Table 2. Results of non-radioisotopic local lymph node assay with isoeugenol, isoeugenol dimers and eugenol used in this study

% tested	Isoeugenol		β-O-4-Dilignol		Dehydrodiisoeugenol		Eugenol	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
0%	1.00	0.10	1.00	0.12	1.00	0.11	1.00	0.10
3%	1.52	0.49	1.02	0.27	1.95	0.42	0.75	0.13
10%	2.43	0.45	1.19	0.30	3.09	0.31[a]	1.46	0.20
30%	6.73	0.88[a]	1.05	0.20	5.37	0.50[a]	3.83	0.68[a]
EC3 (%)	12.7		>30		9.4		22.8	

Results represent mean values and standard errors in four mice

The stimulation index (SI) was calculated by dividing the mean value obtained in each treatment group by that of the control group.

[a] Significantly different from the concurrent vehicle control (0%) at $P < 0.05$ (Dunnett's test).

10% and 30% dehydrodiisoeugenol were 1.95 ± 0.42, 3.09 ± 0.31 and 5.37 ± 0.50, respectively. Stimulation indices for 3%, 10% and 30% eugenol were 0.75 ± 0.13, 1.46 ± 0.20 and 3.83 ± 0.68, respectively. Concentrations of 10% or greater exposure of dehydrodiisoeugenol, 30% of isoeugenol and eugenol caused a significant increase in SIs compared with concurrent vehicle controls (Dunnett's multiple comparison tests, $P < 0.05$). Whereas β-O-dilignol did not show any lymph node cell proliferative activity in all concentrations tested. The EC3 values for isoeugenol, dehydrodiisoeugenol and eugenol were calculated using a standard method and found to be 12.7, 9.4 and 22.8, respectively. With regard to the test groups given β-O-dilignol, SI values did not exceed a value of 3 even in the highest dose group, and the EC3 value for this chemical was estimated as >30% for the purposes of comparison.

Discussion

Allergic contact dermatitis is regarded as a serious side effect of chemical products. Eugenol and isoeugenol are components of clove oil and nutmeg oil, and are fragrance chemicals with a spicy scent. They are extensively used in perfumes and deodorants, moreover they are expected to make whitening and antibacterial effects in the cosmetic and dermatological fields (Yamazaki *et al.*, 1998; Yamazaki *et al.*, 2000). However, these chemicals are known to be human contact sensitizers. Isoeugenol is a much stronger sensitizer than eugenol and classified as a moderate allergen, human class 2 (Basketter *et al.*, 2000). Meanwhile eugenol is classified as a weak allergen, human class 3. Isoeugenol has been reported to induce positive responses in the standard LLNA, other murine predictive tests and guinea pig prediction tests (Barratt and Basketter, 1992; Basketter *et al.*, 2005; Hilton *et al.*, 1996). The decreased sensitization potency of eugenol by dimerization with the addition of a hydroxyl group was previously reported (Takeyoshi *et al.*, 2004). Isoeugenol is an important cosmetic ingredient similar to eugenol, but isoeugenol is a stronger human sensitizer than eugenol. Therefore the possibility of reducing the sensitization potency of isoeugenol by dimerization was examined as well as a case of eugenol.

The guinea pig maximization test is a conventional skin sensitization test method, and it has been widely used for regulatory purposes. However, this method is not suitable for quantitative sensitization potency prediction because GPMT requires an independent dose selection for intradermal and topical applications. The LLNA is recognized as a reliable sensitization test, and it is currently accepted by many authorities for regulatory purposes. From the point of view of animal welfare, the LLNA is a preferable test method and it is the first choice sensitization test for the REACH program in the EU. In addition, LLNA can give a quantitative endpoint and it would be suitable for sensitization potency prediction. However, the standard LLNA requires the use of radioisotopes for detecting the lymph node cell proliferative response, and it requires specialized test facility and handling procedures. Meanwhile, non-RI LLNA employed in this study is totally based on the standard LLNA protocol except for using BrdU instead of ^{3}H-thymidine, and it can provide sensitizing potency information as well as standard LLNA (Takeyoshi *et al.*, 2004, 2005). So these two types of major skin sensitization tests were conducted.

The present investigations have shown that this chemical also tested positive in the non-RI LLNA and the GPMT. The derivation of EC3 values provides an opportunity to compare these forms of the assay with respect to sensitivity. In one series of investigations, the EC3 values for isoeugenol and eugenol were recorded as values of approximately 1.3% and 13%, respectively (Basketter *et al.*, 2000). In this study using the non-RI LLNA reported here, the EC3 values for isoeugenol and eugenol were calculated as 12.7% and 22.8%, respectively. The EC3 values obtained in the non-RI LLNA for these chemicals were somewhat higher than those in the standard LLNA. However, the EC3 in non-RI LLNA

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DOI: 10.1002/jat

for isoeugenol was lower than that for eugenol, thus the quantitative relationship of EC3 values are regarded as the same as the standard LLNA, and the values would be a parameter for the relative sensitization potency. The GPMT was also conducted with the same test chemicals. The sensitization rate for isoeugenol and eugenol were 100% and 20%, respectively. Thus isoeugenol was classified as an extreme sensitizer according to the classification criterion of this method. Similarly eugenol was classified as a moderate sensitizer. The results of non-RI LLNA and guinea pig maximization tests for isoeugenol and eugenol were consistent with the results reported previously.

In this study two types of isoeugenol dimer were tested with different bond-structures in addition to their monomer. Dehydrodiisoeugenol, one of the isoeugenol dimers, showed a 50% sensitization rate in the guinea pig maximization test and was classified as a moderate sensitizer. In the non-RI LLNA, this chemical induced dose dependent lymph node cell proliferation, and its EC3 value was estimated as 9.4%. Meanwhile, the other type of isoeugenol dimer, β-O-dilignol, turned out to have a 0% sensitization rate in the guinea pig maximization test, and was classified as a weak sensitizer. This chemical caused no lymph node cell proliferation, and its EC3 value was estimated as >30%.

Dehidrodiisoeugenol is a dimer in which the monomers are covalently bonded each other, so it would not be easily broken down to monomers in the skin. β-O-Dilignol is a dimer in which the monomers are bonded with ether bond. β-O-Dilignol may be broken down easier than dehydrodiisoeugenol. However, β-dilignol exerts less sensitization potency than its monomer, isoeugenol. It shows that the degradation of β-dilignol in the skin is not significant in this study.

In this study, dimerization of isoeugenol also yielded dimers with different sensitization potencies. Isoeugenol and eugenol are both important cosmetic ingredients, so the reduction of sensitization potency achieved by the dimerization may lead to the development of safer cosmetic ingredients. Isoeugenol dimers are not currently used for fragrance chemicals. However, these two isoeugenol dimers still retain their anti-oxidative and antibacterial effects similar to its monomer (unpublished data). Accordingly the dimerization of isoeugenol may yield a promising candidate for cosmetic ingredients with a low sensitization risk. This trial may contribute to a novel strategy to develop safer cosmetic ingredients.

In addition, when these two types of isoeugenol dimer were applied to the DEREK prediction system (Lhasa Limited, Leeds, UK), both dimers were predicted as 'PLAUSIBLE'. More data accumulation would be necessary, but the case studies for eugenol and isoeugenol dimers would provide valuable information to improve the prediction accuracy of SAR systems. A reduction of false-positives in SAR systems would contribute to effective screening of the promising cosmetic and pharmaceutical candidate chemicals.

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DOI: 10.1002/jat

An Evaluation of Performance Standards and Non-radioactive Endpoints for the Local Lymph Node Assay

The Report and Recommendations of ECVAM Workshop 65[a]

David Basketter,[1] Amanda Cockshott,[2] Emanuela Corsini,[3] G. Frank Gerberick,[4] Kenji Idehara,[5] Ian Kimber,[6] Henk Van Loveren,[7] Joanna Matheson,[8] Annette Mehling,[9] Takashi Omori,[10] Costanza Rovida,[11] Takashi Sozu,[12] Masahiro Takeyoshi[13] and Silvia Casati[11]

[1]St John's Institute of Dermatology, St Thomas' Hospital, London, UK; [2]Health and Safety Executive, Bootle, UK; [3]Department of Pharmacological Sciences, University of Milan, Milan, Italy; [4]Procter & Gamble Company, Miami Valley Innovation Center, Cincinnati, OH, USA; [5]Daicel Chemical Industries Ltd, Himeji, Japan; [6]Faculty of Life Sciences, The University of Manchester, Manchester, UK; [7]National Institute of Public Health and the Environment, Bilthoven, The Netherlands; [8]Consumer Product Safety Commission, Bethesda, MD, USA; [9]Cognis GmbH, Dusseldorf, Germany; [10]Kyoto University School of Public Health, Kyoto, Japan; [11]ECVAM, IHCP, European Commission Joint Research Centre, Ispra, Italy; [12]Osaka University, Osaka, Japan; [13]CERI, Saitama, Japan

Preface

This is the report of the 65th of a series of workshops organised by the European Centre for the Validation of Alternative Methods (ECVAM).

The main objective of ECVAM, as defined in 1993 by its Scientific Advisory Committee (ESAC), is to promote the scientific and regulatory acceptance of alternative methods which have scientific relevance and which reduce, refine or replace the use of laboratory animals. One of the first priorities set by ECVAM was the implementation of procedures that would enable it to become well-informed about the state-of-the-art of non-animal test development and validation, and of opportunities for the possible incorporation of alternative methods into regulatory procedures. It was decided that this would be best achieved through a programme of ECVAM workshops, each addressing a specific topic, and at which selected groups of independent international experts would review the current status of various types of *in vitro* tests and their potential uses, and make recommendations about the best way forward.

A Workshop on *An Evaluation of Performance Standards and Non-radioactive Endpoints for the Local Lymph Node Assay* was held at ECVAM on 25–27 September 2007, under the chairmanship of David Basketter. The workshop was attended by experts from academia, industry, national organisations, and national and international validation authorities. At present, the local lymph node assay (LLNA) involves the use of radiolabelled thymidine as part of the standard protocol. The aim of the workshop was to review the status of methods which employ non-radioactive endpoints for the LLNA and to consider Performance Standards for their eventual assessment. At the end of the report are listed recommendations that should be considered for progressing toward the validation of relevant and reliable methods.

Key Definitions

To ensure the Performance Standards are applied appropriately, it is necessary to define their domain of applicability. For this purpose, the workshop participants debated in depth what could be considered to represent minor or major modifications to the standard LLNA. The following definitions were agreed:

Minor changes: those that maintain full compliance with OECD Test Guideline (TG) 429 (1), and that incorporate potential changes already foreseen in OECD TG 429. For a change to be considered minor, there is a requirement that the endpoint measured is still one of lymph node cell proliferation.

Address for correspondence: David Basketter, 2 Normans Road, Sharnbrook, Bedfordshire MK44 1PR, UK.
E-mail: *david.basketter@ukonline.co.uk*
Requests for reprints: Silvia Casati, ECVAM, IHCP, Joint Research Centre, European Commission, Via E Fermi, 2749, I-21027 Ispra (VA), Italy.
E-mail: *silvia.casati@jrc.it*

[a]This document represents the agreed report of the participants as individual scientists.

Major changes: those that incorporate modifications to the standard LLNA that are broader in scope and of greater substance than those defined as being *minor*. Changes of this type would normally trigger a more thorough validation exercise, but should be considered on a case-by-case basis.

Introduction

The regulatory background

The approach used for the identification of chemicals with a significant degree of skin sensitisation potential is well characterised in the EU, and will soon be within the Globally Harmonised System (GHS; 2). The relevant European legislation includes the Dangerous Substances Directive, *Directive 67/548/EEC* (3), and the Dangerous Preparations Directive, *Directive 1999/45/EC* (4). With the advent of the legislation related to the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) system (5), further emphasis has been placed on the use of the most up-to-date methods, as well as ensuring that decisions are made by using all the available data, and with the minimum of additional animal testing. However, for confirmatory testing, the LLNA is the method of choice within the REACH system.

The tests traditionally used for the identification of chemicals possessing the intrinsic ability to cause skin sensitisation are the guinea-pig maximisation test (GPMT; 6), the Buehler occluded patch test (7) and the LLNA (8). The first two of these use a combination of the induction and elicitation phases in the guinea-pig, with the extent of sensitisation induction being determined as a function of the (erythematous) response to topical challenge. In contrast, the LLNA quantifies the induction response in mice by measuring proliferation in the lymph nodes which drain the site of topical application. The capacity of these methods to identify skin sensitisation hazard has only been formally validated for the LLNA (9–12). However, both within this validation process and via the publication of other datasets, the guinea-pig methods are also recognised to be of sufficient sensitivity and specificity (13–15).

For the purposes of hazard identification, skin sensitisation assays are interpreted in the same manner. In simple terms, if the results in the LLNA are positive (i.e. the stimulation of proliferation in test group lymph nodes is at least 3-times greater than in the concurrent vehicle-only-treated controls), or if at challenge $\geq 30\%$ of the guinea-pigs are positive in a maximisation test, or if $\geq 15\%$ of the guinea-pigs are positive in the Buehler test, then the substance is regarded as a skin sensitiser. The substance can then be classified formally and labelled, according to the EU system, as "R43: May cause sensitisation by skin contact". Thus, labelling can be applied to a chemical substance exclusively on the basis of data from a single animal test. Human experience can only be taken into account if it exists, and even then, it is normally not used to overturn the conclusion from positive animal data (16).

Ultimately, basic hazard identification is not sufficient for protection of human health; it merely represents the first step. Risk assessment and risk management are the processes that deliver human health protection. To permit this, some experts have proposed that, ideally, the relative potencies of skin sensitising chemicals should be determined and considered. The measurement of skin sensitisation potency has been the subject of much discussion in recent years, and expert groups in the EU (17), in European industry (18) and in the World Health Organisation (19) have made closely similar recommendations. Essentially, they all recommended that the optimal strategy is to determine the threshold positive concentration, the EC3 value, in the LLNA. It would not be appropriate to go into any detail of this measurement here, as it has been thoroughly reviewed elsewhere (20). However, what is important, is to appreciate its value for characterising skin sensitisation hazard and facilitating risk assessment (21, 22).

Background to performance standards

Prior to the acceptance of a new test method for regulatory testing, validation studies are conducted to assess its predictive capacity (the ability of the test method to correctly predict or measure the biological effect of interest, also referred to as accuracy) and its reliability (the extent of its intra-laboratory and inter-laboratory reproducibility). The LLNA underwent such a formal assessment before being adopted for use at the regulatory level. However, there might be cases for which a comprehensive validation exercise could be avoided and a simplified procedure applied. General criteria have been established by the validation authorities, and accepted at international level, to identify these cases and provide guidance for their assessment.

The concept of Performance Standards has been introduced as a way to streamline the validation process for test methods that are functionally and structurally similar to existing and adequately-validated test methods (23–25). As defined by the OECD (26), the purpose of Performance Standards is to communicate the basis by which new test methods, both proprietary (i.e. copyrighted, trademarked, registered) and non-proprietary, can be determined to have sufficient accuracy and reliability for specific testing purposes. These Performance

Standards, based on validated and accepted test methods, can be used to evaluate the accuracy and reliability of other analogous test methods (also referred to as "me-too" tests) that are based on the same or similar scientific principles and that measure or predict the same biological or toxic effects.

Performance Standards should be provided by the Management Team of a validation study, and, as appropriate, used in the TGs issued for new test methods. The three main elements of Performance Standards are:

a) The essential structural, functional, and procedural elements of a validated test method that should be included in the protocol of a proposed mechanistically and functionally similar test method. These components include the unique characteristics of the test method, critical procedural details and quality control measures. Adherence to the essential test method components will help to ensure that a proposed test method is based on the same concepts as the corresponding validated test method.

b) A list of recommended reference chemicals that are used to assess the accuracy and reliability of a proposed mechanistically and functionally similar test method. These chemicals are a representative subset of those used to demonstrate the reliability and the accuracy of the validated method.

c) Accuracy and reliability values, which represent the comparable performance requisites that should be achieved by the proposed test method when evaluated by using the list of reference chemicals.

So far, Performance Standards have been developed only for *in vitro* methods, i.e. for *in vitro* skin corrosion testing (27, 28) and *in vitro* skin irritation testing (29). In both cases, they should be used to evaluate the performance of human skin models which are similar to those that have already been validated.

Background to the LLNA

The LLNA identifies chemicals that have skin sensitising potential (8; 30–32). The assay measures sensitising activity as a function of proliferative responses induced in auricular lymph nodes following the repeated topical exposure of mice to several concentrations of the test chemical. In the standard LLNA, the proliferation of draining lymph node cells (LNCs) is measured by using the incorporation of ^{3}H-thymidine and subsequent β scintillation counting. For this purpose, mice are injected intravenously (via the tail vein) with a source of ^{3}H-thymidine, five days after the initiation of exposure to the test chemical.

This approach to measuring the proliferative activity of LNCs was based on studies in which the sensitivity and specificity of various read-outs for lymph node activation and lymphocyte turnover were compared. Of the endpoints considered, the incorporation of radiolabelled thymidine was found to provide the most robust and most reliable correlation with skin sensitising potential.

Although the standard LLNA, which incorporates this approach for the determination of LNC activation and proliferation, has provided a useful and reliable method for identifying skin sensitising chemicals, it is acknowledged that it would be beneficial to have available a version of the LLNA that does not require the use of radioisotopes. For this reason, there has been interest in exploring other relevant read-outs for the assay, including alternative strategies for the measurement of LNC turnover.

Among the approaches that have been explored are:

a) the direct measurement of changes in draining lymph weight and/or cellularity (33, 34);

b) the measurement of other endpoints, such as induced changes in the concentration of ATP, that can serve as surrogates of altered lymph node cellularity;

c) the measurement of induced changes in the relative number of lymphocyte phenotypes found in draining lymph nodes, e.g. alterations in B lymphocytes ($B220^+$) number or in the representation of discrete T-lymphocyte sub-sets (CD62L/CD44; 35–37);

d) the characterisation of the elaboration by LNCs of cytokines, such as interleukin-2 (IL-2), a T-lymphocyte growth factor (38–40);

e) the use of non-radioactive methods for the determination of cell turnover in draining lymph nodes, e.g. the use of BrdU (41–43);

f) the use of radioisotypes other than ^{3}H-thymidine, such ^{125}I-uridine (44); and

g) the use of *in vitro*, rather than *in vivo*, radiolabelling of LNCs (45).

However, the challenge is to ensure that such alternative approaches, employing novel read-outs, have sensitivity, specificity, and overall accuracy and reliability, comparable to the standard LLNA. It is for this purpose, and for verifying the acceptability of the performance characteristics of modified assays, that the proposed Performance Standards described here were developed. However, it must be

emphasised that such Performance Standards are intended for providing an accelerated validation only for versions of the LLNA that involve only minor modifications to the standard assay as described in OECD TG 429 (1).

Both ECVAM and ICCVAM (46) have been working on developing Performance Standards for the LLNA. Their two draft documents were presented and discussed at the ECVAM workshop.

Against this background, the workshop participants debated and suggested the criteria and a list of reference chemicals that should be considered in developing Performance Standards for the LLNA. Consideration was given, not only to the performance of the test as it was assessed in the peer review of the LLNA, but also to the experience gained from the use of the test during the years that have followed that evaluation.

The proposed Performance Standards, as outlined in this workshop, were subsequently considered by ECVAM and ICCVAM as a contribution to the ongoing process of harmonisation of the two documents.

Proposed Performance Standards

Essential test method components

For these Performance Standards to apply, the modified LLNA must comply fully with the OECD TG 429 (1), with the exception of the way in which the lymph node cell proliferation is measured. Ideally, there should be no alteration to the prediction model, but it is accepted that this may be necessary, dependent on how the proliferation is measured. Significant changes to the prediction model may, of course, trigger the need for a more substantial assessment than is provided for by this proposed set of Performance Standards.

Recommended reference chemicals

Reference chemicals should be used to assess whether the performance of modified LLNA methods is comparable with that of the standard LLNA. A set of 20 chemicals, which comprise 13 sensitisers, 5 non-sensitisers, 1 false positive and 1 false negative in the standard LLNA, are suggested for the purpose (Table 1). For the selection of the relevant substances, the following criteria have been considered:

a) The chemicals should be readily available from one or more commercial suppliers.

b) Ideally, for each of the selected chemicals, LLNA and guinea-pig data (from either the GPMT or the Buehler test) should be available, as well as evidence for the elicitation of contact sensitisation in humans.

c) For the majority of the chemicals, there should be a clear consensus about the presence or absence of skin sensitising potential, where the results of all the existing predictive test results match the human data. The exceptions to this are sodium lauryl sulphate (SLS) and nickel sulphate, which have been selected as being false positive and false negative, respectively, in the standard LLNA.

d) There should be no doubt that the skin sensitising activity is attributable to the defined chemical structure, rather than to a contaminant.

e) Defined EC3 values should be available for the selected chemicals.

f) The selected chemicals should not be unstable or require unusual storage or transport conditions.

Within the pool of chemicals selected, attempts have been made to represent the variety of chemical classes and properties associated with skin sensitisation, although it must be recognised that, with a limited number of chemicals, not all the variables can be fully represented.

Furthermore, the list of chemicals has been selected to reflect the range of skin sensitisation potencies which are known to exist, but with emphasis on those chemicals considered to show weak or moderate potency, since these will provide the best assurance of the retention of test sensitivity, commensurate with the absolute minimum of additional animal testing.

In additional, in any evaluations of this type, it is important to define, in advance, whether a chemical is sufficiently sensitising to be formally classified according to the current regulations as a skin sensitiser. Only if this is the case, should it be identified as a positive in a predictive test, with the consequence that the existence of positive human data (e.g. in clinical case reports) is not, of itself, sufficient to mean that a chemical should be regarded as a substance which should test positive in a predictive assay.

The number of chemicals proposed should be sufficient to permit an assessment of the performance of modified versions of the LLNA, while minimising the number of animals required for such an evaluation.

Performance requisites: predictive capacity and reliability

Predictive capacity is defined as the capacity of a method to predict the accepted reference values

Table 1: Reference chemicals recommended for the evaluation of modified LLNA methods for the identification of skin sensitisation hazard

Chemical name	CAS No.	Physical form	Vehicle	EC3 LLNA	Value	n	Reference
Benzoquinone	106-51-4	Solid	AOO	+	0.01[a]	1	Gerberick *et al.* (54)
2,4-Dinitrochlorobenzene	97-00-7	Liquid	AOO	+	0.04[b]	13	Basketter *et al.* (20)
4-Phenylenediamine	106-50-3	Solid	AOO	+	0.11[b]	10	Basketter *et al.* (20)
Isoeugenol	97-54-1	Liquid	AOO	+	1.5[b]	31	Basketter *et al.* (20)
2-Mercaptobenzothiazole	149-30-4	Solid	DMF	+	1.7[a]	1	Gerberick *et al.* (54)
Diethyl maleate	141-05-9	Liquid	AOO	+	3.9[b]	2	Basketter *et al.* (55) Ryan *et al.* (56)
α-Hexyl cinnamic aldehyde	101-86-0	Liquid	AOO	+	9.9[b]	15	Basketter *et al.* (20)
Eugenol	97-53-0	Liquid	AOO	+	10.1[b]	4	Basketter *et al.* (20)
Citral	5392-40-5	Liquid	AOO	+	13[a]	1	Gerberick *et al.* (54)
Phenyl benzoate	93-99-2	Solid	AOO	+	20[a]	1	Gerberick *et al.* (54)
Cinnamic alcohol	104-54-1	Solid	AOO	+	21[a]	1	Gerberick *et al.* (54)
Imidazolidinyl urea	39236-46-9	Solid	DMF	+	24[a]	1	Gerberick *et al.* (54)
Ethyl acrylate	140-88-5	Liquid	AOO	+	32.4[b]	2	Dearman *et al.* (57) Warbrick *et al.* (58)
Methyl salicylate	119-36-8	Liquid	AOO	–			Gerberick *et al.* (54)
Isopropanol	67-63-0	Liquid	AOO	–			Gerberick *et al.* (54)
Salicylic acid	69-72-7	Solid	AOO	–			Gerberick *et al.* (54)
Lactic acid	50-21-5	Solid	DMSO	–			Gerberick *et al.* (54)
Hexane	110-54-3	Liquid	AOO	–			Gerberick *et al.* (54)
Sodium lauryl sulphate	151-21-3	Solid	DMF	False positive			Gerberick *et al.* (54)
Nickel sulphate	10101-98-1	Solid	DMSO	False negative			Basketter & Scholes (59)

[a]single EC3 values; [b]mean EC3 values; AOO = acetone/olive oil (4:1 v/v); DMF = dimethylformamide; DMSO = dimethylsulphoxide. + = sensitiser in the standard LLNA; – = non-sensitiser in the standard LLNA.

(47). This is also referred to as the accuracy of a test method. The predictive capacity of a proposed test method should, when evaluated by using the recommended reference chemicals, at least meet or exceed that of the validated reference method. For this purpose, the threshold concentration at which a test chemical is positive in a new test method must fall within the range of 0.5–2.0× the published threshold (EC3) values from the standard LLNA in the relevant vehicle.

Reliability refers to the extent to which a test method can be performed reproducibly within and between laboratories and over time, when conducted by using the same protocol (26).

For the assessment of the inter-laboratory reproducibility, it is recommended that, when tested in at least three different laboratories, the threshold concentrations at which both α-hexyl cinnamic aldehyde (HCA) and 2,4-dinitrochlorobenzene (DNCB) are positive, fall within a range of 0.5× and 2.0× the published threshold (EC3) values in the standard LLNA.

Intra-laboratory reproducibility should be demonstrated by using HCA on four separate occasions over at least a 12-week period. Again, the threshold concentration should fall between 0.5× and 2.0× that of the expected threshold (EC3) value in the standard LLNA.

Alternative Endpoints to the Standard LLNA: Examples

This section describes three examples of alternative endpoints that are currently being developed for the LLNA.

1. A non-radioactive (non-RI) method, in which ATP content measurement is used as the endpoint

Principle

ATP is the principal energy source for all living organisms, and the amount of ATP determined is

known to correlate with the number of living cells (48, 49). Therefore, ATP content is considered to be a possible alternative endpoint to ^{3}H-thymidine incorporation as an index of cell number. An alternative LLNA involving the use of this endpoint as a non-RI method, termed the LLNA-DA (LLNA modified by Daicel, based on ATP content), has been developed (by Daicel Chemical Industries Ltd, Niigata, Japan; 50, 51). Measuring ATP contents has several advantages. The procedure for determining ATP content is easy and rapid, with a wide dynamic range for its stimulation index (SI).

Description of the method

In the protocol for the LLNA-DA, groups of female CBA/JNCrlj mice are treated by the topical application of 25μl of the test chemical (or the vehicle control) to the dorsum of both ears on days 1, 2, 3, and 7. Pretreatment with 1% SLS is carried out 1 hour before each application. On day 8, the auricular lymph nodes are excised. After recording the lymph node weight, single cell suspensions in phosphate-buffered saline are immediately prepared for each individual animal. The ATP content is determined by a luciferin–luciferase assay after appropriate sample dilution. For the determination of the ATP content, several measurement kits are commercially available (e.g. the Via Light™ HS Kit [Lonza Rockland, Inc., Rockland, ME, USA] and CheckLite™ 250 Plus [Kikkoman Corporation, Chiba, Japan]). A SI relative to the concurrent vehicle control is derived, and a SI of 3 is set as the cut-off value.

Summary of results

Thirty-one well-known chemicals were tested by the lead laboratory, and clear dose–response results were observed for weak sensitisers, as well as for strong or extreme sensitisers. The performance of this method showed good agreement with that of the standard LLNA or other test systems (Table 2). The accuracy of LLNA-DA compared to the standard LLNA was 93% (28/30), LLNA-DA *versus* guinea-pig tests was 80% (20/25), and LLNA-DA *versus* human tests was 79% (15/19). Similarly to the LLNA, the LLNA-DA uses a SI of 3 to discriminate between positive and negative responses. The EC3 values of the LLNA-DA, calculated from the positive tested chemicals, were compared with those of the original LLNA (Figure 1). This demonstrated that the EC3 values obtained with LLNA-DA were very similar to those obtained with the standard LLNA.

To evaluate inter-laboratory reproducibility, two large scale inter-laboratory validation studies were conducted in Japan. The results from 17 laboratories, with 14 chemicals under blind conditions in the two studies, showed that this method is sensitive, with little intra-laboratory variation. In the first study, acceptably small inter-laboratory variations in SI values were obtained for 10 chemicals, with the exception of two metallic salts. In the second study, with five chemicals, including three metallic salts, acceptably small variations for all the chemicals were obtained.

Conclusions

Compared with the standard LLNA, the LLNA-DA not only has a modified endpoint measurement, but also requires an adjustment to the dosing schedule and a pretreatment with SLS — changes which are necessary to enable the SI cut-off value of the LLNA-DA to retain that of standard the LLNA. As a consequence, this method cannot be considered to be a minor modification of the standard LLNA. However, as described above, the evidence concerning its sensitivity and reliability suggests that this approach looks promising.

2. A non-RI modification of the LLNA, based on BrdU incorporation

Principle

This modification of the LLNA has been developed as an alternative to the standard LLNA. It is based on BrdU (5-bromo-2′-deoxyuridine) incorporation in place of ^{3}H-thymidine incorporation, to measure lymph node cell proliferation (41).

Description of the method

The method based on BrdU incorporation is practically identical to the standard LLNA methodology, apart from the use of BrdU and colorimetric detection, for which a single intraperitoneal injection (5mg/mouse per injection) of BrdU was made on day 4. This administration schedule was found to be the most effective labelling protocol for yielding maximum SI values, based on preliminary study data with several different protocols. Approximately 24 hours after the BrdU injection, the auricular lymph nodes were removed, weighed, and stored at -20°C until analysed by using an enzyme-linked immunosorbent assay (ELISA) to measure the level of BrdU incorporation. In the current form of the test, cellular proliferative responses are measured by using a commercial BrdU detection

Table 2: Comparison of the concordances for 31 chemicals

Chemicals	LLNA-DA	LLNA*	GPMT/BA*	HMT/HPTA*
2,4-Dinitrochlorobenzene	+	+	+	
p-Phenylenediamine	+	+	+	+
Toluene diisocyanate	+	+		
Glutaraldehyde	+	+		
$K_2Cr_2O_7$	+	+	+	+
Phthalic anhydride	+	+	+	
Trimellitic anhydride	+	+		
Formaldehyde	+	+	+	+
Cinnamic aldehyde	+	+	+	+
Isoeugenol	+	+	+	+
Cobalt chloride	+	+	+	+
Eugenol	+	+	+	+
Resorcinol	+	+	–	+
Benzocaine	+	+/–	+	+/–
Abietic acid	+	+	+	+
α-Hexyl cinnamic aldehyde	+	+	+	
Mercaptobenzothiazole	–	+	+	+
Citral	+	+	+	+
Hydroxycitronellal	+	+	+	+
Imidazolidinyl urea	+	+	+	+
SLS	+	+	–	–
$NiSO_4$	–	–	+	+
Benzalkonium chloride	+	–	–	+
Propyl paraben	–	–	–	+/–
Diethylphthalate	–	–		
1-Bromobutane	–	–		
Methylsalicylate	–	–	–	–
Chlorobenzene	–	–	–	
Lactic Acid	–	–	–	
Hexane	–	–		–
Isopropanol	–	–	–	

*GPMT = guinea-pig maximisation test; BA = Buehler assay; HMT = human maximisation test; HPTA = human patch test allergen. *Results of the LLNA, GPMT/BA and HMT/HPTA are taken from references 18, 20, 54 and 60. + = sensitiser; – = non-sensitiser; +/– = ambiguous results.*

kit (e.g. one provided by Roche Diagnostics, Indianapolis, IN, USA; Cat. No. 11647229001). To perform the BrdU ELISA, the lymph nodes were crushed, passed through a #70 nylon mesh, and individual LNCs were suspended in 15ml of physiological saline. The cell suspension was added to the wells of a flat-bottomed microplate, in triplicate. After fixation and denaturation of the LNCs, anti-BrdU antibody was added to each well, and after rinsing, a substrate solution containing tetramethylbenzidine (TMB) was added and allowed to produce the chromogen. Absorbance at 370nm, with a reference wavelength of 492nm, was defined as the BrdU labelling index (41).

Summary of results

This method can display clear dose-related responses, and the potency class prediction is obtained with similar concentrations of test chemicals to those required in the standard LLNA (42, 43).

However, the potential lower sensitivity of non-RI alternative methods was also recognised as an issue. To evaluate the assay performance of this non-RI LLNA, 23 well-known chemicals, categorised as human contact allergens from class 1 to class 5, were tested, and the results were analysed to identify the best prediction model (Table 3).

Consequently, the conditions that set the cut-off point as SI > 1.5, with statistical significance between the treated and concurrent vehicle control or the cut-off point as SI > 1.5 with > 3 SD of the mean SI of the concurrent vehicle control, were adopted as the best endpoints for this non-RI LLNA method. By using these decision criteria, the highest concordance and lowest number of false negatives in the non-RI LLNA were obtained, as compared with the standard LLNA (52).

In addition, a novel approach to predicting the sensitisation potency of chemicals by comparison with known human contact allergens has been proposed as a useful application of this method (Table 4; 43). In this approach, nine well-known chemicals, categorised as human contact allergens from class 1 to class 5, were tested by the non-RI LLNA, with the reference allergens, 2,4-dinitrochlorobenzene (DNCB) as the class 1 human contact allergen, isoeugenol as the class 2 human contact allergen, and HCA as the class 3 human contact allergen. All the chemicals were assigned to the correct or adjacent classes.

Conclusions

The results suggested that this new strategy for a non-RI LLNA could provide both hazard identification and sensitisation potency data, which are prerequisites for judging the sensitisation risk for humans represented by new chemical products. These results indicate that a non-RI LLNA based on the BrdU incorporation, may be one of the promising alternatives to the standard LLNA.

3. The measurement of cell numbers and ear thickness as alternative non-radioactive endpoints in the LLNA

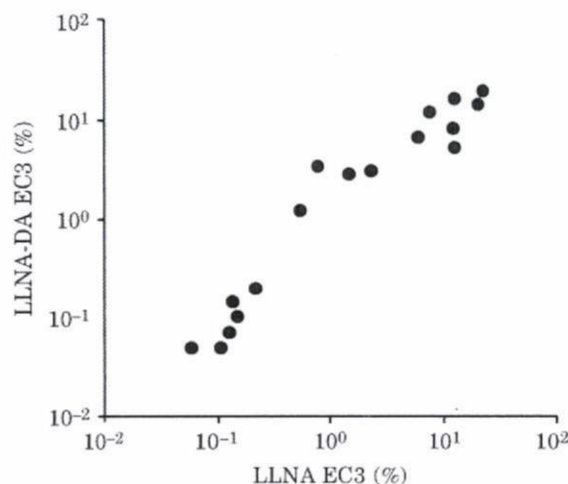
Principle

In the LLNA, lymphocyte proliferation in the auricular lymph node is measured by analysing ^{3}H-thymidine incorporation in the lymph node as a whole, after intravenous injection in the test mice. Based on the notion that the process of proliferation is induced within the lymph node, and

Table 3: Positive (+) and negative (–) classifications by different SI cut-off values in the non-RI LLNA based on BrdU incorporation

	Cut-off SI value																		
Chemical name	**3.0**	**2.9**	**2.8**	**2.7**	**2.6**	**2.5**	**2.4**	**2.3**	**2.2**	**2.1**	**2.0**	**1.9**	**1.8**	**1.7**	**1.6**	**1.5**	**1.4**	**1.3**	**RI**
2,4-Dinitrochlorobenzene	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1,4-Benzoquinone	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Diphencyclopropenone	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glutaraldehyde	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1,4-Phenylenediamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2-Mercaptobenzothiazole	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+
Cinnamic ldehyde	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Isoeugenol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1,3-Aminophenol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3-(4-Isopropylphenyl) isobutyraldehyde	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+
Citral	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Eugenol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hydroxycitronellal	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+
α-Hexyl innamic ldehyde	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Isopropyl yristate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4-Chloroaniline	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Aniline	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	–
2-Hydroxypropylmethacrylate	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Glycerol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Isopropanol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Phthalic acid iethyl ster	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Propylene lycol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Dimethyl sophthalate	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Figure 1: Correlation of EC3 values in the standard LLNA and the LLNA-DA



that the proliferation of lymphocytes results in increased numbers of lymphocyte after application of the sensitiser on the ear, assessment of lymphocyte numbers rather than lymphocyte proliferation as the read-out, has been proposed (33, 34). Many studies show that the ear-draining lymph nodes increase in size after the application of sensitisers on the ear. In addition, the number of lymphocytes is also increased after such treatment. There is good correlation between lymph node cell number counts and ^{3}H-thymidine incorporation (Figure 2).

Description of the method

This alternative non-RI test is based on the standard LLNA described in OECD TG 429 (1). Instead of assessing proliferative reactions directly, the method involves the removal of the lymph nodes, the preparation of single cell suspensions according to routine procedures, and the subsequent counting of cell numbers by using automated cell-counting devices. However, the proposed test also involves a number of other deviations from the standard LLNA: the use of BALB/c or NMRI mice, the excision of lymph nodes at day 4 instead of day 6, and the use of DAE433 as a vehicle. Also, a cut-off at a stimulation index of 1.4, subject to the provision of statistical significance, is used.

Some chemicals that have irritant capacity, also appear to be capable of inducing cell proliferation, and thus increase numbers of cells in the lymph nodes draining the ears on to which the test chemical is applied. Therefore, an additional endpoint on irritancy, i.e. the measurement of ear thickness, was added to this non-RI approach. Ear thickness can be measured in the same mice in which the modified LLNA is carried out. At the same time as when the lymph nodes are removed, punch biopsies of the ears are made and weighed (53). An increment in weight amounting to 1.2-times the control value, and which is statistically significant, is considered a positive result. The classification of chemicals is then based on both types of information (Table 5).

Summary of results

In a collaborative study, carried out by nine laboratories in Europe with 12 chemicals (including four irritants and one fully negative control) and BALB/c mice, the proposal to use lymph node cell numbers as the read-out for assessing the sensitising capacities of chemicals was put to the test (33, 34). The majority of the laboratories correctly identified the chemicals that were characterised as sensitisers, on the basis of statistically-significant increases in cell numbers. The results for HCA are shown in Table 6. The results from a sin-

Table 4: Classification criteria for the sensitisation potency of chemicals tested by the non-RI LLNA based on BrdU incorporation

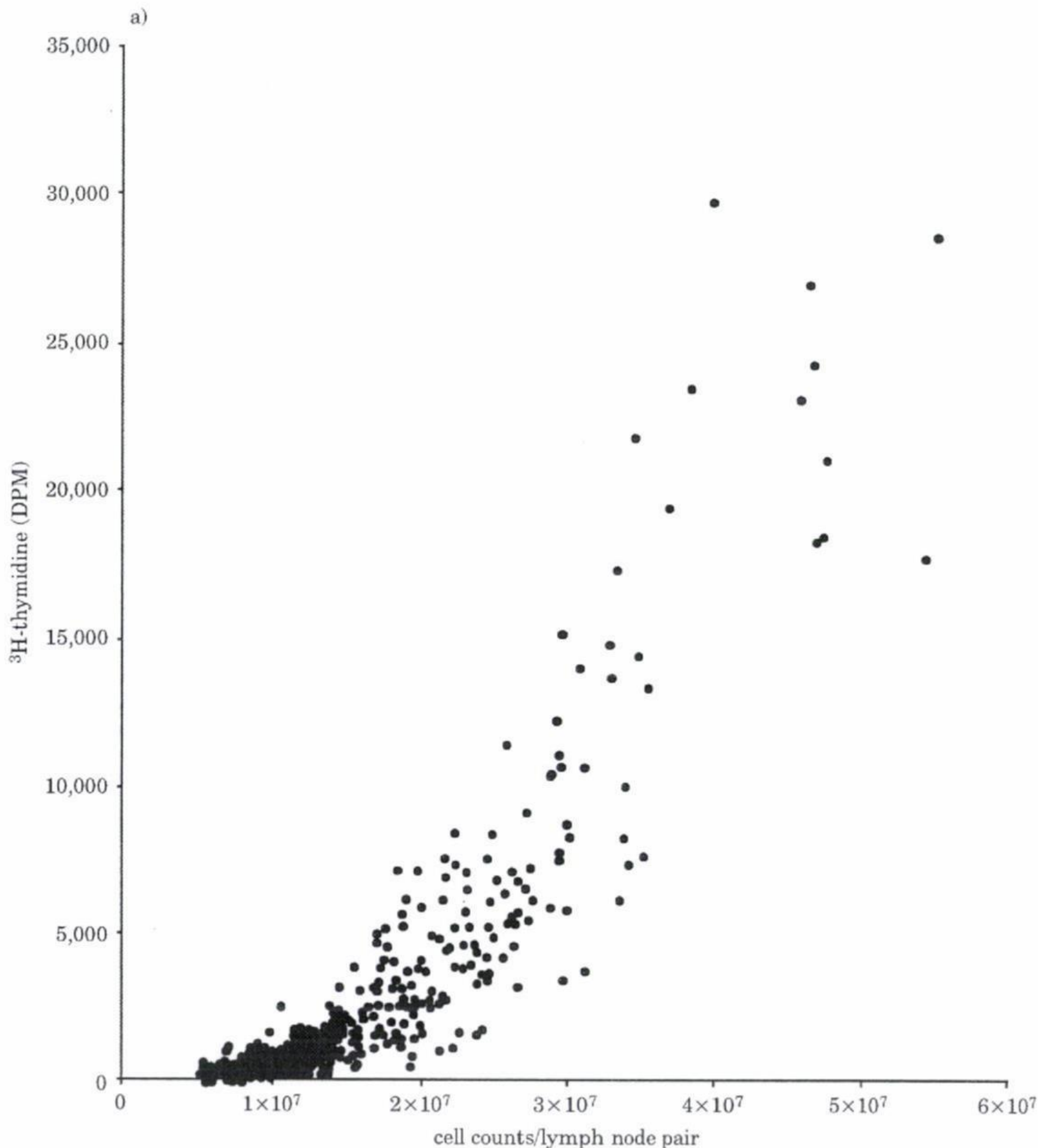
Human class	Requirements	Sensitisation class
1	SI for 2% test chemical ≥ SI for 2% DNCB	Strong sensitiser
2	SI for 2% test chemical < SI for 2% DNCB SI for 10% test chemical ≥ SI for 10% isoeugenol	Moderate sensitiser Moderate sensitiser
3	SI for 10% test chemical < SI for 10% isoeugenol SI for 50% test chemical ≥ SI for 50% HCA	Weak sensitiser Weak sensitiser
4–5	SI for 50% test chemical < SI for 50% HCA	Extremely weak or non-sensitiser

gle laboratory indicated that the assessment of potency by using this method was reproducible.

Conclusions

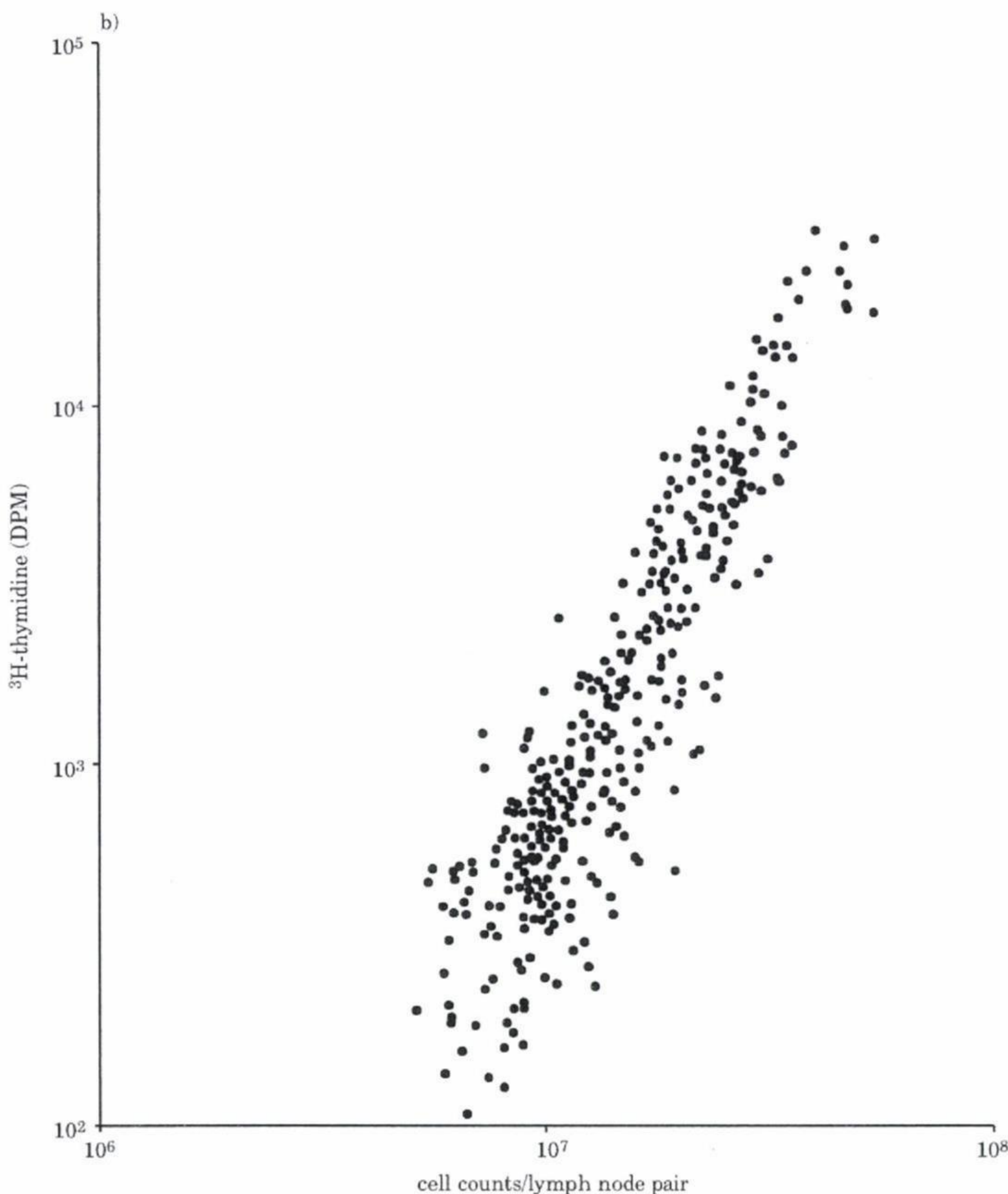
This proposed non-RI LLNA uses cell number as a correlate of cell proliferation, but, as other modifications to the standard LLNA were also made, the method constitutes a major change. There is value in the proposed test, in that it would, if validated, eliminate the use of a radioactive label. Moreover, the measurement of ear thickness may provide a means of providing further information that can help to discriminate true sensitisers from chemicals that induce increase lymphocyte numbers or proliferation in lymph nodes for other reasons. With regard to the counting of cell numbers,

Figure 2: Lymph node cell proliferation: cell counts compared with ^{3}H-thymidine uptake values



Data kindly provided by BGIA, Bonn, Germany. (b) shows an expansion of the data points in the bottom-left area of graph (a).

Figure 2: continued



Data kindly provided by BGIA, Bonn, Germany. (b) shows an expansion of the data points in the bottom-left area of graph (a).

further experience needs to be gained with this approach, in a setting where there are no other deviations from the standard LLNA, and formal comparisons of the same endpoints in the same animals are performed. Further experience in the interpretation of ear thickness measurements is also required, so that criteria can be set for judging when a positive ear swelling response can reverse the conclusion of sensitisation, based on proliferation of cells (numbers) in the LLNA. It should be noted that the additional endpoint of measuring ear thickness could also be used in combination with other (non-radioactive) modifications of the LLNA.

Table 5: A classification scheme for evaluating tests based on cell counting and the measurement of ear thickness

Endpoints	Results							
Lymph node weight	+	+	+	+	–	–	–	–
Lymph node cell counts	+	+	–	–	+	+	–	–
Assessment I	A/I	A/I	I	I	A	A	–	–
Acute skin reaction	–	+	+	–	–	+	+	–
Assessment II	–	I	I	–	–	I	I	–
Overall assessment	A	A/I	I	I?	A	A/I	I	–

+ = statistically-significant increase; A = allergen; I = irritant; Assessment I = evaluation on the basis of lymph nodes only; Assessment II = evaluation on the basis of ear thickness only.

Summary and Conclusions

The LLNA is becoming the skin sensitisation assay of first choice for many regulatory authorities. Consequently, there is an increasing drive to replace its radiolabel-based endpoint. A number of alternatives to the standard LLNA, which do not employ radiolabel, have already been developed. However, several of these efforts involve major changes to the standard LLNA protocol, so a substantial validation process will be required, to ensure that they retain the degree of sensitivity and specificity afforded by the standard method (1). Where the changes to the LLNA are only minor, retaining the same endpoint measure (proliferation in draining auricular lymph nodes), ECVAM and some other authorities (e.g. ICCVAM) have decided to consider the establishment of Performance Standards to allow for a streamlined validation process.

The ECVAM draft Performance Standards have been reviewed in this workshop, and recommendations have been made, which cover both strategic issues, such as the harmonisation of standards between validation authorities, and suggestions for the selection of chemicals and how the modified LLNA protocols and associated prediction models should perform in relation to them. In addition, three examples of modified LLNAs which avoid the use of radiolabelling have been considered (but not reviewed in detail). These have been presented in a common format, to display the principles on which they are founded, the current status of their evaluation, and brief conclusions on their performances. Whilst some of them may be relatively close to acceptability for use, the workshop participants agreed that none of them have yet been adequately validated as substitutes for the standard LLNA, either because they do not fit the criteria of "minor change" or because their data package is at present insufficient.

Recommendations

1. Wherever possible, Performance Standards should be harmonised between validation authorities.

2. Minor modifications to the assay can be assessed by using Performance Standards such as those recommended in this report.

Table 6: Comparisons of observed stimulation indices between different laboratories, for HCA in the non-RI LLNA based on cell counting

	Lab. 1	Lab. 2	Lab. 1	Lab. 2	Lab. 3	Lab. 4	Lab. 5	Lab. 6	Lab. 7
Vehicle	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
3% HCA	1.05	1.20	0.92	1.03	1.21	1.06	1.05	1.30	0.98
10% HCA	1.11	**1.90**	**1.69**	1.15	**1.53**	1.29	1.24	**1.64**	**1.64**
30% HCA	**1.59**	**2.01**	**1.57**	**2.02**	**2.37**	**1.90**	**2.03**	**2.10**	**3.10**

Statistically-significant stimulation indices are marked in bold; laboratories 1 and 2 performed repeat experiments.

3. Minor modifications to the OECD TG 429 should be assessed by using a carefully selected set of chemicals and against a clear set of criteria, both of which have been detailed in this report.

4. For modifications to the OECD TG 429 which are not regarded as minor, it may also be the case that the information needed would not be as comprehensive as for a completely new test. Such data requirements should be judged on a case-by-case basis.

5. The progress that has been made in developing realistic alternative read-outs for the standard LLNA should be welcomed, and there should be further investment in research in this area.

Acknowledgements

The participation and contributions of Dr William S. Stokes, Director of the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina, USA, are gratefully acknowledged. This acknowledgement does not necessarily indicate agreement with or endorsement of the workshop report.

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FULL PAPER *Clinical Pathology*

Gene Expression Changes Induced by Type IV Allergy-Inducible Chemicals in Dendritic Cells

Rie TARAMA[1)], Hirotomo KATO[1)]*, Youichi ISHIKAWA[1)], Hideki MIYAURA[2)], Masahiro TAKEYOSHI[2)] and Hiroyuki IWATA[1)]

[1)]*Department of Veterinary Hygiene, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753–8515 and*
[2)]*Chemical Assessment Center, Chemicals Evaluation and Research Institute, Saitama 345–0043, Japan*

(Received 21 December 2007/Accepted 19 February 2008)

ABSTRACT. In the present study, the changes of gene expression profile in dendritic cell (DC)-derived DC2.4 cells sensitized with two allergenic chemicals were analyzed by microarray analysis to develop a basis for an *in vitro* assessment system of type IV allergenic chemicals. Consequently, 26 genes were significantly up-regulated, and 53 were down-regulated in both groups. Interestingly, some of up-regulated genes were associated with the maturation process of DCs. A set of genes was further evaluated by real-time reverse transcription-polymerase chain reaction to identify the gene expression changes specifically induced by type IV allergy-inducible chemicals in DC2.4 cells, and 2 possible candidates, syndecan-1 (Sdc1) and smoothened (SMO) genes were identified. Thus, up-regulation of Sdc1 gene and down-regulation of SMO gene in DC2.4 cells may be diagnostic markers for the screening of type IV-allergenic chemicals.
KEY WORDS: chemical, dendritic cell, microarray analysis, type IV allergy.

J. Vet. Med. Sci. 70(7): 673–680, 2008

Allergic contact dermatitis (ACD), a type IV allergy, is one of the most common inflammatory diseases of the skin with unknown genetic basis and is often an occupationally related disorder in industrialized countries with an important socio-medical impact [14, 28]. At present, many chemicals are considered to have allergenic potency and thus risk assessment of such chemical substances is important. ACD has been intensively studied, and the development of an allergic hypersensitivity reaction in the skin is considered to be processes depending on the induction of specific T-lymphocyte responses [23]. At the initial step, chemical allergens exposed on the skin are recognized by Langerhans cells (LCs), the principal DC residing in the epidermis and known to play a key role in the development of ACD. Following an encounter with a chemical allergen, LCs are activated and subsequently migrate from the skin to the draining lymph nodes, undergoing a maturation process during the journey [20, 22].

For many years, guinea pigs have been applied for the hazard analysis of skin-sensitizing chemicals [9, 26]. Recently, the local lymph node assay (LLNA) was developed in mice as an alternative approach based on the characterization of initial proliferative responses in draining lymph nodes caused by chemicals exposure [16, 21, 34, 39], and this method is now widely used for estimation of the allergenic potency of chemical substances. Although these approaches are sensitive and reliable, more advantageous ways in terms of cost performance, safety, readiness and animal welfare are expected. More recently, *in vitro* assay for chemical substances with allergenic potency is extensively explored by using cultured cells, especially macrophages [5, 41]. In these experiments, up-regulation of several molecules including cell surface markers were reported to be induced by chemical exposure, suggesting that these markers may be candidates for evaluating chemicals with allergenic potency [5, 41]. However, as well documented, LCs, a family of DCs, are the main antigen presenting cell in the epidermis and have central roles on the induction of allergic skin disorders [4, 38]. In this regard, assessment of LC/DC responses to chemical exposures should be reasonable to know accurate mechanisms of ACD development. Although several studies evaluated the effect of chemicals on DCs by use of *in vitro* differentiated primary DCs, it is disadvantageous because of the limited numbers in the source, donor-to-donor variability and cost for obtaining these cells.

In the present study, the changes of gene expression profile in an established DC line sensitized with allergenic chemicals were analyzed by microarray analysis and real-time reverse transcription-polymerase chain reaction (RT-PCR) to develop a basis for an *in vitro* assessment system of type IV allergy-inducible chemicals.

MATERIALS AND METHODS

Chemicals: Chemical substances, 2,4-dinitrochlorobenzene (DNCB; Wako Pure Chemical Industries, Osaka, Japan), *p*-benzoquinone (BQ; Kanto Chemical, Tokyo, Japan), citral (Cit; Nacalai Tesque, Kyoto, Japan), trimellitic anhydride (TMA; Kanto Chemical) and dextran (Dex; Kanto Chemical) were dissolved in DMSO to a concentration of 10 $\mu g/ml$ as a stock solution and further diluted with cell culture medium for use. DNCB, BQ and Cit are known to be strong sensitizers and type IV allergy-inducible chemicals [15, 18, 24]. TMA is a respiratory sensitizer and a strong inducer of type I allergy. This chemical is also

* CORRESPONDENCE TO: KATO, H., Department of Veterinary Hygiene, Faculty of Agriculture, Yamaguchi University, 1677–1 Yoshida, Yamaguchi 753–8515, Japan.
e-mail: katoh@yamaguchi-u.ac.jp

known as an irritant on the skin [10, 17]. Dex is reported to be a non-sensitizer on the skin [7, 39].

Cells: The cell line DC2.4, derived from C57BL/6 mice, was kindly provided by Dr. Kenneth L. Rock (Division of Lymphocyte Biology, Dana Farber Cancer Institute, Boston, MA, U.S.A.). The DC2.4 cells have a dendritic morphology, express DC-specific markers, MHC molecules and co-stimulatory molecules, and have phagocytic activity as well as antigen-presenting capacity [35]. The cells were maintained in DMEM (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS; Cansera International Inc., Ontario, Canada), 4 mM L-glutamine and 50 μM 2-mercaptoethanol at 37°C under 5% CO_2 in air.

Sensitization of DC2.4 cells and RNA extraction: DC2.4 cells were plated at 1.0×10^5 cells/m*l* and incubated for 12 hr. The cells were treated with chemicals in non-toxic doses (0.1 μg/m*l* for DNCB, BQ and Cit, 1.0 μg/m*l* for TMA and Dex) and harvested after cultivation for 24 hr. Total RNA was extracted with RNeasy mini kit (QIAGEN K. K., Tokyo, Japan).

Microarray analysis: Total RNA from control and BQ-treated DC2.4 cells was reverse transcribed into cDNA using Fluorescent Direct Label Kit (Invitrogen, Carlsbad, CA). cDNA samples were labeled with Cy3-dCTP or Cy5-dCTP (AmershamPharmacia Biotech, Piscataway, NJ) and purified with MinElute Mini Kit (QIAGEN K. K.). Microarray was performed with Mouse cDNA Microarray Kit (Agilent Technologies, Palo Alto, CA), and the data was analyzed by Feature Extract software (Agilent Technologies). Microarray analysis of DNCB-treated DC2.4 cells was performed with Filgen Array Mouse 32K kit (Filgen Technologies), and the data was analyzed by GenePix 4000B microarray laser scanner (Axon Instruments, Union City, CA).

Quantification of the gene expression by real time RT-PCR: The real time RT-PCR was performed with the QuantiTect SYBR Green RT-PCR kit (QIAGEN K.K.) in accordance with the manufacture's instructions. Briefly, 25 μ*l* of a PCR mixture in a tube containing each target gene-specific primer pair at 0.5 μM, 1 × QuantiTect SYBR Green RT-PCR Master Mix, 0.25 μ*l* of QuantiTect RT Mix, and 0.2 μg of total RNA was subjected to reverse transcription at 50°C for 30 min. After inactivation of reverse transcriptase at 95°C for 15 min, real-time PCR amplification was performed with 40 cycles of denaturation (94°C for 15 sec), annealing (55°C for 30 sec), and polymerization (72°C for 30 sec). As an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was assessed by real-time RT-PCR. The target genes and their primer sequences addressed in this study were described in Table 1. For the compensation of accidental errors among samples, relative gene expression numbers were calculated as raw values divided by the amount of GAPDH in the same samples.

RESULTS

Sensitivity of DC2.4 cells to chemical exposures: To determine the dose of chemicals for *in vitro* sensitization to DC-derived DC2.4 cells, the cells were exposed to various concentrations (0–100 μg/m*l*) of chemicals for 24 hr, and cell viabilities were determined by trypan blue dye exclusion assay. The viability of DC2.4 cells was largely affected when exposed to DNCB, BQ and Cit at the concentration of 5–10 μg/m*l* although their cytotoxicity was low at 1–5 μg/m*l* and almost background level at 0–0.5 μg/m*l*. On the other hand, the effect of TMA or Dex on the viability of DC2.4 cells was minimal through the examined concentrations (0–100 μg/m*l*) (Fig. 1). Based on these findings, we decided to use these chemicals at the concentration of 0.1 μg/m*l* on DNCB, BQ and Cit, and 1.0 μg/m*l* on TMA and Dex for subsequent experiments.

Microarray analysis of gene expression changes in DC2.4 cells induced by chemical exposures: To determine the typical gene expression changes in DC2.4 cells induced by type IV allergenic chemical exposure, DC2.4 cells were exposed to DNCB or BQ, both of which are known as strong sensitizers of contact hypersensitivity, for 24 hr and subjected to cDNA microarray analysis. In the DNCB-treated DC2.4 cells, 957 genes were up-regulated with fold changes of +1.3 to +4.4 and 1933 genes were down-regulated with fold changes of –1.3 to –9.0 when compared with control samples. On the other hand, in the BQ-treated cells, 790 genes had fold increases in expression over control ranging from +1.3 to +3.9, and 726 genes showed a down-regulation with fold changes of –1.3 to –2.2. When we overlapped these two data, 26 genes were up-regulated, and 53 were down-regulated in both groups. The changed genes represented a wide range of cellular processes including signal transduction, protein modification/synthesis and transcription. A list of genes which were up-regulated in DC2.4 cells

Table 1. Primer sequenses used for real-time RT-PCR

Gene name	Gene symbol	Forward primer	Reverse primer
glyceraldehyde-3-phosphate dehydrogenase	GAPDH	5'-TGAACGGGAAGCTACACTGG-3'	5'-TCAGATCCACGACGGACACA-3'
Cytokine inducible SH2-containing protein 2	Socs2	5'-CAGTCAAACAGGATGGTACTGG-3'	5'-AGTCTTGTTGGTAAAGGCAGTC-3'
Syndecan-1 precursor	Sdc1	5'-TTCATTGTGGGGAGGTCTAC-3'	5'-AAGTTCTCACACAGGCTCTT-3'
Tubulin alpha-6 chain	Tuba6	5'-CTGATGGAGCGGCTCTCTGT-3'	5'-AAGCTGCTCATGGTAGGCTTTC-3'
Lymphocyte antigen 86 precursor (MD-1 protein)	LY86	5'GGAGAATATCAGCTCTTGCTGG-3'	5'-CCAGGCAACTTCAAGGAAGTG-3'
smoothened	SMO	5'-TTGGATGCAGACTCGGACTT-3'	5'-CCAGAAAAGGGCACTCATTGG-3'
Hairy/enhancer-of-split related with YRPW motif 1	Hey1	5'-CTTCGGACATCACCCACACA-3'	5'-GTCCCAACACACCTGGGATT-3'
phosphatidylinositol 3-kinase, C2 domain containing, alpha	Pik3c2a	5'-CCTTTGCTGGGTACATGATGAC-3'	5'-GGAAGGTTAACTGCTCGCTTT-3'
polypeptide epidermal growth factor receptor pathway substrate 8	Eps8	5'-AACTGGCCAGTTCGGTACTC-3'	5'-GGAGTTGACTTGAAAGGCATGG3'
exonuclease 1.	Exo1	5'-GCTGGCTGAAGATGACCTGTT-3'	5'-AGCTCAGATTGTGCATCCCATT-3'
N-acylsphingosine amidohydrolase 2; neutral/alkaline; neutral/alkaline ceramid	Asah2	5'-AACAACCATGTCGGGACGAA-3'	5'-GAGGAAGGTTTGATGGGTCTGG-3
carboxypeptidase D	Cpd	5'-CAACTTCACCCTTGTTCGATCT-3'	5'-CAGTTCCAACTGGAGCGTTT-3'

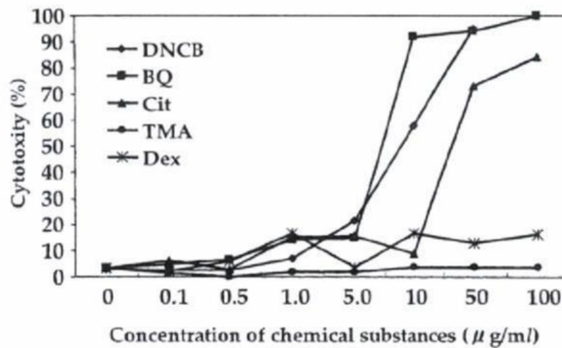

Fig. 1. Sensitivity of DC2.4 cells to chemical exposures. DC2.4 cells were exposed to chemicals at various concentrations (0–100 μg/ml) for 24 hr, and cell viabilities were determined by trypan blue dye exclusion assay.

after exposure to DNCB or BQ for 24 hr are presented in Table 2 along with their accession number and fold-change relative to the control cell cultures. The genes down-regulated are also shown in Table 3.

Real-time RT-PCR analyses of gene expression changes in DC2.4 cells induced by chemical exposures: In order to determine the reproducibility of the gene expression changes observed in microarray analysis, a set of genes was selected for evaluation by real-time RT-PCR. In the previous study, we assessed *in vivo* changes of gene expression in mouse ear sensitized with a type IV allergy-inducible chemical, BQ (data not shown). The microarray data from DNCB or BQ-treated DC2.4 cells were compared with that from type IV chemical-exposed mouse ears, and up- or down-regulated genes in all the experiments were explored. As the result, 3 up-regulated (Socs2, Sdc1 and Tuba6) and 8 down-regulated (Eps8, Exo1, Asah2, Cpd, LY86, SMO, Hey1 and Pik3c2a) genes showed strict changes in all the experiments,

Table 2. List of genes that were up-regulated by 24 hr exposure to DNCB and BQ

Accession no.	Gene name	Gene symbol	Fold-change	
			DNCB	BQ
Cytokine				
M27960	Interleukin-4 receptor alpha chain	Il4ra	1.3	1.5
AK078708	LPS-induced TNF-alpha factor	Litaf	1.6	1.3
Protein modification/synthesis				
AJ578468	defensin beta 14	NM_183026	1.7	1.4
U80019	proline dehydrogenase (oxidase) 2	Prodh2	1.3	1.4
AK054050	amine oxidase (flavin-containing)	Maob	1.3	1.5
L07645	Histidine ammonia-lyase	Hal	1.5	1.3
AK007058	L-lactate dehydrogenase C chain	Ldh3	1.8	2.7
AI323038	Cathelin-related antimicrobial peptide	Camp	1.4	1.4
NM_008185	Glutathione S-transferase theta 1	Gstt1	1.6	1.4
AK076002	proteasome 26S non-ATPase subunit 11	Psmd11	1.7	2.4
Siganal transduction				
AK036928	MAP kinase kinase 4	Map2k4	1.5	1.7
U88327	Cytokine inducible SH2-containing protein 2	Socs2	1.7	1.3
AK122347	Dedicator of cytokinesis protein 10	Dock10	2.7	1.5
Cell surface receptors/membrane proteins				
AF237914	membrane-spanning 4-domains.	Ms4a4c	1.7	1.4
NM_011519	Syndecan-1 precursor	Sdc1	1.6	1.3
BC057133	Chloride channel protein 3	Clcn3	1.4	1.4
Transcription factors/activators/repressors				
L09600	nuclear factor. erythroid derived 2	Nfe2	1.4	1.7
AK131183	Nuclear factor erythroid 2 related factor 1	Nfe2l1	1.7	1.7
Cell cycle/proliferation/death				
K014411	Synaptonemal complex protein 2	SCP2	1.6	1.5
Others				
BC040397	3 beta-hydroxysteroid dehydrogenase	Hsd3b2	2.1	1.9
U12147	Laminin alpha-2 chain	Lama2	1.4	1.4
AK081350	phosphatidylinositol 3-kinase. catalytic.	Pik3cb	2.3	1.7
M13441	Tubulin alpha-6 chain	Tuba6	1.3	1.6
BC010745	tubulointerstitial nephritis antigen	Tinag	1.4	1.7
L29468	Cofilin. muscle isoform (Cofilin 2)	Cfl2	1.6	1.3
XM 354745	Proliferin 1 precursor	Mrpplf3	1.3	1.4