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Effect of repeated and prolonged exposure to low concentrations of Low Molecular Weight chemicals on local lymph node responses

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PREFACE

The local lymph node assay (LLNA) is currently used to determine the sensitizing potency of low molecular weight chemicals. A chemical is considered a sensitizer (allergen) when it induces a reaction with a stimulation index of 3 or higher in the LLNA. This threshold for identification of a sensitizer might be considered as starting point for risk analysis. However, not all chemicals show a similar behavior in the skin. Some chemicals may accumulate locally and thus have a prolonged persistence in the skin. In this study the local lymph node stimulation was investigated after repeated and prolonged exposure to doses of formaldehyde and formaldehyde donors below the threshold for sensitization.

RAPPORT IN HET KORT

Effect van herhaalde en langdurige blootstelling aan lage concentraties van laag moleculaire verbindingen op de lokale lymfklier reacties

De resultaten van de lokale lymfkliertest zijn niet voor alle stoffen bruikbaar als uitgangspunt voor een kwantitatieve risicoanalyse. Dit onderzoek beschrijft wat er gebeurt na een herhaalde blootstelling van de huid aan een concentratie van een allergene stof onder de drempelwaarde die gebruikt wordt in de zogenaamde lokale lymfkliertest. Bij 3 van de 5 onderzochte stoffen (formaldehyde, 2-chloro-N-acetamide, quartenium-15) bleek langdurige blootstelling van de huid beneden de drempelwaarde toch een positieve reactie op te roepen. Bij de andere twee stoffen (paraformaldehyde, hexamethylenetetramine) werd slechts een geringe verhoging waargenomen ten opzichte van een kortdurende blootstelling.

Soms roepen stoffen die in contact komen met de huid al allergische reacties op bij doseringen die in een veel gebruikt standaardtestsysteem nog geen effecten laten zien.

De lokale lymfkliertest, die veel gebruikt wordt om contactovergevoeligheid van (sensibiliserende) stoffen te kunnen bepalen, kent een grenswaarde waarboven een stof als contactallergeen beschouwd wordt. Testen met doseringen lager dan deze grenswaarde, geven geen sensibilisatie meer in deze standaardtest. Er zijn echter stoffen die na langdurige blootstelling van de huid aan een lagere dosis toch tot sensibilisatie zullen leiden. Om tot een betere categorisering van stoffen te komen dan simpel “wel/geen” allergeen, was de vraag aan de orde of, net zoals voor sommige andere testsystemen, drempelwaarden uit de lokale lymfkliertest gebruikt kunnen worden voor de risico-evaluatie van allergene stoffen. Deze risico-evaluatie zou dan kunnen leiden tot de vaststelling van veilige blootstellingsniveaus.

Trefwoorden:

allergie, local lymph node assay, langdurige blootstelling, laag moleculaire stoffen.

ABSTRACT

Effect of repeated and prolonged exposure to low concentrations of Low Molecular Weight chemicals on local lymph node responses

The results of the local lymph node assay are not for all compounds useful as starting point for a quantitative risk assessment. This study describes the effects after repeated exposure of the skin to a concentration of a sensitizer below the threshold used in the local lymph node assay. Positive reactions were induced by three of the five investigated compounds (formaldehyde, 2-chloro-N-acetamide, quaternium-15) after long term exposure of the skin to concentrations below the threshold. For two other compounds (paraformaldehyde, hexamethylenetetramine) only a minor increase was observed when the response was compared to the short term exposure schedule. Compounds contacting the skin may induce allergic responses at dosages which do not induce responses in commonly used standard test assays. The local lymph node assay, which is now commonly used for the determination of sensitizing potency of chemicals, uses a threshold above which a compound is considered a contact sensitizer. Tests with doses below this threshold do not result in positive responses in this standard assay. However, there are compounds which induce sensitization after prolonged exposure of the skin to low doses. In order to develop an improved categorization than “yes/no” sensitizer, the question was raised whether the threshold used in the local lymph node assay could be used for the risk assessment of allergenic compounds similar to other test systems. This risk assessment might then result in the determination of safe exposure levels.

Key words:

allergy, local lymph node assay, long term exposure, low molecular weight chemicals.

Contents

SUMMARY.....	6
1. INTRODUCTION.....	7
2. ANIMALS, MATERIALS AND METHODS.....	9
3. RESULTS.....	12
4. DISCUSSION.....	13
REFERENCES.....	16
ANNEX 1 TABLES AND FIGURES.....	18

SUMMARY

Effect of repeated and prolonged exposure to low concentrations of Low Molecular Weight chemicals on local lymph node responses

In the Local Lymph Node Assay (LLNA) the EC3 value, the effective concentration inducing a threefold increase of lymph node responses compared to controls, is considered a threshold value for sensitization. It might be argued that this threshold could be used for risk assessment using the EC3 value as a benchmark dose. This implies that there should be no sensitization or lymph node reaction when an animal is exposed to doses below this threshold. We investigated local lymph node responses after repeated and prolonged exposure to low doses of test chemicals which are known to persist in the skin. In a short term local lymph node assay of five days the EC2 value was determined of five formaldehyde donors. After repeated and prolonged exposure (13 times for 8 weeks) to a dose of EC2 draining lymph node responses were determined by measuring the cellular proliferation. For all test chemicals investigated enhanced lymph node responses were obtained in the long term exposure when compared to short term exposure, while three of five chemicals induced responses above $SI=3$. Our results show that repeated and prolonged exposure to doses below the EC3 value can induce reactions above the sensitization cut-off point of $SI=3$ in draining lymph nodes.

So, when discussing the possible use of a bench mark approach, and using the EC3 or the lower confidence interval (L05) as a possible threshold for skin sensitization, one should be aware that in interpretation of the results of the LLNA for risk assessment also the characteristics of the chemical under investigation should be considered.

Key words:

allergy, local lymph node assay, long term exposure, low molecular weight chemicals.

1. INTRODUCTION

In addition to the guinea pig maximization test and guinea pig Buehler test, now also the local lymph node assay is recognised as stand alone test for the determination of sensitizing potential of low molecular weight chemicals (OECD 2002). In this assay the chemical is applied to the dorsum of the ear of mice and local lymph node cells are isolated for determination of their proliferative activity in response to the exposure. (Basketter and Scholes, 1992; Kimber et al., 1995; Loveless et al., 1996). In the LLNA the chemical under investigation is generally investigated in a dose response manner. The threefold stimulation of the cellular proliferation in the draining auricular lymph node compared to vehicle control animals is considered the cut-off point. When a chemical induces a stimulation index of 3 or higher it is considered a sensitizer. This figure was empirically determined by comparing the murine results with knowledge of human sensitizers (Kimber and Basketter, 1992), and later confirmed by statistical analysis (Basketter et al., 1999a). The LLNA has the advantage over the GPMT that quantitative results are obtained.

In addition to using the SI of three as cut-off point for sensitization this value can also be used for comparing the potency of various chemicals with each other (Van Och, 2000; De Jong et al., 2002b). The dose response relation in the LLNA can be used to calculate the critical effect dose (CED) the concentration (dose) resulting in a stimulation index of three (Slob, 2002), also designated EC3 the effective concentration inducing a stimulation index of 3 or higher ($SI \geq 3$). The uncertainty distribution of the determined CED can be quantified by using the bootstrap method (Slob and Pieters, 1998; Slob, 2002). The uncertainty distribution or confidence interval (L05-L95) of the calculated EC3 presents an indication on the quality of the data obtained in the LLNA.

The EC3 value can be used for comparing (ranking) various chemicals for their sensitizing potency (Basketter et al., 1999a,b; Van Och et al., 2000; De Jong et al., 2002b). Based on such ranking chemicals should be chosen with a high EC3 value which is indicative for a low(er) sensitizing potency. In addition, when considering risk analysis such a value of EC3 might be of use for the risk assessment of individual

chemicals rather than looking at them as a group consisting of sensitizers and non-sensitizers. The EC3 value could then be considered as a possible maximal limit for the presence of individual chemicals in products. This might be a valuable use of the data obtained in the LLNA. However, the question remains whether the threshold of an EC3 is the level of choice. What happens when an animal is exposed repeatedly to concentrations below the EC3. In addition, not all chemicals disappear as easily and quickly from the skin. Some chemicals show a persistence in the skin over a prolonged period of time. Formaldehyde is well known for its use as preservative in a number of products, and a well known sensitizer (Sasseville, 2004). The mechanism of action is crosslinking of proteins and other structures in the tissue. This leads to a prolonged persistence of formalin in the skin. In this study we investigated the local lymph node stimulation after repeated and prolonged exposure to low doses of formalin and formalin donors.

2. ANIMALS, MATERIALS AND METHODS

Animals. Young adult (6-8 weeks of age) female BALB/c mice were obtained from the Central Animal Facility of the Institute. The animals were bred under specified pathogen-free (SPF) conditions. During the experiments the animals were housed barrier maintained under conventional conditions in light-, humidity-, and temperature controlled rooms. All animals were housed in macrolon cages. The mice were fed chow pellets (Hope Farms, Woerden, the Netherlands) and water ad libitum.

All other husbandry conditions were maintained according to all applicable provisions of the following national laws: Experiments on Animals Decree, and Experiments on Animals Act. All animal experiments were performed according to all applicable national laws, and had permission from the ethics committee on animal experimentation of our Institute (RIVM).

Chemicals. The chemicals investigated belonged to the group of formaldehyde donors, including formaldehyde (CAS no. 50-00-0, Brunswig Chemie, Amsterdam, the Netherlands), paraformaldehyde (CAS no. 30525-89-4, Merck, Darmstadt, Germany), Quartenium-15 (1-(cis-3-chloroallyl)3,5,7-triaza-1-azonia adamantane chloride, CAS no. 51229-78-8, Sigma-Aldrich, Zwijndrecht, the Netherlands), 2-Chloro-N-(hydroxymethyl)acetamide (CAS no. 2832-19-1, Sigma-Aldrich,), and hexamethyleneamide (CAS no. 100-97-0, Sigma-Aldrich). All chemicals were dissolved in 4:1 acetone/olive oil (AOO). For each chemical a dose response experiment was performed for determination of the EC3 value. Chemicals were administered at various concentrations dissolved in AOO. The EC3 value was calculated as described previously (Van Och et al., 2000; De Jong et al., 2002b, Slob et al., 2002).

Experimental design. The LLNA was performed as previously described (De Jong et al., 2002a,b). In order to enhance possible low responses of weak sensitizers animals were pretreated on the dorsum of the ears with sodium dodecyl sulfate 1% (SDS, Merck) one hour before administration of the chemicals (De Jong et al., 2002a). The treatment schedule for short term experiments was as follows: administration of

chemicals on skin of the dorsum of the both ears (25µl each) on days 0-1-2. At day 5 following start of treatment animals were sacrificed and draining (auricular) lymph nodes (LN) were excised. For the long term experiments the treatment schedule was: administration on days 0-1-2 followed by a treatment once weekly for 7 weeks, and a three days treatment at days 56-57-58 followed by isolation of the LN cells at day 61. Isolated left and right LNs from each mouse were weighed, and single cell suspensions prepared using a cell strainer (Falcon, Franklin Lakes, NJ, USA). Cells were washed twice and suspended in RPMI 1640 (Gibco, Grand Island, NY, USA) culture medium supplemented with 10% inactivated fetal calf serum (PAA, Linz, Austria), 100 IU/ml penicillin, and 100µg/ml streptomycin, referred to as supplemented medium. Cells were counted in a Coulter Counter (Coulter Electronics, Mijdrecht, the Netherlands) and adjusted to a concentration of 1×10^7 cells/ml. When necessary, cell suspensions of several animals were pooled in order to obtain cell concentrations of 1×10^7 cells/ml, notably so for vehicle (AOO) treated controls.

Lymphocyte stimulation test. LN cell suspensions, 2×10^6 cells per well in 150 µl RPMI 1640 supplemented medium were cultured in triplicate in round bottomed 96 wells microtiter plates (Greiner, Alphen aan de Rijn, the Netherlands). An aliquot of 10 µl of ^3H -methylthymidine (Amersham International, Buckinghamshire, UK) (^3H -TdR, 37kBq/well, 3.7 MBq/ml, specific activity 185 GBq/mmol) was added to the wells of the cell culture directly after initiation of culture. Cultures were maintained for 24h at 37°C in a humidified atmosphere of 5% CO_2 in air. The cellular DNA was harvested on glass fiber filters using an automatic cell harvester (Harvester 96[®], Tomtec, Orange, CT, USA), scintillation liquid was added, and incorporation of ^3H -TdR was measured by liquid scintillation in a Betaplate counter (1205 Betaplate[™] Wallac, Turku, Finland). Proliferation per animal was determined by calculating the ^3H -TdR incorporation for the total cell number harvested (left and right lymph nodes combined). The ^3H -thymidine incorporation of cells of treated animals were compared to cells of vehicle AOO (acetone : olive oil, 4:1) treated animals.

Statistical analysis. The EC3 (effective concentration inducing a threefold increase in ^3H -thymidine incorporation in the harvested lymph node cells of treated animals compared to vehicle treated animals) was estimated by the benchmark approach, by fitting a nonlinear regression model to the data of all individual animals as described

previously (Van Och et al., 2000; De Jong et al., 2002b; Slob et al., 2002). In short, a non linear regression analysis was used applying a series of mathematical models for the evaluation of the responses. The choice of the model for deriving the EC3 follows from a procedure of applying likelihood ratio tests on the members of the following nested family of models:

model 1: $y = a$

model 2: $y = a \exp(bx)$

model 3: $y = a \exp(bx^d)$

model 4: $y = a(c - (c - 1)\exp(bx))$

model 5: $y = a(c - (c - 1)\exp(bx^d))$,

where y is the response, and x denotes the applied concentration. The parameter a represents the level of the response at concentration zero, and b can be considered as the parameter reflecting the efficacy of the chemical. At high doses models 4 and 5 level off to the value ac , so the parameter c can be interpreted as the maximum relative change, compared to the background. Models 3 and 5 have the flexibility to mimic threshold-like responses. For a detailed description see Slob and Pieters (1998), and Slob (2002). Additionally, an estimate of the uncertainty (90%-confidence interval) associated with the estimated EC3 was determined using a (parametric) bootstrap method (Slob and Pieters, 1998).

3. RESULTS

Table 1 shows the results obtained with the chemicals in the short term local lymph node assay. The following order of increasing sensitizing potency can be derived from our data: hexamethylenetetramine < quartenium 15 < 2-Chloro – N – acetamide < paraformaldehyde < formaldehyde. Indicating formaldehyde as the most potent sensitizer. Besides the EC3 value as sensitizing threshold we also derived an EC2 dose for further investigations with a repeated exposure protocol for a prolonged time period. An example of the derivation of both EC2 and EC3 critical effective doses for formaldehyde is presented in Figure 1. The results of the prolonged exposure experiments are presented in Table 2. For all chemicals investigated in all experiments the prolonged repeated exposure protocol (61 days) to the EC2 dose resulted in an enhanced stimulation index when compared with the short (5 days) protocol. For three of the five chemicals investigated (2 – Chloro – N – acetamide, formaldehyde, and quartenium 15) the repeated exposure to an EC2 dose resulted in a stimulation index above the sensitising threshold of 3.

4. DISCUSSION

The threefold stimulation of cellular proliferation, the $SI \geq 3$, in the draining auricular lymph node compared to vehicle control animals is considered the cut-off point for determining whether a chemical is considered a sensitizer or not (Kimber and Basketter, 1992; Basketter et al., 1999a). In this the LLNA is primarily used for hazard identification (Dearman and Basketter, 1999). The quantitative results and the dose response relationship in the LLNA offers the possibility for comparing skin sensitizers based on the calculated EC₃, the effective concentration inducing a SI of 3. This comparison offers the possibility for ranking chemicals according to their sensitizing potency, and thus choosing chemicals for production processes and/or products with a high EC₃ value resulting in a low chance for inducing a sensitization of exposed populations. Although such ranking of chemicals offers credibility for the choice of chemicals with a high EC₃ value, it has to be clear that small differences in EC₃ values would have little biological significance (Dearman and Basketter, 1999). As the results in the LLNA are obtained in a dose response evaluation one might consider to use the data for risk assessment for chemicals. So, besides ranking the data also can be used to classify chemicals in groups according to high and low or (almost) no risk for sensitization. This offers the possibility for a more reliable labeling of products containing sensitizers. Classes for sensitizers have previously been determined for chemicals investigated in the GPMT, and are now under discussion for chemicals investigated in the LLNA, such as $EC_3 \leq 0.2\%$ for the extreme sensitizers, $EC_3 > 0.2\%$ but $\leq 2\%$ for strong sensitizers, and $EC_3 > 2\%$ for the moderate sensitizers.

Previously we demonstrated that for Benzocaine, TMTD (tetramethylthiuramdisulfide), and DNCB (2,4-dinitrochlorobenzene) that repeated exposure to doses below the EC₃ value did not induce responses above the $SI=3$ (Van Och et al., 2003). Only for DNCB, and only with doses above the EC₃ value proliferative lymph node responses were observed with a stimulation index above 3. This suggested that the EC₃ indeed might be considered as a real threshold below which there is no sensitization. If this is so, then the EC₃ value could be applied for risk assessment for individual chemicals. It might be considered whether it should not only be used for categorisation by determining classes of sensitizers, but it might be

considered for risk assessment based on bench marking for individual chemicals. This would have an impact on the current rules of labeling as now a product/mixture should be labeled if it contains more than 1% of a sensitizer. The EC3 value as threshold would be then be the new level for indication of a hazard, and could be individually applied to each chemical. Then there is no need for labeling for an allergic hazard if a chemical would be present well below its EC3 threshold. In order to include a possible safety margin one might use the lower value of the 90% confidence interval (L05) which would indicate that there is still a 5% chance of a positive response in the LLNA, in others words “getting sensitized”, when exposed to a concentration below this L05 value. In contrast, also in the LLNA some false negative and positive responses can occur (ICCVAM, 1999). Also for chemicals which were chosen for their capacity to induce hypersensitivity in man (Knudsen et al., 2002a,b), for some chemicals a rather (unrealistic) high EC3 or no EC3 value could be determined (De Jong et al., 2002b).

However, previously the prolonged studies were only performed for 3 well known compounds (Van Och et al., 2003). One of the more serious risks of sensitization would be the prolonged presence of chemicals in the skin. In order to determine whether there is indeed no sensitization possible below the threshold of SI=3, we investigated a group of chemicals which are known to persist in the skin. We used for this evaluation the dose with an EC2 inducing stimulation indices around SI=2. All test chemicals induced an enhanced response after the repeated and prolonged exposure compared to a short term exposure, while three out of five induced lymph node responses above SI=3. Our results show that the concentration that induces an EC3 in the LLNA is not an absolute threshold. Although for two of three compounds no responses above the SI=3 were obtained after the repeated and prolonged exposure, the responses were higher than those after short term exposure (Table 2). This implies that there are certain characteristics of chemicals, such as skin persistence, that have an impact on the risk assessment of these kind of compounds. In conclusion, the quantitative data obtained in the LLNA offer the possibility for use in a bench mark approach for risk assessment of individual chemicals for sensitization, with either the EC3 value or the L05 lower level of the 90% confidence interval as parameter. However, when discussing the possible use of a bench mark approach, and using the EC3 or the lower confidence interval (L05) as a possible threshold for skin sensitization, it has to be considered that not only the results in the

LLNA are of importance but also the characteristics of the chemical under investigation. We demonstrated that repeated and prolonged exposure to doses below the EC3 value can induce reactions above the sensitization cut-of point of SI=3 in draining lymph nodes.

REFERENCES

Basketter DA, Lea LJ, Cooper K, Stocks J, Dickemns A, Pate I, Dearman RJ, Kimber I. (1999a) Threshold for classification as a skin sensitiser in the local lymph node assay: a statistical evaluation. *Food Chem Toxicol* 37, 1167-1174.

Basketter DA, Lea LJ, Dickens A, Briggs D, Pate I, Dearman RJ, Kimber I (1999b) A comparison of statistical approaches to the derivation of EC3 values from local lymph node assay dose responses. *J Appl Toxicol* 19, 261-266.

Basketter DA, Scholes EW (1992) Comparison of the local lymph node assay with the guinea pig maximisation test for the detection of a range of contact allergens. *Food Chem Toxicol* 32, 543-547.

Dearman RJ, Basketter DA, Kimber I (1999) Local Lymph node assay: use in hazard identification and risk assessment. *J Applied Toxicol* 19, 299-306.

De Jong WH, Tentij M, Spiekstra SW, Vandebriel RJ, Van Loveren H (2002a) Determination of the sensitising activity of the rubber contact sensitizers TMTD, ZDMC, MBT and DEA in a modified local lymph node assay and the effect of sodium dodecyl sulfate pretreatment on local lymph node responses. *Toxicology* 176, 123-134.

De Jong WH, Van Och FMM, Den Hartog Jager CF, Spiekstra SW, Slob W, Vandebriel R, Van Loveren H. (2002b) Ranking of allergenic potency of rubber chemicals in a modified local lymph node assay. *Toxicol Sci* 66, 226-232.

ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods, NIH Publication No.99-4494 (1999) The murine local lymph node assay: a test method for assessing the allergic contact dermatitis potential of chemicals/compounds. Internal NIH Publication. National Institutes of Environmental Health Sciences, National Institutes of Health, US Public Health Service, Department of Health and Human Services, National Toxicology Program, Research Triangle Park, NC, USA.

Kimber I, Basketter DA (1992) The murine local lymph node assay: a commentary on collaborative studies and new directions. *Food Chem Toxicol* 30, 165-169.

Kimber, I., Hilton, J., Dearman, R.J., Gerberick, G.F., Ryan, C.A., Basketter, D.A., Scholes, E.W., Ladics, G.S., Loveless, S.E., and House, R.V. (1995) An international evaluation of the murine local lymph node assay and comparison of modified procedures. *Toxicology* 103, 63-73.

Knudsen, B.B., Hametner, C., Seycek, O., Heese, A., Koch, H.-U., and Peters, K.-P. (2000a) Allergologically relevant rubber accelerators in single use medical gloves. *Contact Dermatitis* 43, 9-15.

Knudsen, B.B., Hametner, C., Seycek, O., Heese, A., Koch, H.-U., and Peters, K.-P. (2000b) Bioavailability of rubber accelerators in rubber gloves and patch test reactivity. *Dermatosen* 48, 127-133.

Loveless SE, Ladics GS, Gerberick GF, Ryan CA, BAsketter DA, Scholes EW, House RV, Hilton J, Dearman RJ, Kimber I. (1996) Further evaluation of the local lymph node assay in the final phase of an international collaborative trial. *Toxicology* 108, 141-152.

OECD (1992) Guideline 429. Guideline for the testing of chemicals. Skin sensitisation: local lymph node assay. OECD, Paris, France.

Sasseville D (2004) Hypersensitivity to preservatives *Dermatol Ther* 17, 251-263.

Slob W, Pieters MN. A probabilistic approach for deriving acceptable human intake limits and human health risks from toxicological studies: General framework. *Risk Anal* 18, 787-798, 1998.

Slob W. Dose response modelling of continuous endpoints. *Toxicol Sci* 66, 298-312, 2002.

Van Och FMM, Slob W, De Jong WH, Vandebriel R, Van Loveren H. (2000) A quantitative method for assessing the sensitizing potency of low molecular weight chemicals using a local lymph node assay: employment of a regression method that includes determination of the uncertainty margins. *Toxicology* 146, 49-59.

Van Och FMM, Vandebriel R, De Jong WH, Van Loveren H. (2003) Effect of prolonged exposure to low antigen concentration for sensitization. *Toxicology* 184, 23-30.

ANNEX 1 Tables and Figures

Table 1 EC2 and EC3 values of cellular proliferation in lymph nodes after exposure to formaldehyde donors

Chemical	Concentrations ^a Exposure (%)	EC3 (L05 – L95) ^b	EC2 (L05 – L95)
2-Chloro-N-acetamide	0.16, 0.62, 2.5, 10	16.0 (9.0 – 75.1)	10.1 (5.7 – 47.4)
Formaldehyde	0.06, 0.23, 0.92, 1.85	0.96 (0.75 – 1.32)	0.6 (0.5 – 0.8)
Hexamethylenetetramine	2.5, 5, 10, 20	30.6 (18.7 – 84.2)	19.3 (11.8 – 53.1)
Paraformaldehyde	0.06, 0.23, 0.92, 1.85	3.15 (1.84 – 11.23)	2.0 (1.2 – 7.1)
Quartenium-15	0.62, 25, 10, 20	20.8 (15.8 – 30.4)	13.1 (10.0 – 19.2)

a) Chemicals were administered on the dorsum of both ears (25µl per ear) at days 0-1-2 one hour after SDS 1% treatment, and cellular proliferation in draining lymph nodes was determined at day 5.

b) EC2 and EC3 values were calculated using non linear regression analysis as described in the Animals, Materials and Methods section.

Table 2 Local lymph node reactions after short term and repeated exposure to low concentrations (EC2) of sensitizers.

Chemical	Concentration ^a (%)	Short protocol ^b (3x) Stimulation Index	Repeated protocol ^c (3x-7x-3x) Stimulation Index
2-Chloro-N-acetamide	10.1	1.31 ^d 1.83	4.82 3.85
Formaldehyde	0.6	2.24	6.99
Hexamethylenetetramine	19.3	4.00 0.66	5.78 1.38
Paraformaldehyde	2.0	0.74 1.36	0.97 1.68
Quartenium-15	13.1	1.52	9.42

a) Animals were treated with the effective concentration inducing a stimulation index of 2 (EC2) in the local lymph node assay one hour after SDS 1% treatment.

b) Animals were treated on days 0-1-2 and lymph node cell proliferation was determined on day 5.

c) Animals were treated on days 0-1-2, 7-14-21-28-35-42-49, and 56-57-58 and lymph node cell proliferation was determined on day 61.

d) Results are expressed as stimulation index compared to AOO treated controls.

Formaldehyde model 2 in terms of CED

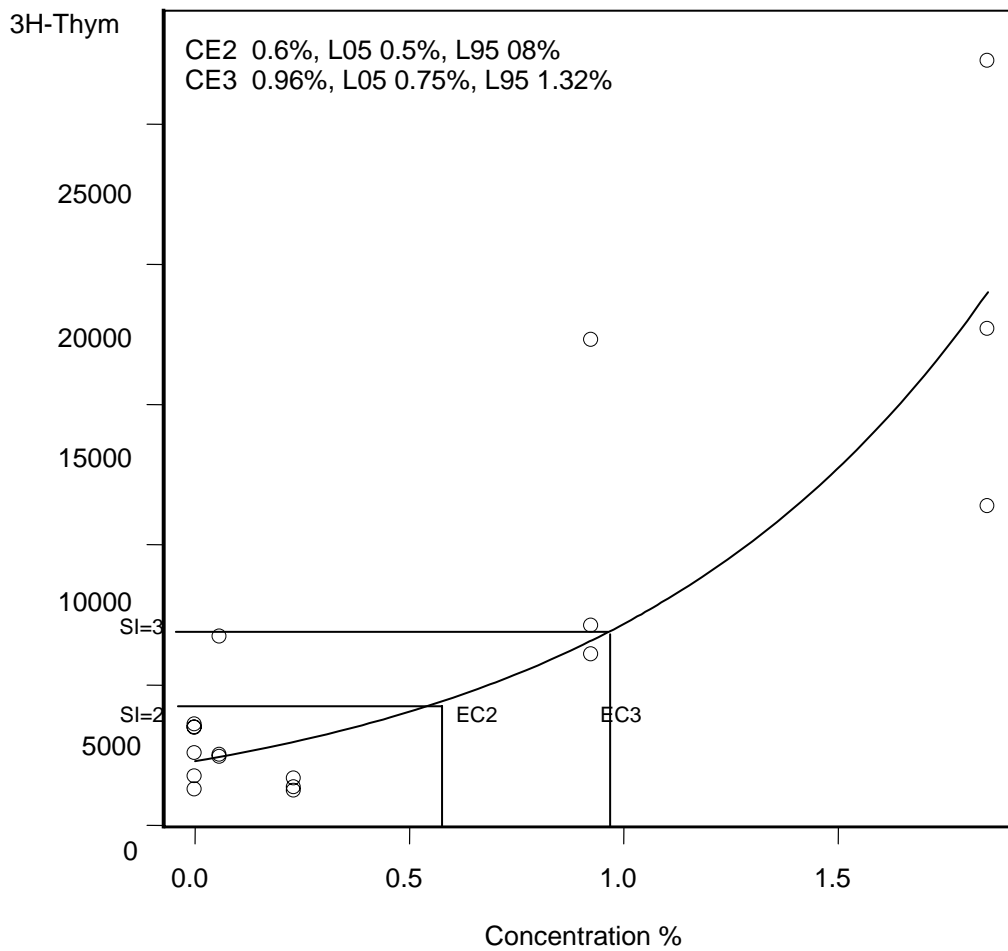


Figure 1 Example of determination of EC2 and EC3 values using the bench mark approach. Results are presented in cpm as a function of concentration for formaldehyde together with fitted regression function.