sulfate	2.5/5.0/10.0	DMF
Butylglycidyl ether	10.0/25.0/50.0	AOO
Chlorpromazine	10.0/25.0/50.0	DMF
Formaldehyde	5.0/10.0/25.0	AOO
Penicillin G	10.0/25.0/50.0	DMSO
Nickel sulfate	0.5/1.0/2.5	DMSO
Cinnamic aldehyde	5.0/10.0/25.0	AOO
Cobalt chloride	0.5/1.0/2.5	DMSO
2-Mercaptobenzothiazole	10.0/25.0/50.0	DMF
Citral	10.0/25.0/50.0	AOO
Aniline	10.0/25.0/50.0	AOO
Neomycin	5.0/10.0/25.0	DMSO
Sulfanilamide	10.0/25.0/50.0	DMF
Benzocaine	10.0/25.0/50.0	AOO
Hydroxycitronellal	10.0/25.0/50.0	AOO
Methyl/propyl paraben	5.0/10.0/25.0	AOO
p-Aminobenzoic acid	2.5/5.0/10.0	AOO
Geraniol	12.5/25.0/50.0	AOO
Methyl salicylate	5.0/10.0/25.0	AOO
6-Methyl coumarin	5.0/10.0/25.0	Acetone
Resorcinol	5.0/10.0/25.0	DMF
Salicylic acid	5.0/10.0/25.0	AOO
Sodium dodecyl sulfate	5.0/10.0/25.0	DMSO

DMF = dimethyl formamide DMSO = dimethyl sulfoxide
AOO = acetone–olive oil 4:1 (v/v)
*In ascending order of concentration, expressed as % (w/v).

Chemicals. The chemicals tested in the LLNA and their sources are listed in Table 3.

RESULTS

The results of the tests in both sensitization assays are summarized in Table 4. In the HMT, 23 of the chemicals were found to be skin sensitizers in humans; seven were without effect (Ford *et al.*, 1988; Kligman, 1966; RIFM 1974a,b, 1975, 1976 and 1978). For the 23 sensitizers, responses were spread evenly across a wide range (from 4 to 100% of subjects positive).

The LLNA results are shown as the ratio of proliferation in the test group at each concentration relative to the vehicle control. When this ratio is 3 or greater and the data are not incompatible with a biological dose response, the result is regarded as positive. On this basis, 18 of the 23 HMT positive chemicals were judged to be potential skin sensitizers in the LLNA, one (benzocaine) gave an equivocal result and four were found not to be positive. Aniline was regarded as positive by 'weight of evidence'—it reached a T:C ratio of 2.9 at the top concentration and there was clear dose response. Of the seven chemicals which proved negative in the HMT, six were also negative in the LLNA whereas the seventh, sodium dodecyl sulfate (SDS), gave apparently positive results. Previously, this chemical has failed to

Table 3. Test chemicals for the LLNA and their source

Chemical	Source*	Chemical	Source*
p-Aminobenzoic acid	Sigma	Aniline	Aldrich
Benzocaine	Sigma	Beryllium sulfate	Fluka
Butylglycidyl ether	Aldrich	Chlorpromazine	Aldrich
Cinnamic aldehyde	Quest	Citral	Quest
Cobalt chloride	Aldrich	Diethylenetriamine	Aldrich
Formaldehyde	BDH	Geraniol	Quest
Glyoxal	Aldrich	Hydroxycitronellal	Quest
2-Mercaptobenzothiazole	Aldrich	Mercuric chloride	Aldrich
6-Methyl coumarin	Aldrich	Methyl salicylate	Sigma
Neomycin	Sigma	Nickel sulfate	Sigma
Penicillin G	Sigma	*p*-Phenylene diamine	Aldrich
Potassium dichromate	Aldrich	Propyl paraben	Chesebrough Ponds
Resorcinol	Aldrich	Salicylic acid	Aldrich
Sodium dodecyl sulfate	BDH	Sulfanilamide	Lancaster
Tetrachlorosalicylanilide	Ciba-Geigy	Thioglycerol	Fluka

*Aldrich Chemical Co., Gillingham, Dorset, UK; BDH Chemicals Ltd, Poole, Dorset UK; Chesebrough Ponds, Trumbull, CT, USA; Ciba-Geigy, Basel, Switzerland; Fluka AG, Glossop, Derbyshire, UK; Lancaster Synthesis, Morecambe, Lancashire, UK; Quest International, Ashford, Kent, UK; Sigma Chemical Co., Poole, Dorset, UK.

Table 4. Classification of sensitization potential

		LLNA	
Chemical	HMT%	T:C ratio	Result
Glyoxal	100	18.1/13.6/12.2	+
Potassium dichromate	100	3.5/10.2/10.4	+
p-Phenylene diamine	100	12.8/16.5/23.3	+
Thioglycerol	100	6.7/10.0/10.0	+
Tetrachlorosalicylanilide	95	11.2/14.4/18.0	+
Mercuric chloride	92	19.9/11.8	+
Diethylenetriamine	84	6.4/12.1	+
Beryllium sulfate	82	8.4/7.1/9.4	+
Butylglycidyl ether	79	1.4/2.2/5.6	+
Chlorpromazine	75	11.8/13.7/8.9	+
Formaldehyde	72	3.7/4.0/5.8	+
Penicillin G	60	1.5/3.8/8.9	+
Nickel sulfate	48	1.1/1.5/1.5	−
Cinnamic aldehyde	44	12.5/18.4/15.4	+
Cobalt chloride	40	3.2/3.7/2.8	+
2-Mercaptobenzothiazole	37	4.5/4.6/5.5	+
Citral	32	2.1/5.0/9.3	+
Aniline	28	1.4/1.8/2.9	+*
Neomycin	28	1.0/0.9/1.0	−
Sulfanilamide	20	1.0/1.0/0.9	−
Benzocaine	19	1.7/2.0/0.9	+/−
Hydroxycitronellal	8†	1.7/3.2/6.7	+
Methyl/propyl paraben	4	1.4/1.0/1.3	−
p-Aminobenzoic acid	0	1.1/0.9/1.0	−
Geraniol	0	0.9/1.2/2.6	−
Methyl salicylate	0	1.3/1.0/0.8	−
6-Methyl coumarin	0	0.8/1.0/0.8	−
Resorcinol	0	2.2/2.2/2.7	−
Salicylic acid	0	0.8/1.5/2.5	−
Sodium dodecyl sulfate	0	3.2/4.0/4.2	+

LLNA = lymph node assay HMT = human maximization test
T:C ratio = test:control ratio
*Regarded as positive on a 'weight of evidence' basis.
†Data taken from Ford *et al.* (1988). Hydroxycitronellal gave variable results in nine studies. For consistency, the highest result obtained by Kligman has been given.

cause significant lymph node activation (Kimber and Weisenberger, 1989).

DISCUSSION AND CONCLUSIONS

Classically, the skin sensitization potential of chemicals has been determined by using guinea pig tests. Consequently, when validating alternative assays, there is a strong temptation to judge the merits of the new assay against the 'gold standard' of a guinea pig assay such as the maximization test (Magnusson and Kligman, 1970). However, technical aspects of test conduct and species differences between guinea pigs and humans inevitably mean that no such gold standard exists. Guinea pig data may not always mirror precisely and quantitatively the extent of the hazard to humans. An illustrative example of this has been reported for sulfanilic acid (Basketter *et al.*, 1992). In general, though, it is difficult to compare predictive tests with human data, since the former relate to

hazard identification, whereas the latter represent problems arising from the expression of that hazard (risk). However, for skin sensitization, we are in the fortunate position of having a definitive human dataset. Almost three decades ago, Kligman used a rigorous protocol in groups of human volunteers to assess the sensitization potential of more than 90 chemicals and preparations (Kligman, 1966). Subsequently this dataset has been supplemented by RIFM who commissioned human maximization tests from Kligman. In the present work, therefore, we have compared HMT results with those generated in the LLNA, taking 23 chemicals identified as positive in the HMT and seven chemicals found negative.

Of the 23 chemicals positive in the HMT, 18 were clearly positive in the LLNA, while one gave equivocal results and four were definitely negative. The chemical which yielded equivocal results was benzocaine. Data obtained with this material have been discussed elsewhere (Basketter and Scholes, 1992; Basketter *et al.*, 1993). Other workers have obtained positive results (Kimber and Weisenberger, 1989; J. Montelius, personal communication, 1993). The four 'false negatives' in the LLNA were nickel, sulfanilamide, neomycin and the paraben esters. Of these, the allergen of most concern is nickel. Nickel is well known as a human contact allergen for which it is difficult to obtain significant and reproducible positive results in animal models (Kimber *et al.*, 1990; Wahlberg, 1989), although this may depend heavily on the procedure used (Turk and Parker, 1977). However, it should be remembered that the importance of any allergen as a human contact sensitizer is a function of both intrinsic potential and exposure. In this equation, it is probable that it is the extent and nature of exposure to nickel which makes it such a frequent contact allergen in humans.

Two of the HMT positives found negative in the LLNA are less worrying. Sulfanilamide gave only a slight response in a concurrent GPMT (1/10 test animals exhibited weak erythema—data not shown). Thus sulfanilamide would not classify as a skin sensitizer when interpreted according to current EEC guidelines. The paraben esters are extremely weak sensitizers in humans (Andersen *et al.*, 1992) and are negative in guinea pig tests (Goodwin *et al.*, 1981). Consequently it can be concluded that the LLNA failed to detect only one expected contact allergen, neomycin. In our hands, this chemical gave a 30% positive response in the GPMT and was thus on the borderline of EEC classification as a skin sensitizer (Basketter and Scholes, 1992).

Of the seven chemicals found not to be human contact sensitizers in the HMT, six were negative in the LLNA. The seventh, SDS, on this occasion gave a positive result. Previously SDS has failed to produce a significant response (Kimber and Weisenberger, 1989). Two points need to be considered. First, it is difficult to demonstrate weak sensitizing properties of irritant chemicals using standard guinea pig methods (see for example Botham *et al.*, 1991). Secondly, SDS has been shown to cause Langerhans' cell migration from the epidermis to draining lymph nodes, presumably by stimulating the production by keratinocytes of tumour necrosis factor-α (Cumberbatch *et al.*, 1993). It has been suggested that this may result in an increased flux of 'environmental antigens' to the draining lymph node with a consequent transient increase in cell proliferation (Kimber and Basketter, 1992).

In conclusion, the data presented in this paper show that the LLNA identifies important proven human contact allergens. It should be remembered that, although the LLNA may not detect some weaker contact allergens identified in the most sensitive guinea pig assays, the classification thresholds set by the EEC mean that these chemicals need not be positive in a suitable alternative assay. The LLNA offers refinement and reduction in the severity of procedure compared with guinea pig assays, especially the GPMT. As such, it represents a viable and desirable alternative to guinea pig tests.

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