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THE NON-RADIOACTIVE LOCAL LYMPH NODE ASSAY: SKIN SENSITISATION AND IRRITATION TESTING

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This study was performed to investigate whether alternative endpoints of the local lymph node assay — non-radioactive (LLNA-nR) like the ear weight and count cells would give additional information about the sensitizing/irritating potential of a tested substance. The legislation of REACH — Registration, Evaluation and Authorisation of Chemicals (Regulation EC/1907/2006) prefers alternative testing for skin sensitization and irritation. We have performed classic testing methods for skin irritation (Draize rabbit test), for skin sensitization (Maximization test on guinea-pig, GPMT) and alternative method LLNA-nR with verified skin sensitizers, skin irritants and with some compounds which were intended to be subjected to registration process according to REACH and other legislation rules.

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It was possible to divide the tested chemicals into three groups according to results of classic testing methods described above: (1) contact irritants as well as contact allergens; (2) contact irritants; (3) contact allergens. Then all the chemicals have undergone the testing in LLNA-nR method with two end-points: lymph node cell count as a measure of sensitizing potential and the ear disc weight as a measure of irritation potential. Unfortunately our hypothesis turned out to be non-realistic. With the chemicals when both LLNA-nR end-points gave positive results it was not possible to differentiate between the cases when the tested chemical had both irritation and sensitization potentials and the case when the substance had only strong irritation potential, which could induce the cell proliferation in the draining lymph node and further testing would be needed. Then the intention to reduce the number of animals used in this type of testing was not satisfied especially because recently we have only the classic GMPT test for further discrimination testing. In our opinion the advantage of this procedure is the fact that it is simple enough especially for routine testing.

Introduction

Cutaneous toxicity may have several forms – those of greatest prevalence being allergic contact dermatitis (ACD) and irritant contact dermatitis (ICD) [1]. Although the skin elicited reactions are usually indistinguishable with respect to macroscopic or histopathological appearance, the pathogeneses for ACD and ICD are clearly very different, with the former, but not the latter, being dependent upon the initiation of a primary cutaneous immune response and skin sensitization [2]. It is well established that antigen presentation to the T-cells are essential in the mechanism of ACD. In contrast ICD is believed to activate the immune cascade independent of the antigen presentation pathway, by stimulating release of proinflammatory mediators and cytokines that directly recruit and activate T cells. The precise mechanism of skin irritancy is still unclear [3].

Traditionally, testing for acute skin irritation has been conducted in animals [4]. Back in the mid 1940s, Draize (1944) published a method for assessing skin corrosion and irritation hazard in rabbits [5]. This method, modified to varying degrees by regulatory authorities in different parts of the world, became the world standard [6].

A variety of animal test methods are available for the identification of chemicals that have the potential to cause skin sensitization and allergic contact dermatitis. Originally, guinea pigs represented the species of choice for skin sensitization predictive tests and two methods using this species, the guinea pig maximization test [7] and the occluded patch test of Buehler [8], have found wide application. More recently, a method in the mouse has been developed, the murine local lymph node assay (LLNA) [9,10], having been endorsed by the Interagency

Coordinating Committee on the Validation of Alternative Methods (ICCVAM) as a stand-alone method for the evaluation of skin sensitizing potential [11]. The end-point is the assessment of cell proliferation through measuring of incorporated radioactivity by scintillation method (LLNA-R). In the case of this method the possibility of false positive results exists caused by non-specific cell activation as a result of inflammatory processes in the skin (irritation). This is based on the fact that inflammatory processes in the skin may lead to non-specific activation of dendritic cells, cell migration and non-specific proliferation of lymph node cells. Measuring of cell proliferation without taking into account the irritating properties of test items thus could lead to false positive results [12].

In the case of the LLNA-nR the end-point is the cell proliferation in draining lymph node assessed by conductometric measuring of cell count. The measurement of cell concentration is used for determination of cell proliferation in toxicology studies, and this proceeding is authorized by OECD Test Guidelines. This end-point is used for the identification of chemicals that have the potential to cause skin sensitization. False positive results with certain skin irritants are disadvantages of non-radioactive as well as radioactive LLNA. The second end-point of LLNA-nR (ear weight) is focused to the determination of irritation effect. Ear weight of the apical area of both ears were prepared and immediately weighed in order to assess the irritation potential of the test chemicals. Irritation is an acute inflammatory reaction which causes increased vascular permeability. The following influx of lymph fluid is responsible for the increase of measured ear weight. We use second end-point for the recognition of false positive results of certain skin irritants. The primary aim of our experiments was to assess the possibility of using LLNA-nR for the determination of allergenic and irritation potential of chemicals with untested dermal toxicity in one test.

The aim of this paper was to test the hypothesis that it would be possible to use the LLNA-nR testing method as single stand-alone method for the evaluation of sensitizing and irritation potential of untested chemicals. There is not only one test for simple determination of two skin toxicity endpoints — sensitization and skin irritation to date.

Materials and Methods

Chemicals: α-hexylcinnamaldehyde (HCA) (Sigma-Aldrich, USA); sodium dodecylsulfate (SDS) (Sigma-Aldrich, USA); dimethylsulfoxide (DMSO) (Sigma-Aldrich,USA); Chloramine B (CH-B) (Bochemie Bohumín, CZ); Biolit UNI (Lybar Velvěty, CZ) and 1-chloro-3,5-dimethyladamantane (DMA) (JSC Olain Farm, CZ) induced skin irritant reaction. Skin-sensitizing potential was proved by GPMT method for HCA, hydroquinone (HCh) (Sigma-Aldrich, USA); CH-B, 2,6-dinitro-4-*tert*-butyl-chlorobenzene (DNTCB) (Synthesia Pardubice, CZ) and 2-

cyclohexyl-1,4-naphtoquinone (cNCh) (VUOS Rybitví, CZ).

Animals: for the test LLNA were used nulliparous and non-pregnant females of laboratory mouse, strain BALB/C, SPF quality. Breeding farm BioTest s.r.o., the Czech Republic. Six animals per group for testing one concentration of chemical were used. The animals were fed standard pellets for laboratory animals, water *ad libitum*. The age of animals at the start of the test was from 8 to 12 weeks. The animal weight deviations were minimal and did not exceed 20 % of average weight. The temperature in the testing room was 22 ± 3 °C, relative humidity was 30-70 %, and lightning was 12 hours light. For skin irritation test were used white albino rabbits, New Zealand strain, body weight ca 3 kg. For maximization test were used albino guinea pigs, BFA strain, age 6-7 weeks. Housing conditions — according to the rules set down in European Convention (1986).

Test methods:

A) Draize test – Skin irritation test was performed according to Method B.3 Acute Toxicity (Dermal), Directive 92/69/EEC. Published in OJ L 383A, 1992; OECD Test Guideline No. 402.

B) Maximisation test on guinea-pigs: classic sensitisation testing method was performed following Method B.6, Skin sensitisation, Directive 96/54/EC, Published in OJ L 248, 1996; OECD Test Guideline No. 406.

C) LLNA-nR according to Ulrich *et al.* (2001): solvent DAE433 (40 % v/v *N,N*-dimethylacetamide, 30 % v/v acetone, 30 % v/v ethanol). Positive controls substances: DNCB, HCA (Sigma – Aldrich, USA). The volume of 25 µl of tested solution (tested substance of specific concentration or vehicle or positive control solution) was applied to the dorsal side of both ears in 3 consecutive days. The first 20 % (w/v) HCA was used as positive control substance and then DNCB 0.5 % (w/v). The concentration test substances were: 30 % (w/v), 3 % (w/v), 0.3 % (w/v). The auricular lymph nodes are excised on day 4th. These nodes are draining the ear area. Both of the nodes were excised and weighed for each animal individually. Both of the lymph nodes were disaggregated by gentle mechanical pushing through 100 µm mesh nylon gauze with pooling of 1 ml PSB (Phosphate Buffered Saline). The cell concentration in the resulting suspension was measured by Coulter tip Celltac α. Preparations of ear weight Punch biopsies 1 cm in diameter of the apical area of both ears were prepared and immediately weighed on analytical scales in order to verify the irritation potential of the test chemicals. The test efficiency evaluated by comparison negative and positive controls. All the samples at all the dosage levels were compared with the negative control.

Two parameters in the test LLNA — leukocyte proliferation (cell concentration in suspension) and weight of ear discs (irritation effect) were evaluated. Software Statgraphic ® Centurion (version XV, USA) was used for the statistical calculations. Each of the two measured parameters was at first statistically evaluated by non-parametric Kruskal-Wallis test for the comparison

of all the measurements with the vehicle control, as global test. Then the non-parametric two-group Mann-Whitney rank test was used for all two-group comparisons (probability level 0.05).

Results

In our laboratory all the compounds were tested by classic testing methods for skin irritation (Draize rabbit test) [4] and for skin sensitization (Maximization test on guinea-pig) [13] and further by alternative LLNA-nR method. The tested chemicals were classified into three groups according to the results obtained in the testing by classical methods (Table II): (*i*) contact irritants as well as contact allergens — included chemicals with both effects (positive Draize test, positive GPMT); (*ii*) contact irritants only — comprised true skin irritants (positive Draize test, negative GPMT); (*iii*) contact allergens only — comprised true skin allergens (negative Draize test, positive GPMT).

Table II Evaluation of sensitizing/irritating potential of tested substances

Groups	Chemicals	Draize test	GPMT	LLNA-nR	
				Ear weight	Cell proliferation
Contact irritant as well as contact allergen	1-chloro-3,5-dimethyladamantane	+	+	+	+
	Chloramine B	+	+	+	+
	α-hexyl-cinnamaldehyde	+	+	+	+
Contact irritant not contact allergen	Biolit UNI	+	–	+	+
	dimethylsulfoxide	+	–	+	+
	sodium dodecylsulphate	+	–	+	+
Contact allergen not contact irritant	2,6-dinitro-4-*tert*-butyl-chlorobenzene	–	+	–	+
	2-cyclohexyl-1,4-naphtoquinone	–	+	–	+
	hydroquinone	–	+	–	+

Table I Summary of LLNA-nR results: lymph node activation (cell count) and skin irritation induced by contact allergens and irritants

		Cell count, $*10^3$ ul^{-1}	SD	Ear weight, mg	S
1-chloro-3,5-dimethyladamantane	vehicle	7.13	2.84	23.72	1.63
	30 %	26.12*	7.28	31.08	3.06
Chloramine B	vehicle	7.20	3.30	23.75	1.44
	0.30 %	7.62	2.21	24.95	0.75
	3 %	10.68	4.55	25.56	1.13
	30 %	16.68*	3.90	29.58*	2.22
α-hexyl-cinnamaldehyde	vehicle	11.14	1.67	24.52	2.46
	10 %	14.04*	13.4	32.40*	5.43
	vehicle	11.08	4.31	26.20	3.01
	20 %	25.83*	6.92	33.50*	2.07
Biolit UNI	vehicle	7.13	2.84	23.72	1.63
	30 %	31.75*	11.5	27.57*	1.44
dimethylsulfoxide	vehicle	8.61	3.09	23.23	0.90
	30 %	14.81*	3.62	26.70*	1.01
sodium dodecylsulphate	vehicle	11.14	1.67	24.52	2.46
	20 %	19.26*	3.78	39.28*	9.12
2,6-dinitro-4-*tert*-butyl-chlorobenzene	vehicle	10.78	2.49	35.12	1.14
	20 %	21.02*	2.88	36.76	1.37
2-cyclohexyl-1,4-naphtoquinone	vehicle	11.08	4.31	26.20	3.01
	7.5 %	14.02	5.09	24.96	1.71
	15 %	15.28	6.17	26.17	2.73
	30 %	23.30*	4.42	28.97	3.49
hydroqinone	vehicle	11.14	1.67	24.52	2.46
	1 %	20.76*	6.56	27.84	1.97

* Statistically significant compared to vehicle treated control; $P < 0,05$.
Contact allergen and irritant dose depending include lymph node cell concentration and ear weight. In 3 consecutive days, six BALB/C mice per group were topically treated with HCh (1

%), cNCh (30 %), DNTCB (20 %), SDS (20 %), DMSO (30 %), Biolit (30 %), HCA (20 %), CH-B (30 %), DMA (30 %), or vehicle (NC-negative control) on the dorsal surface of both ears. On Day 3, local draining lymph nodes of the ears were removed and individual lymph node cell counts, ear weight and lypmh node weight were determined.

These chemicals were then tested in LLNA-nR assay: *Group 1:* 1-chloro-3,5-dimethyladamantane (DMA), Chloramine B (CH-B) and α-hexyl-cinnamaldehyde (HCA) are contact irritants as well as contact sensitizers, these results were confirmed by classic testing methods performed in our laboratory. These substance caused statistically significant increase in both end-points of the LLNA-nR, i.e. on the basis of stand-alone method LLNA-nR it could be possible to agree that they have both irritation and sensitization potentials. *Group 2:* according to the results of classic tests the compounds SDS, DMSO and Biolit UNI belong to the group of irritating chemicals without sensitizing potential. But the LLNA-nR method showed that these chemicals increased LN cell proliferation significantly i.e. using stand-alone method LLNA-nR they would be identified as both irritants and sensitizers in the same way as the chemicals in Group 1. Then such a result will be false positive from sensitization point of view. *Group 3:* using LLNA-nR, end-point ear weight was not increased for hydroquinone (HCh), 2,6-dinitro-4-*tert*-butyl-chlorobenzene (DNTCB), 2-cyclohexyl-1,4-naphtoquinone (cNCh) and Draize test confirmed these negative results. Also the sensitization potential was identified properly (Table I).

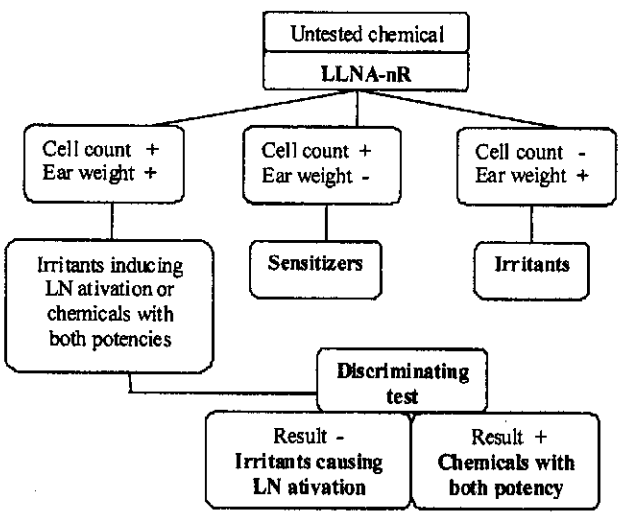

Fig. 1 Scheme of discrimination between sensitizing or irritating potential of chemicals with untested skin toxicity properties

In the case of both end-points of LLNA-nR being positive it would be convenient to use the second test for discrimination of irritants causing LN

activation and chmicals with both effects (Fig.1). This approach to skin reaction testing is not new. A few years ago an Integrated Model for the Differentiation of Skin Reactions (IMDS) was proposed by Homey *et al.* [14] which was claimed to quickly and reliably differentiate between allergenic and irritation potential of chemicals by simple parameters. This model is based on testing procedure similar to LLNA-nR, when instead the weight of ear discs the ear swelling is measured. In our opinion the IMDS procedure is not simple enough especially for routine testing. It depends on establishing of "maximal ear swelling" and "maximal cell count" values which could be different in individual laboratories. These values then serve to calculation of series of the indexes, which are in the end related to each other to form the final dimensionless quantitative measure. Our approach was meant to avoid this procedure and use simply the results of LLNA- nR for further decisions.

Discussion and Conclusion

The LLNA-nR cell proliferation of LN cells determined *via* the count of cells was use as endpoint criteria. As mentioned above a major disadvantage of this LLNA-nR but also of „standard" LLNA [9] is the inability to differentiate between the increase in cell count caused by irritating potential of a chemical and the sensitizing effect. This could lead to false positive results as it has been shown for some (photo-)irritating substances [15-18].

The aim of this paper was to confirm the ability of LLNA-nR testing method for the evaluation of allergenic and irritation potential of untested chemicals. In this case only one test could be used for the determination of two toxic endpoints — sensitization and skin irritation. In such case we can avoid using the rabbit Draize test (very detrimental to experimental animals) for the assessment of skin irritation potential. We have performed experiments with verified skin sensitizers, verified skin irritants and with some compounds which were intended to be subjected to registration process according to REACH or other legislation rules. The data about skin effects of some of the tested chemicals were found in the literature. HCA induces weak-moderate skin sensitization reaction [19,20] and it is recommended as positive control for OECD for guinea pig procedures and the LLNA [21]. SDS causes skin irritation [22] and DMSO displayed considerable lymph node activation potential [23]. Hydroquinone (HCh) induces skin sensitization [24,20]. Chloramine-B (CH-B), Biolit UNI, 2-cyclohexyl-1,4-naphtoquinone (cNCh), 1-chloro-3,5-dimethyladamantane (DMA) and 2,6-dinitro-4-*tert*-butyl-chlorbenzene (DNTCB) were tested in our laboratory for the purpose of chemical registration process and no literature data have been found. All the compounds examined were tested in our laboratory by classic testing methods for skin irritation (Draize rabbit test, OECD 404) and for skin sensitization

(Magnusson and Kligman maximization test on guinea-pig, OECD 406) and the results were in agreement with literature data.

On the basis of the results described above we should conclude, that our presumption that it would be possible to identify the two toxicity endpoints — irritation and sensitization potential by performing only one test, LLNA-nR, turned out to be non-realistic. This identification is possible only in the case of unique combination of partial results of LLNA-nR, e.g. when ear weight will not increase (irritation potential identification), and on the other hand, the cell count will be significantly higher (sensitization potential identification). In the cases when both measurings give positive results it is not possible to differentiate between the cases when the tested chemical have both potentials and the case when the substance has only strong irritation potential. Further testing would be needed for identification of potential false positive sensitization result. Then the intention to reduce the number of animals used in this type of testing will be not satisfied especially when we have only the classic GMPT test. Cell culture represents the most promising alternative method, and many tests of various kinds are in progress, for example the monolayer culture, skin explant culture [25] and three-dimensional skin equivalent culture [26]. Maybe in the future when we could use some special targeted examinations (distinct immune cell-type, single cytokine) as additional endpoint we can return to this idea.

Unfortunately we found no chemicals which induce only increase in ear weight detected by LLNA-nR and are only skin irritants but not sensitizers (by the results of classic testing) as was outlined in the scheme proposed in results (Fig. 1). This study also not involved the compounds with negative results in classic test for both irritation and sensitization. Finding appropriate standards and testing of other substances is an essential condition for further research in this area.

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