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The Respiratory Local Lymph Node Assay as a Tool to Study Respiratory Sensitizers

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The local lymph node assay (LLNA) is used to test the potential of low molecular weight (LMW) compounds to induce sensitization via the skin. In the present study, a respiratory LLNA was developed. Male BALB/c mice were exposed head/nose-only during three consecutive days for 45, 90, 180, or 360 min/day to various LMW allergens. Ear application (skin LLNA) was used as a positive control. Negative controls were exposed to the vehicle. Three days after the last exposure, proliferation was determined in the draining mandibular lymph nodes, and the respiratory tract was examined microscopically. Upon inhalation, the allergens trimellitic anhydride, phthalic anhydride, hexamethylene diisocyanate, toluene diisocyanate, isophorone diisocyanate (IPDI), dinitrochlorobenzene, and oxazolone were positive and showed stimulation indices (SIs) up to 11, whereas trimeric IPDI, formaldehyde, and methyl salicylate were negative (viz. SI < 3). All compounds, except trimeric IPDI, induced histopathological lesions predominantly in the upper respiratory tract. Exposure by inhalation is a realistic approach to test respiratory allergens. However, based on the local toxicity, the dose that can be applied is (generally) much lower than can be achieved by skin application. It is concluded that strong LMW allergens, regardless their immunological nature, besides the skin can also sensitize the body via the respiratory tract. In addition, the contact allergens were as potent as the respiratory allergens, although the potency ranking differed from that in a skin LLNA.

Key Words: **sensitizers; respiratory allergy; contact allergy; LLNA; potency.**

The local lymph node assay (LLNA) is designed to monitor cellular proliferation, as measured by the uptake of tritium-thymidine ($[^3H]TdR$) by the draining lymph node (LN) cells, in order to assess the potential of compounds to induce sensitization via the skin (Kimber and Weisenberger, 1989; Kimber *et al.*, 1994). Chemicals that elicit a stimulation index (SI) of 3 or more—a threefold increase in $[^3H]TdR$ incorporation relative to the control value—in the LLNA are considered skin sensitizers. The EC_3 value being the dose (concentration) inducing stimulation rates of threefold of the control values has been suggested to be used as an indicator of the potency of skin sensitization (Kimber *et al.*, 1995; Loveless *et al.*, 1996). Based on the EC_3 value, chemicals can be ranked and selected for low skin sensitizing potency (De Jong *et al.*, 2002; Van Och *et al.*, 2000). Within the context of REACH, the LLNA is the preferred assay to investigate skin sensitization potential (EC, 2006).

There is evidence that most, if not all, low molecular weight (LMW) respiratory allergens also test positive in the dermal LLNA or other assays for skin sensitization such as the guinea pig maximization or Buehler test (Arts, 2001; Kimber *et al.*, 2007; Van Loveren *et al.*, 2008), and thus, would require labeling for sensitization via the skin (Risk Phrase 43). There are at present no validated assays to test for the potential of compounds to sensitize by inhalation, that is, via the respiratory tract (Risk Phrase 42). Can it be expected that sensitization mechanisms for the skin are similar to the sensitization mechanisms for the respiratory tract? Potential and potency may differ between these two routes because the respiratory epithelium has a less robust mechanical defense than skin and antigen-presenting and immune-regulating mechanisms may be different. To test if an inhalation exposure to allergens could induce proliferation in the LNs, which drain the respiratory tract, and, if so, to test whether potency would be comparable for the inhalation route and dermal route, a so-called respiratory LLNA was developed. The test compounds were known respiratory allergens including trimellitic anhydride (TMA), phthalic anhydride (PA), toluene diisocyanate (TDI), hexamethylene diisocyanate (HDI), and isophorone diisocyanate (IPDI). The responses to these compounds were compared to the responses to the typical contact allergens dinitrochlorobenzene (DNCB), oxazolone (OXA), and formaldehyde (FA) and those of a respiratory irritant methyl salicylate (MS), and a compound of unknown inhalation sensitizing properties namely oligomeric isophorone diisocyanate (trimeric IPDI). The protocol of the respiratory LLNA was identical to the skin LLNA, viz. three days of exposure (days 0, 1, and 2) followed

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TABLE 1
Test and Control Groups

	Inhalation exposure		Dermal exposure	
	Target concentration (mg/m^3)[a]	Negative control[b]	Concentration (wt/vol)[b]	Negative control[b]
TMA	30	Acetone (12)	50% (3)	AOO (3)
PA	15	Acetone (12)	25% (3)	AOO (3)
TDI	7.5 (ca. 1.0 ppm)	Air (12)	1% (3)	AOO (3)
HDI	7.5 (ca. 0.9 ppm)	Air (6)	1% (6)	AOO (6)
IPDI	7.5 (ca. 1.2 ppm)	Acetone (6)	1% (6)	AOO (6)
Trimeric IPDI	15	Acetone (6)	12.5% (3)[c]	AOO (3)
DNCB	30	Acetone (12)	50% TMA (6)	AOO (3)
OXA	15	Acetone (12)	0.1% (3)	AOO (3)
FA	3.6 (ca. 3.0 ppm)	Air (6)	10% (vol/vol)[d] (6)	AOO (6)
MS	30	Air (6)	25% (3)[c]	AOO (3)

[a]Groups of six animals were exposed for 45, 90, 180, or 360 min/day.
[b](*n*): number of animals.
[c]Trimeric IPDI and MS were not expected to increase proliferation, therefore 1% DNCB-exposed groups (each consisting of 3 animals) was included to check the validity of the test procedure.
[d]10% (vol/vol) FA was prepared by adding eight parts of AOO to three parts of FA (37%).

by necropsy on day 5 after which the proliferation activity was measured in the draining LNs. During exposure, the concentration was held constant, but exposure duration was varied in order to obtain an increasing exposure dose for each group of animals. The selection of the draining or local LNs upon an inhalation exposure to be used in the respiratory LLNA is less straightforward than upon dermal application in the skin LLNA because there are many LNs draining the respiratory tract (Van den Broeck *et al*., 2006). Rather than injecting the animals with radioactive label *in vivo*, the draining LNs were excised and the LN cells were cultured with radioactive label *in vitro*. This method was proven to be a good alternative and requires much less radioactive label and waste (De Jong *et al*., 2002; Kimber and Weisenberger, 1989; Van Och *et al*., 2000). The *ex-vivo* procedure allowed us to also measure the cytokine profiles in the local LNs, thus offering an opportunity to reduce the number of animals which is needed in separate LLNA and cytokine assays. The cytokine data will be reported separately (De Jong, Wim H. de Jong, Josje H.E. Arts, Arja de Klerk, Marcel A. Schijf, Janine Ezendam, C. Frieke Kuper, H. van Loveren, submitted).

The present paper describes the performance (sensitization potential and potency) of a selection of LMW allergens in a respiratory LLNA and compares these with data obtained in simultaneously and previously conducted skin LLNAs.

MATERIALS AND METHODS

Animals and maintenance. Male, 6- to 7-week-old, inbred BALB/c mice were purchased from Charles River Deutschland (Sulzfeld, Germany) and acclimatized for at least 5 days before the start of each experiment. They were kept under conventional laboratory conditions and received the standard pelleted diet (RM3 [E] SQC, Special Diet Service, Witham, UK) and unfluoridated tap water *ad libitum*. All animal procedures were approved by the Institute's Commission of Animal Welfare.

Chemicals. The following chemicals were used: TMA (97% purity), toluene 2,4-diisocyanate (TDI; technical grade, 80%), and hexamethylene-1,6-diisocyanate (HDI; 98% purity; all from Aldrich, Brussels, Belgium); 2,4-dinitrochlorobenzene (DNCB; purity at least 98%), OXA (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one; purity at least 90%), MS (purity at least 99%), and raffinated olive oil (all from Sigma, St Louis, MO); PA (purity at least 99%) and FA (pro analysis purity at least 36.5% in H_2O); both from Fluka, Buchs, Switzerland); monomeric and oligomeric IPDI (Degussa GmbH, Marl, Germany; acetone (from Biosolve, Valkenswaard, the Netherlands). Oligomeric IPDI consisted of 67% of trimeric IPDI (the remaining consisted of pentameric, heptameric, and nonameric IPDI) and is therefore called trimeric IPDI during the remainder of the paper.

Experimental design. Groups of male BALB/c mice (six animals per group) were exposed nose-only to one of the various LMW compounds for 45, 90, 180, or 360 min/day on three consecutive days. The dermal route (ear application) was used as a positive control. Negative controls were exposed by ear application of the vehicle (acetone-olive oil, 4:1) or by inhalation to the vehicle (acetone or air) for 360 min/day for 3 days. The animals were necropsied 3 days after the last exposure, several draining LNs were excised, LN cells were harvested, and DNA was labeled with [^{3}H]-thymidine to determine proliferation. In the positive, dermally exposed control group, the auricular LNs were excised. In addition, the respiratory tract was histopathologically examined, and cytokine production (IFN-γ, IL-4, IL-10, and IL-12) in the cultured draining LN cells was measured using a Bio-Plex assay (Biorad Laboratories, Hercules, CA). The results of the cytokine examinations will be reported separately (De Jong *et al*., submitted).

Dermal exposure. Animals received 25 µl/day of a single concentration of each test substance, dissolved in a 4:1 (vol/vol) mixture of acetone and olive oil (AOO) on the dorsum of both ears on three consecutive days (positive skin control), or AOO alone (negative skin control). Target concentrations of the various test chemicals as well as controls and numbers of animals are summarized in Table 1. The target concentrations of DNCB, OXA, PA, TDI, and TMA were based on dermal LLNAs, previously performed in BALB/c mice (Van Och *et al*., 2000).

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TABLE 2
Actual Concentrations, Acetone Concentrations, and Determination of Aerodynamic Particle Size

	Actual concentration ± SD (mg/m^3) (n)[a]	Acetone concentration (g/m^3)		MMAD[b] (μm)	Geometric standard deviations
		Test	Control		
TMA	31.4 ± 6.0 (19)	1.05	0.66	2.0	2.3
PA	14.2 ± 1.5 (17)	0.99	0.77	2.6	4.4
Trimeric IPDI	15.6 ± 6.0 (22)	0.82	0.78	1.9	2.5
DNCB	30.1 ± 3.3 (12)	1.34	1.26	1.9	2.0
OXA	14.8 ± 2.2 (17)	0.60	0.66	2.2	1.9
IPDI	7.5 ± 0.3 (12)	0.29	0.29	NA	NA
HDI	7.3 ± 0.4 (12)	—	—	NA	NA
TDI	7.4 ± 0.6 (12)	—	—	NA	NA
FA	4.0 ± 0.3 (12)	—	—	NA	NA
MS	30.4 ± 0.2 (3)	—	—	NA	NA

[a]Actual concentration was measured 4–8 times per day except for the MS concentration that was calculated over three separate days; mean was given over the 3-day period.

[b]Particle size distribution was measured on the first exposure day; — not used; NA, not applicable as no particles were present in the test atmosphere.

Inhalation exposure and test atmosphere generation and analysis. Mice were exposed to one of the various test materials on three consecutive days for 45, 90, 180, or 360 min/day. Variation in exposure duration rather than in concentration was used to investigate the dose-response relationships. The target concentrations of TMA, TDI, DNCB, and OXA were based on previous inhalation experiments (Arts *et al.*, 1998, 2004; Mommers *et al.*, 2006; or unpublished results).

During exposure, the mice were individually restrained in Battelle tubes, and each tube was then placed into inhalation units (ADG Developments Ltd., Codicote, Herts, UK) for head/nose-only exposure to the test atmosphere. The units had a volume of approximately 50 l. Exposure air flows were at least 0.3 l/min per animal.

DNCB, TMA, OXA, PA, and IPDI (monomer and trimer) test atmospheres were generated as aerosols from daily freshly prepared solutions of the chemicals in acetone. Acetone was used to help generate respirable particles of these compounds normally consisting of very large noninhalable particles. The solutions were delivered to a compressed air-driven nebulizer (Schlick type 970/S, Coburg, Germany) by a motor-driven syringe pump (WPI type SP22i, World Precision Instruments, Sarasota, FL). Air flow to the nebulizer was regulated by a mass flow controller (Bronkhorst Hi Tec, Ruurlo, the Netherlands). The aerosolized acetone evaporated completely before reaching the animals nose. The final acetone concentrations in air were below 600 ppm (1.4 g/m^3; viz. below 0.06% in air; Table 2), which is considered to be far below a level inducing sensory irritation (Alarie, 1973; De Ceaurriz *et al.*, 1981; Schaper and Brost, 1991). The corresponding controls were exposed to similar low levels of acetone in air for 360 min/day (Table 2). Test atmospheres of the more volatile substances TDI, HDI, MS, and FA were generated by evaporation. The chemicals were delivered to a glass evaporator by a motor-driven syringe pump (WPI type SP22i, World Precision Instruments). The temperature in the evaporator was controlled by circulating heated water (at 60°C and 70°C, in case of TDI and HDI, and of MS and FA, respectively). The corresponding controls were exposed to clean air only for 360 min/day. Thus, test atmospheres, except for the presence of test compound, consisted of air or air with maximally 0.06% acetone, which is a completely different situation when using different vehicles in the dermal LLNA.

The temperature and relative humidity (RH) were recorded during exposure. The mean temperature was 21.6 ± 0.4°C for control atmospheres and 22.0 ± 0.4°C for the test atmospheres. The RH was 42.8 ± 3.7% and 47.5 ± 7.4% for the control and test atmospheres, respectively, except in experiments with the chemically very reactive compounds TDI and HDI where animals were exposed under dry air conditions (mean RH of 3.3 ± 0.8%).

The concentrations of DNCB, TMA, OXA, PA, and trimeric IPDI in the test atmosphere were determined gravimetrically by filter sampling at 5 l/min. TDI, HDI, and monomeric IPDI concentrations were analyzed according to method MDHS 25/3 (White, 2006). In short, samples of the test atmosphere (1.0 l/min) were adsorbed onto 1-(2-methoxyphenyl) piperazine–coated glass fiber filters, followed by solvent desorption and analysis using reversed phase high-performance liquid chromatography (RP-HPLC) with UV detection at 242 nm. In addition, the concentrations of TDI, HDI, and MS were monitored using a total carbon analyzer (Ratfisch RS55T, Munich, Germany). The MS concentrations were calculated based on the nominal concentration and the complete evaporation of the test solution. FA concentrations were analyzed according to method MDHS 78. Samples of the test atmosphere (1.5 l/min) were adsorbed onto 2,4-DNPH-silica cartridges (Waters Chromatography, Etten-Leur, the Netherlands), followed by solvent desorption, and RP-HPLC analysis with UV detection at 365 nm. Actual concentrations are indicated in Table 2; these were close to the target concentrations.

Particle size distributions of DNCB, OXA, TMA, PA, and trimeric IPDI in the test atmospheres were determined using a 10-stage cascade impactor (Andersen, Atlanta, GA). The mass median aerodynamic diameter (MMAD) and geometric standard deviations of these chemicals are depicted in Table 2. The particle size measurements indicated that aerosol particles were of respirable size. The other compounds (TDI, HDI, IPDI, MS, and FA) were present in the gaseous phase, and therefore no particles were present.

Local effects, body and organ weights, and necropsy. All animals were observed at least once daily and weighed shortly before the first treatment and just prior to necropsy. At necropsy, 5 days after the first exposure, animals were anesthetized with Nembutal, killed by exsanguination from the abdominal aorta, and examined grossly for abnormalities. Draining LNs were excised, and the nose and lungs with trachea and larynx were removed. The lungs (with trachea and larynx) were weighed in case of TMA, PA, TDI, DNCB, and OXA exposure.

Local LN activation. The pulmonary and dermal sensitizing potency of the chemicals was investigated in a modified LLNA using *ex vivo* labeling of the proliferating LN cells (De Jong *et al.*, 2002; Kimber and Weisenberger, 1989; Vandebriel *et al.*, 2000; Van Och *et al.*, 2002). In a preliminary 3-day test

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at a high concentration of 250 mg/m^3 TMA, it was examined which LNs were enlarged. These were, as expected, the auricular LNs upon dermal treatment and, not entirely expected, the mandibular LNs following inhalation exposure. Therefore, these LNs were taken out in all further exposures, and when enlargement of other LNs was noted at macroscopic observation of the highest dosage group, these were collected additionally in all groups. This was especially the case for the cervicalis posterior LNs in animals exposed by inhalation to HDI and IPDI and also for the auricular LNs following inhalation exposure to TDI, HDI, IPDI, and OXA. For a better comparison, the auricular LNs were therefore taken out following all exposures except for DNCB which was the first study.

The cells of left and right LNs were pooled for each animal and suspended in 5 ml RPMI-1640 (Gibco, Life Technologies, Breda, the Netherlands) supplemented with 5% heat-inactivated fetal calf serum (FCS; Integro, Zaandam, the Netherlands), 100 U/ml penicillin, and 100 μg/ml streptomycin (referred to as supplemented medium). Single-cell suspensions were prepared in supplemented medium under aseptic conditions by pressing the LNs through a 70-μm nylon cell strainer (Falcon, Franklin Lakes, NJ). Cells were washed twice (10 min, 300 g, 4°C), resuspended in 1 ml supplemented medium with 10% FCS, and counted using a Coulter Counter (Z2, Coulter Electronics, Mijdrecht, the Netherlands). After counting, the concentration of the cell suspensions was adjusted to 1×10^7 cells/ml. When necessary, cell suspensions of a few animals were pooled to obtain concentrations of 1×10^7 cells/ml, notably for vehicle-treated controls. To assess lymphocyte proliferation, cell suspensions (2×10^6 cells in 200 μl supplemented medium per well) were seeded in triplicate in round-bottomed 96-well microtiter plates (Greiner, Alphen aan de Rijn, the Netherlands). A 10-μl aliquot of [^{3}H]-methylthymidine ([^{3}H]-TdR; 37 kBq per well or 3.7 MBq/ml, specific activity 185 GBq/mmol; Amersham Int., Buckinghamshire, UK) was added to the wells immediately after the initiation of culture. Cultures were maintained at 37°C for 20–24 h 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), scintillation liquid was added, and [^{3}H]-TdR incorporation was measured by liquid scintillation counting in a β-plate counter (1205 Betaplate, Wallac, Turku, Finland). Proliferation per animal was determined by calculating the incorporation of [^{3}H]-TdR for the total cell number harvested (left and right LNs combined). The mean [^{3}H]-TdR incorporation was calculated per experimental group. SIs were calculated by dividing the mean [^{3}H]-TdR incorporation per group by the mean [^{3}H]-TdR incorporation of the (vehicle-treated) control group. ED_3 values (stimulation rates of threefold the control values in relation to the respiratory exposure dose) were derived by plotting SI values against total dose, followed by linear interpolation between two points on the SI axis, immediately above and below SI = 3, similar to the calculation of the EC_3 value after dermal exposure. Subsequently, ED_3 values were calculated using the equation: $ED_3 = c + [(3 - d)(b - d)] \times (a - c)$, where (a, b) and (c, d) represent data points above and below an SI value of 3, respectively (Basketter *et al.*, 1999).

Airway histopathology. Neutral, phosphate-buffered 4% (vol/vol) FA was used to inflate and preserve the lungs and to preserve the nose and larynx. Next, these organs were embedded in paraffin wax, and 5 μm sections were stained with hematoxylin and eosin. Cross sections of the nasal tissues were prepared at four different levels. The larynx was sectioned longitudinally at three levels including the epiglottis, the ventral pouch, and the arythenoid projections. Each lung lobe was cut at one median sagittal level. All tissues of animals exposed for 360 min/day (high dose group) were examined microscopically. In addition, tissues of all DNCB-exposed animals were examined to investigate dose-response relationships by increasing exposure duration at a constant concentration.

Statistics. SIs in mandibular and auricular LNs following inhalation exposure were statistically analyzed after logarithmic transformation, using a one-way ANOVA, followed by a two-sided Dunnett's test. A p value <0.05 was considered to be statistically significant. First, outliers were detected using Grubb's test and rejected for further analysis.

RESULTS

Local effects, body and organ weights, and necropsy.

The inhalation exposures were relatively well tolerated by the animals, and no abnormalities were noted during each day's exposure. Clinical signs noted after exposure were piloerection and hunched posture (TMA, OXA, PA, TDI), blepharospasm (OXA, PA, IPDI), dyspnoea (TMA, PA), sluggishness (PA), and leanness (TMA; data not shown). One animal exposed to FA (180-min group) and one control animal exposed to clean air (during the study with TDI) died on the first day of exposure, and one animal exposed to OXA (360-min group) died on day 4. The animal exposed to OXA may have died because of the inhaled dose; the FA-exposed death was considered not to be test-compound related, just like the control animal.

Slight to moderate losses in body weight between day 0 and day 5 were seen following exposure to TDI, HDI, and OXA (all four test groups), TMA, PA, and FA (90-, 180- and 360-min exposure groups), DNCB (180- and 360-min exposure groups), and IPDI (360-min exposure group only). No changes in body weights were observed following exposure to trimeric IPDI and MS (data not shown).

Lung weights were determined in animals exposed to DNCB, OXA, TMA, PA, and TDI. Slight to strong increases in relative lung weight were observed at necropsy in animals exposed to DNCB and OXA (all four test groups), TMA and PA (90-, 180- and 360-min exposure groups), and TDI (360-min exposure group only; data not shown). Because mean absolute lung weights were 140–150 mg in almost all groups, the increase in relative lung weight was largely explained by the decrease in body weight.

Findings at necropsy generally consisted of rales (TMA), visually increased breathing rate (TMA, TDI), air-filled intestines (TMA, PA, OXA, TDI), leanness (HDI, IPDI), and stained lungs (IPDI). No data were available for DNCB-exposed animals, and no changes were generally observed in animals exposed to FA, trimeric IPDI, or MS (data not shown). The aforementioned changes generally showed a clear exposure duration-related increase in severity and/or incidence.

Local LN activation.

The mandibular LNs draining the nasal tissues following inhalation exposure to TMA, PA, TDI, HDI, IPDI, DNCB, and OXA were enlarged and showed statistically significant increases in SIs, which were in excess of 3 in many groups. Clear dose (concentration × time)–response relationships were obtained for HDI and DNCB, and to a lesser extent for IPDI; bell-shaped curves were seen in the case of TMA, TDI, and OXA. The response to PA, though positive, was variable. No increases were observed following inhalation exposure to FA, trimeric IPDI, and MS (Table 3 and Fig. 1).

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TABLE 3
Proliferation Data in Mandibular LNs Following Inhalation Exposure ([^{3}H]-TdR incorporation in cpm)

Chemical	Vehicle exposure	45 min/day	90 min/day	180 min/day	360 min/day
TMA	1177 ± 168[a] (0/11)[b,c]	2367 ± 304 (0/6)	6642 ± 1486 (4/6)	2696 ± 437 (1/5)[c]	2403 ± 241 (0/5)[c]
PA	1471 ± 151 (0/11)[c]	4238 ± 843 (3/6)	5352 ± 801 (4/5)[c]	3330 ± 223 (0/5)[c]	4289 ± 760 (2/6)
TDI	1867 ± 230 (0/11)	6075 ± 1244 (2/6)	6417 ± 1830 (2/6)	10,248 ± 2514 (4/6)	5961 ± 438 (5/6)
HDI	811 ± 41 (0/5)[c]	3792 ± 844 (4/6)	4906 ± 769 (6/6)	5307 ± 917 (6/6)	7952 ± 825 (6/6)
IPDI	2429 ± 455 (0/5)[c]	6831 ± 2033 (2/6)	8354 ± 2407 (3/5)[c]	10,729 ± 1291 (5/5)[c]	9670 ± 1854 (4/5)
Trimeric IPDI	1585 ± 354 (0/6)	1626 ± 219 (0/6)	1869 ± 391 (0/6)	1399 ± 204 (0/6)	1172 ± 215 (0/6)
DNCB	2808 ± 335 (0/6)	6831 ± 1684 (2/5)	11,129 ± 2635 (4/6)	14,686 ± 4231 (4/6)	30,254 ± 3383 (5/5)[c]
OXA	1248 ± 118 (0/11)	8858 ± 1726 (6/6)	12,032 ± 2686 (6/6)	4439 ± 1146 (3/6)	1100 ± 347 (0/4)[c]
FA	2403 ± 120 (0/6)	2155 ± 276 (0/6)	3306 ± 733 (0/5)[c]	3931 ± 978 (0/5)	2079 ± 767 (0/5)[c]
MS	2558 ± 303 (0/6)	2094 ± 248 (0/6)	3184 ± 473 (0/6)	3262 ± 501 (0/6)	3369 ± 544 (0/6)

[a] Mean ± SEM.
[b](n/n): Number of animals positive (i.e., SI $\geq$ 3)/total number of animals.
[c]One outlier was detected using Grubb's test and rejected for further analysis.

Highly significant, dose (concentration $\times$ time)–related increases in SI values (with SI values up to 100) were seen in the auricular LNs following inhalation exposure to TDI, HDI, IPDI, and OXA. The SI values in the auricular LNs were much higher than those in the mandibular LNs. No increases in SI values in auricular LNs were seen following inhalation exposure to TMA, PA, or to FA, trimeric IPDI, and MS (Table 4 and Fig. 2). The auricular LNs following DNCB exposure, unfortunately, were not measured. Although the cervicalis posterior LNs seemed to be enlarged following exposure to HDI and IPDI, low SI values of about 3 ± 2 were observed, indicating borderline stimulation (data not shown).

The SIs of the positive controls (dermal exposure) are indicated in Table 5. The SI values in the auricular LNs were very high. This was due to the concentrations tested that were generally much higher than EC_3 values of several of these compounds (Van Och et al., 2000) to minimize test failure. There was some variation in controls that were carried out twice (TMA, DNCB; Table 5). For some of the compounds, the negative control had a high tritium uptake resulting in lower SI values for the test groups and positive control. This is why the SI value for the auricular LNs following dermal exposure to TDI (positive control) is low in comparison to HDI and IPDI, whereas absolute tritium uptake was similar in these groups (Table 4).

To investigate possible differences in sensitizing potency between routes of exposure, ED_3 values following inhalation exposure were compared to EC_3 values obtained following dermal exposure. The total dose by inhalation (3 days) was calculated and compared to that obtained by dermal application for 3 days. The total inhalation dose was calculated using the mean actual concentration (mg/m^3), the duration of exposure (min) at which an SI value of 3 was obtained, the mean body weight between day 0 and day 5 (g), and a standard ventilation rate of 1.5 l/kg for mice. The total dose by dermal application (3 days) was calculated using the applied volume (25 μl per ear) based on published EC_3 values for five of the tested compounds by Van Och *et al.* (2000). Absorption was assumed to be 100% for both routes. With regard to potency ranking (Table 6), the dermal ranking would be: OXA, DNCB, diisocyanates, acid anhydrides, whereas the potency ranking following inhalation exposure is different: diisocyanates/OXA, acid anhydrides, DNCB. Interestingly, the contact allergens were as potent as the respiratory allergens to sensitize via the respiratory tract, as EC_3 and ED_3 values (assuming 100% absorption) were of the same order of magnitude regardless the exposure route (the maximum difference was a factor 10 in the case of PA, whereas OXA showed no difference at all).

Airway histopathology.

Trimeric IPDI was the only compound that did not induce histopathological lesions in the respiratory tract following exposure for 360 min/day. All other compounds induced lesions in the nasal tissues, and five of these also induced lesions in the larynx. No histopathological changes were observed in the lungs (Table 7). Only at a very high concentration of 250 mg/m^3 TMA, which was used during preliminary testing to examine which LNs should be collected, histopathological changes were observed in the lungs. These changes consisted of slight mononuclear cell infiltrate (data not shown).

Severe lesions in the nasal tissues were induced by OXA and TDI, whereas HDI and IPDI induced moderate lesions in the nasal tissues. DNCB, PA, and FA were slightly to moderately, and TMA and MS were only slightly toxic to the nasal tissues. The concentrations used for the different compounds varied to some extent, and therefore, compounds could not be compared fully on the basis of the severity of the lesions. The changes consisted of inflammatory cell infiltration, epithelial degeneration, hyperplasia, and, in severe cases, ulceration. Exudative rhinitis was seen in all animals exposed to TDI and in three out of six animals exposed to IPDI. DNCB and OXA were also moderately toxic to the larynx, whereas

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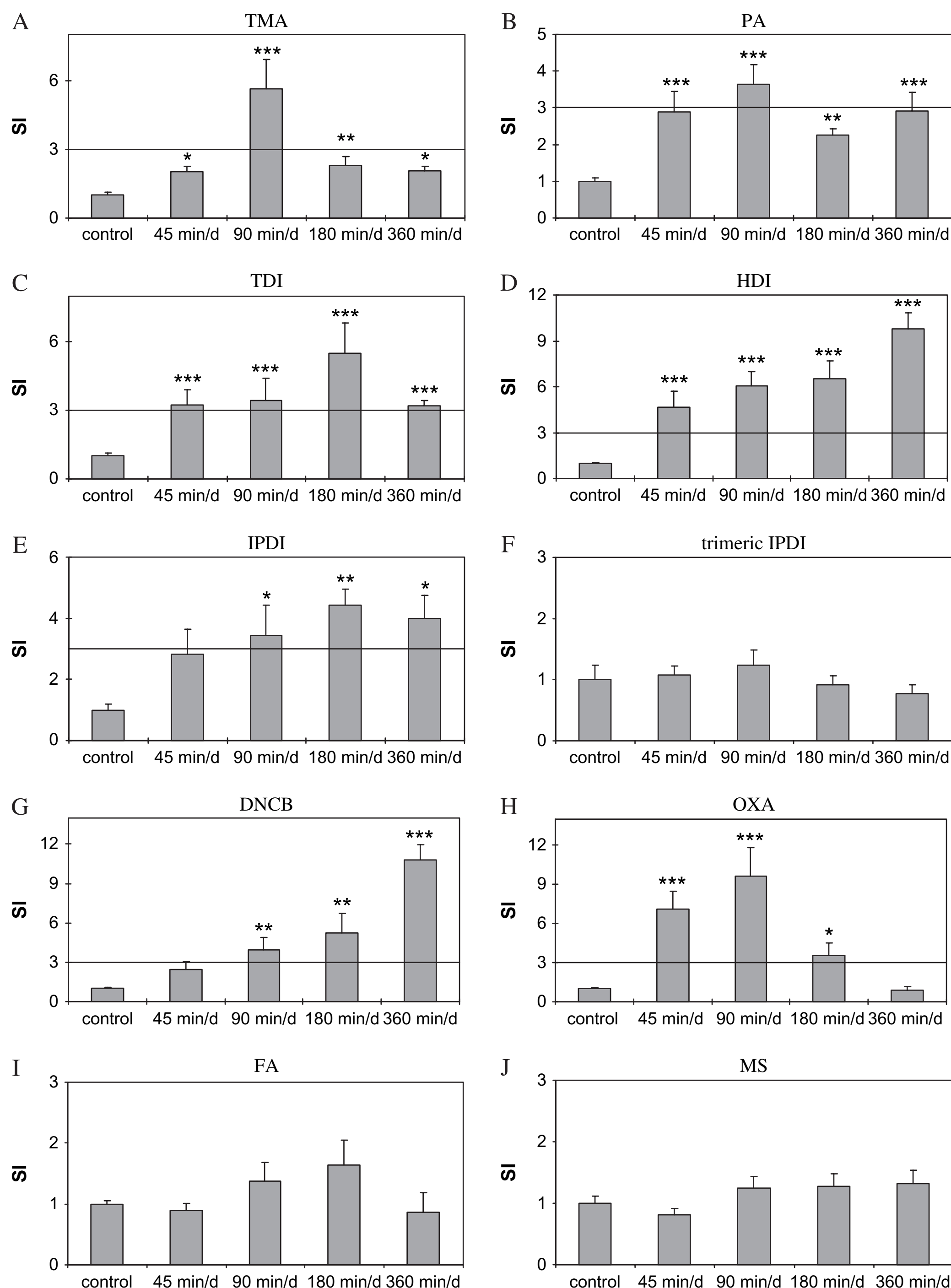

FIG. 1. SI values in mandibular LNs upon head/nose-only exposure to TMA, PA, TDI, HDI, IPDI, trimeric IPDI, DNCB, OXA, FA, and MS. Results are expressed in means ± SEM. Solid line indicates an SI value of 3. Statistical analysis was performed after logarithmic transformation, using a one-way ANOVA followed by a two-sided Dunnett's *t*-test; $*p < 0.05$, $**p < 0.005$, $***p < 0.001$.

TMA, PA, and TDI induced only slight to moderate laryngeal lesions (Table 7).

The histopathological changes in nasal tissue and larynx induced by DNCB were exposure duration-related (Table 8).

DISCUSSION

The LLNA in rodents is used as a test for predicting skin sensitization potential of compounds in humans. A chemical

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TABLE 4
Proliferation Data in Auricular LNs Following Inhalation Exposure ([^{3}H]-TdR incorporation in cpm)

Chemical	Vehicle exposure	45 min/day	90 min/day	180 min/day	360 min/day
TMA	630 ± 67[a] (0/11)[b,c]	502 ± 64 (0/6)	438 ± 32 (0/6)	474 ± 43 (0/6)	339 ± 91 (0/6)
PA	1593 ± 191 (0/12)	1334 ± 95 (0/5)[c]	2565 ± 509 (1/6)	1412 ± 331 (0/6)	1332 ± 150 (0/6)
TDI	6822 ± 2469 (1/11)	4394 ± 767 (0/6)	13,464 ± 3257 (1/6)	35,923 ± 5180 (5/6)	99,562 ± 23,265 (6/6)
HDI	1174 ± 212 (0/6)	1014 ± 86 (0/6)	34,655 ± 7390 (6/6)	11,2347 ± 7559 (6/6)	127,634 ± 8980 (6/6)
IPDI	1369 ± 160 (0/6)	41,339 ± 16,654 (6/6)	50,567 ± 15,350 (6/6)	91,928 ± 21,646 (6/6)	116,347 ± 33,582 (5/5)
Trimeric IPDI	751 ± 97 (0/6)	947 ± 121 (0/6)	1677 ± 105 (0/5)[c]	915 ± 129 (0/6)	700 ± 100 (0/6)
DNCB	1583 ± 253 (0/11)[c]	NM	NM	NM	NM
OXA	721 ± 71 (0/10)[c]	2148 ± 576 (3/6)	1025 ± 295 (1/6)	18,871 ± 5000 (6/6)	22,876 ± 10,864 (3/5)
FA	1650 ± 119 (0/6)	1542 ± 230 (0/6)	1929 ± 404 (0/6)	2573 ± 401 (0/5)	1378 ± 205 (0/6)
MS	2332 ± 464 (0/6)	2543 ± 270 (0/6)	2107 ± 166 (0/5)[c]	2547 ± 471 (0/6)	1727 ± 367 (0/6)

[a]Mean ± SEM.
[b](*n*/*n*): Number of animals positive (i.e., SI $\geq$ 3)/total number of animals, NM = not measured.
[c]One outlier was detected using Grubb's test and rejected for further analysis.

that induces a proliferation index (SI) of 3 or more in the ear draining (auricular) LNs is regarded a sensitizer. In the present study, all allergens, including the respiratory allergens, were tested positive following dermal application. Therefore, the present results support the view (Arts, 2001; Kimber *et al.*, 2007; Van Loveren *et al.*, 2008) that the skin LLNA can also detect respiratory sensitizers.

In analogy to the skin LLNA, a respiratory LLNA (exposure by inhalation) was developed, and cellular proliferation was determined in the respiratory tract draining LNs. Also in analogy to the skin LLNA, we hypothesized that a chemical that induced an SI of 3 or more in the respiratory tract draining LNs should be regarded a respiratory tract sensitizer.

The first challenge encountered in the respiratory LLNA was the identification of the LNs to be harvested. In contrast to the skin LLNA, in which the auricular LNs are the draining LNs, there are several groups of LNs draining the respiratory tract (Van den Broeck *et al.*, 2006). However, the mandibular LNs turned out to be the LNs that were always grossly enlarged upon inhalation exposure, despite the different physicochemical characteristics of the allergens tested. This may be explained by a distance-related decrease in amounts of test material deposited in the respiratory tract following inhalation exposure. This assumption is supported by the results of the histopathological examination of the respiratory tract (Tables 7 and 8), showing most effects on the nasal tissues. In addition, allergic rhinitis almost always precedes asthma, indicating that also in humans allergens predominantly impact on the upper respiratory tract tissues (Gautrin *et al.*, 2006; Malo, 2005). Therefore, it is advised to investigate the mandibular LNs in the respiratory LLNA, when testing compounds of unknown sensitizing potential.

Interestingly, inhalation exposure to the vapors TDI, HDI, IPDI, and the nebulized OXA following dissolution in acetone stimulated the auricular LNs as well, inducing even higher SI values in the auricular than in the mandibular LNs. The mandibular LNs drain on the posterior cervical LNs and not on the auricular LNs, and more distant LNs are not expected to show higher responses than LNs closer to the route of exposure. This was not observed in the case of TMA and PA. Therefore, it was concluded that head/nose-only exposure to the diisocyanates and OXA is very effective for uptake of the allergen via the skin, most probably via the sparsely haired ears. This may be (partly) due to the higher lipophilicity of the diisocyanates when compared to the acid anhydrides, viz. the predicted octanol-water partition coefficients (mean ± SD; using online software: http://www.vcclab.org/lab/alogps) are 1.13 ± 0.46, 1.35 ± 0.45, 2.40 ± 1.31, 2.73 ± 1.54, 3.31 ± 1.64 for TMA, PA, HDI, TDI, and IPDI, respectively. An experimental logP of 1.60 was reported for PA by Hansch *et al.* (1995). The predicted logP for OXA is somewhere in between, viz. 2.03 ± 0.46.

Another problem was how to design an efficient and cost-effective exposure regime in a respiratory LLNA suitable for investigating dose-response relationships. It was expected that the total dose per unit area rather than the concentration would be critical for sensitization (Gerberick *et al.*, 2001; Menné and Calvin, 1993; White *et al.*, 1986). Therefore, duration rather than concentration was varied, viz. groups of animals were exposed for 3 days to a fixed concentration during different periods of time. Using this procedure, dose-response relationships were not always linear, but sometimes bell-shaped (Fig. 1). It remains to be investigated whether this is due to the variation in duration rather than concentration.

All allergens, including DNCB and OXA, but except FA induced lymphocyte proliferation in the mandibular LNs (Table 3 and Fig. 1). FA is considered an allergen of rather modest potency by some (Andersen *et al.*, 1985, 1995; Arts *et al.*, 1997; Hilton *et al.*, 1998), which was also confirmed in the present study following dermal application, but relatively potent by others (Frankild *et al.*, 2000). In a range of compounds, EC_3 values varied from 0.01% for OXA to about

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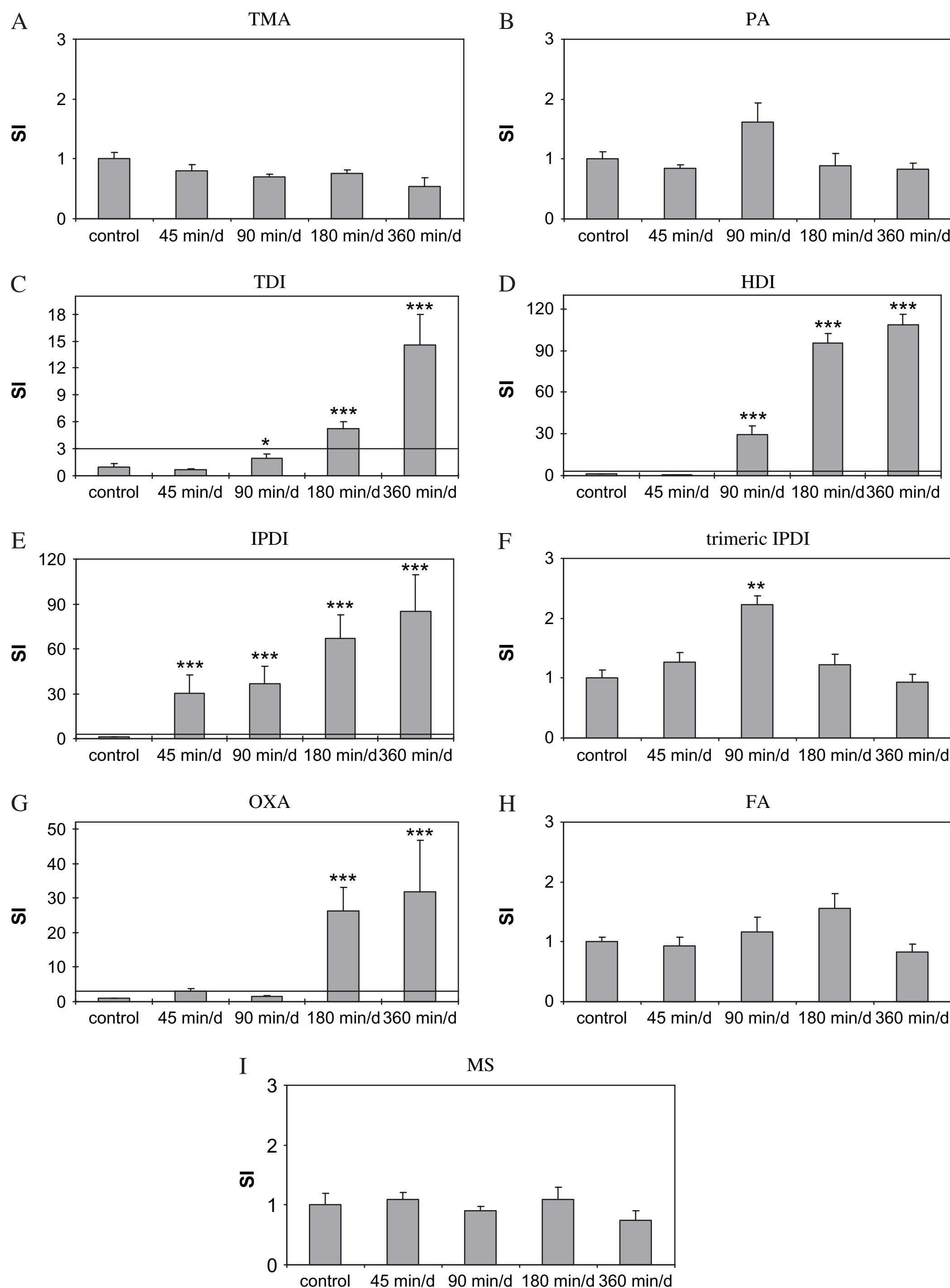

FIG. 2. SI values in auricular LNs upon head/nose-only exposure to TMA, PA, TDI, HDI, IPDI, trimeric IPDI, OXA, FA, and MS. Results are expressed in means ± SEM. Solid line indicates an SI value of 3. Statistical analysis was performed after logarithmic transformation, using a one-way ANOVA followed by a two-sided Dunnett's *t*-test; $*p < 0.05$, $**p < 0.005$, $***p < 0.001$.

44% for isoprobyl myristate, whereas the EC_3 value for FA was reported to be 0.4% (Basketter *et al.*, 2002). De Jong *et al.* (2007) observed an EC_3 value for FA of 0.9%. In our study, a relative high dermal concentration of 10% FA induced an SI value of only 10 in the auricular LNs, which was low in comparison to most of the other allergens. The inhalation concentration of 3 ppm (3.6 mg/m^3) was considered slightly irritating, based on the induction of slight nasal respiratory

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TABLE 5
SIs in Auricular LNs of Positive (dermal) Controls

Chemical	Concentration in % (wt/vol)	Number of animals tested	SI auricular LN[a]
TMA	50	3	130 ± 22
PA	25	3	93 ± 21
TDI	1	3	46 ± 6.3
HDI	1	6	285 ± 18
IPDI	1	6	415 ± 41
Trimeric IPDI[b]	12.5	3	1.0 ± 0.3
DNCB[c]	50	6	188 ± 30
OXA	0.1	3	52 ± 14
FA	10[d]	6	10 ± 1.7
MS[e]	25	3	2.3 ± 0.2

Note. 10% (vol/vol) FA was prepared by adding eight parts of AOO to three parts of FA (37%).
[a]Mean ± SEM.
[b]Three additional control animals were exposed to 1% DNCB (SI = 61 ± 3.5).
[c]In this experiment TMA (50% wt/vol) was used as the positive control.
[d]% (vol/vol).
[e]Three additional control animals were exposed to 1% DNCB (SI = 15 ± 3.9).

epithelial hyper/metaplasia following repeated exposure (reviewed by Arts *et al.*, 2006) and the nasal squamous hyper/metaplasia in three out of six mice following exposure for 360 min/day for 3 days in the present study (Table 7). However, although this concentration was irritating to the respiratory tract, it may still have been insufficient to induce sensitization. It could also mean that for moderately strong sensitizers, aerosolization rather than evaporation would be required to obtain sufficiently high concentrations in the respiratory tract.

The compound of unknown sensitizing potential trimeric IPDI was negative in the respiratory and skin LLNA. This may indicate that trimeric IPDI is a nonsensitizer. This is in accordance to the finding that the biuret or isocyanurate type homopolymers of HDI, unlike its monomeric form, did not induce sensitization by intradermal injection or via repeated inhalation exposures (Pauluhn *et al.*, 2002). On the other hand, the concentrations used in the present study might still have been too low, although trimeric IPDI was tested at a higher concentration than monomeric IPDI both via inhalation and dermal application. Trimeric IPDI (IPDI isocyanurate) was also negative in the Buehler test (Zissu *et al.*, 1998). However, it was tested at an induction concentration of 10% only, whereas other polyisocyanate prepolymers, viz. those of TDI and HDI, were concomitantly tested at much higher induction levels (30, 50, or 100%) and were found positive. Nonetheless, this means that these polyisocyanate prepolymers appear to be far less potent allergens than their monomeric counterparts.

The potency ranking in the respiratory LLNA differed from that in the skin LLNA. The typical respiratory allergens (the diisocyanates and the acid anhydrides) were more potent than DNCB via inhalation; the typical contact allergens OXA and DNCB were the most potent following dermal exposure. OXA was very potent via both routes, which supported our observation that OXA induced respiratory allergy in the Brown Norway rat model (Mommers *et al.*, 2006), besides the well-known skin allergy (Asherson *et al.*, 1983; De Sousa and Parrott, 1969; Kimber and Weisenberger, 1989; Parrott and De Sousa, 1966).

The total dose calculated to induce an SI of 3 (assuming 100% absorption) was, therefore, slightly lower in the skin than in the respiratory tract for DNCB, similar in the skin and in the respiratory tract for OXA; and lower in the respiratory tract than in the skin for TMA, PA, and TDI. The maximum difference was a factor 10 in the case of PA (Table 6).

SI values in the auricular LNs following dermal exposure were generally much higher (up to 415; Table 5) than the SI values in the mandibular LNs following inhalation exposure (up to 11; Fig. 1). This is most likely due to the higher amount of chemical that can be applied to the skin versus the airways as the presently tested inhalation concentrations already induced histopathological changes at various degrees (Tables 7 and 8). In addition, the total dose that reached the airway epithelium may have been much lower due to the altered breathing frequency and pattern induced by the allergen-induced irritation to the respiratory epithelium, thereby significantly reducing the calculated respiratory ED_3 values that assumed a normal breathing rate and 100% absorption (Table 6). Evidence of this is presented by animals that had air in the gastrointestinal tract suggestive of extensive irritation

TABLE 6
Ranking, Total Dose, and ED_3/EC_3 Values by the Inhalation and Dermal Route

Inhalation			Dermal		
Ranking	Interpolated ED_3 value ($C \times t$)	Calculated ED_3 (dose) (μg)	Ranking	EC_3 value (%)	Calculated EC_3 (dose) (μg)
HDI	7.3×25	18	OXA	0.013	20
OXA	14.8×17	19	DNCB	0.044	66
TDI	7.4×41	28	TDI	0.109	164
IPDI	7.5×59	44	TMA	0.218	327
PA	14.2×52	63	PA	0.357	536
TMA	31.4×57	156			
DNCB	30.1×62	173			

Note. The total dose by inhalation (3 days) was calculated using the mean actual concentration (mg/m^3), the interpolated (in some cases the extrapolated) duration of exposure (min) at which an SI value of 3 was obtained, the mean body weight between day 0 and day 5 (g), and a ventilation rate of 1.5 l/kg for mice. The total dose by dermal application (3 days) was calculated using the applied volume (25 μl per ear) based on published EC_3 values by Van Och *et al.* (2000). Absorption was assumed to be 100% for both routes. FA, trimeric IPDI, and MS were negative and therefore not included in this Table. For the dermal route, an EC_3 value of 0.40% FA was reported by Basketter *et al.* (2002) and 0.9% FA by De Jong *et al.* (2007).

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TABLE 7
Histopathology of the Respiratory Tract of Mice Exposed by Inhalation to the Different Test Compounds for 360 min/day

	Compounds (mg/m^3)									
Organs and lesions	TMA (30)	PA (15)	HDI (7.5)	TDI (7.5)	IPDI mono (7.5)	DNCB (30)	OXA (15)	FA (3.6)	IPDI trimeric (15)	MS (30)
Nasal cavity (number of animals) and overall score	(6) +[a]	(6) ++	(6) +++	(6) ++++	(6) +++	(6) ++	(6) ++++	(6) ++	(6) –	(6) +
Slight to moderate mixed inflammatory cell infiltration	–	2	4	–	4	5	6	1	–	–
Olfactory epithelial degeneration/necrosis										
Slight	–	–	2	4	1	–	–	–	–	4
Moderate	–	–	3	2	4	–	–	–	–	–
Respiratory epithelial degeneration										
Slight	–	–	–	4	1	–	–	–	–	5
Moderate	–	–	–	2	–	–	–	–	–	–
Slight squamous epithelial degeneration	–	–	–	–		–	3	–		
Squamous metaplasia/hyperplasia										
Slight	–	2	–	–	1	6	3	1	–	–
Moderate	–	–	–	–	3	–	–	2	–	–
Slight goblet cell hyperplasia	6	–	–	–	–	–	–	–	–	–
Ulceration										
Slight to moderate	–	–	2	4	3	–	2	–	–	–
Severe	–	–	–	2	–	–	1	–	–	–
Exudative rhinitis	–	–	–	6	3	–	–	–	–	–
Slight to moderate respiratory epithelial hyperplasia	–	–	6	6	–	–	–	–	–	–
Larynx (number of animals) and overall score	(6) ++	(6) ++	(6) –	(6) ++	(6) –	(6) ++++	(6) ++++	(4) –	(6) –	(6) –
Mixed inflammatory cell infiltration										
Slight	2	–	–	–	–	2	3	–	–	–
Moderate	–	–	–	–	–	–	1	–	–	–
Squamous metaplasia/hyperplasia										
Slight to moderate	3	2	–	1	–	4	4	–	–	–
Severe	–	–	–	–	–	1	–	–	–	–
Moderate ulceration/necrosis	–	–	–	–	–	–	2	–	–	–
Slight respiratory epithelial hyperplasia	–	–	–	3	–	–	–	–	–	–
Slight mononuclear cell infiltrate										
Lungs (number of animals) and overall score	(6) –	(5) –	(6) –	(6) –	(6) –	(6) –	(6) –	(6) –	(6) –	(5) –

[a]The overall score in histopathological lesions is expressed as follows: + = slight; ++ = slight to moderate; +++ = moderate to severe; ++++ = severe. Ulceration gave additional weight to the score.

and partial mouth breathing for ventilation. In addition, it is known that in mice under normal breathing conditions, the deposition of aerosol particles of about 2 micron MMAD may be approximately 50% in the nasal region and an additional 15–20% in the lower airways. Therefore, when the data are presented as EC_3 or ED_3 values (Table 6), viz. expressed in terms of delivered dose, the respiratory LLNA seems to be more sensitive than the traditional assay in view of the most probably much lower absorption value than 100% in the respiratory tract as described above. This may have important implication for risk assessment purposes.

In summary, the present results show that the respiratory LLNA can detect allergens. The respiratory LLNA can be used to test if a compound can sensitize the body via the respiratory

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TABLE 8
Histopathology of the Respiratory Tract of Mice Exposed by Inhalation to 30 mg/m^3 DNCB for Different Exposure Durations Per Day

(min/day)	DNCB (45)	DNCB (90)	DNCB (180)	DNCB (360)
Nasal cavity (number of animals) and overall score	(6) –	(6) –	(6) ++	(6) ++
Slight to moderate mixed inflammatory cell infiltration	–	–	6	5
Slight squamous epithelial degeneration	–	–	5	–
Squamous metaplasia/ hyperplasia Slight	–	–	–	6
Larynx (number of animals) and overall score	(6) –	(6) +++	(6) +++	(6) ++++
Slight to moderate mixed inflammatory cell infiltration	–	–	–	2
Squamous metaplasia/ hyperplasia				
Slight to moderate	–	5	5	4
Severe	–	–	–	1
Slight mononuclear cell infiltrate	–	2	1	0
Lungs (number of animals) and overall score	(6)	(6)	(6)	(6)
Slight mononuclear cell infiltrate				

Note. The overall score in histopathological lesions is expressed as follows: ++ = slight to moderate, +++ = moderate to severe, ++++ = severe.

tract, at what concentrations, and under which conditions. It may especially be used in case of highly volatile chemicals. Overall, in analogy to the dermal LLNA, the present results suggest that moderate to strong LMW allergens, regardless their immunological nature, cannot only sensitize the body via the skin but also via the respiratory tract. In addition, the contact allergens tested were as potent as the respiratory allergens to sensitize via the respiratory tract, which may pose a risk for human health if these compounds are inhaled.

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REFERENCES

Alarie, Y. (1973). Sensory irritation of the upper airways by airborne chemicals. *Toxicol. Appl. Pharmacol.* **24,** 279–297.

Andersen, K. E., Boman, A., Vølund, A., and Wahlberg, J. E. (1985). Induction of formaldehyde contact sensitivity: Dose-response relationship in the guinea pig maximization test. *Acta Derm. Venereol.* **65,** 472–478.

Andersen, K. E., Vølund, A., and Frankild, S. (1995). The guinea pigs maximization test—With a multiple dose design. *Acta Derm. Venereol.* **75,** 463–469.

Arts, J. H. E. (2001). In *Respiratory Allergy Induced by Low Molecular Weight Chemicals in Rats*. Utrecht University, Utrecht, the Netherlands; PhD thesis

Arts, J. H. E., de Koning, M. W., Bloksma, N., and Kuper, C. F. (2004). Respiratory allergy to trimellitic anhydride in rats: Concentration-response relationships during elicitation. *Inhal. Toxicol.* **16,** 259–269.

Arts, J. H. E., Dröge, S. C. M., Spanhaak, S., Bloksma, N., Penninks, A. H., and Kuper, C. F. (1997). Local lymph node activation and IgE responses in Brown Norway and Wistar rats after dermal application of sensitizing and non-sensitizing chemicals. *Toxicology* **117,** 229–237.

Arts, J. H. E., Kuper, C. F., Spoor, S. M., and Bloksma, N. (1998). Airway morphology and function of rats following dermal sensitization and respiratory challenge with low molecular weight chemicals. *Toxicol. Appl. Pharmacol.* **152,** 66–76.

Arts, J. H. E., Rennen, M. A. J., and de Heer, C. (2006). Inhaled formaldehyde: Evaluation of sensory irritation in relation to carcinogenicity. *Reg. Toxicol. Pharmacol.* **44,** 144–160.

Asherson, G. L., Colizzi, V., and Watkins, M. C. (1983). Immunogenic cells in the regional lymph nodes after painting with the contact sensitizers picryl chloride and oxazolone: Evidence for the presence of IgM antibody on their surface. *Immunology* **48,** 561–569.

Basketter, D. A., Evans, P. E., Gerberick, G. F., and Kimber, I. (2002). Factors affecting thresholds in allergic contact dermatitis: Safety and regulatory considerations. *Contact Derm.* **47,** 232–233.

Basketter, D. A., Lea, L. J., Dickens, A., Briggs, D., Pate, I., Dearman, R. J., and Kimber, I. (1999). A comparison of statistical approaches to the derivation of EC_3 values from local lymph node assay dose responses. *J. Appl. Toxicol.* **19,** 261–266.

De Ceaurriz, J. C., Micillino, J. C., Bonnet, P., and Guenier, J. P. (1981). Sensory irritation caused by various industrial airborne chemicals. *Toxicol. Lett.* **9,** 137–143.

De Jong, W. H., De Klerk, A., Ter Beek, M., Veenman, C., and Van Loveren, H. (2007). Effect of prolonged repeated exposure to formaldehyde donors with doses below the EC_3 value on draining lymph node responses. *J. Immunotoxicol.* **4,** 239–246.

De Jong, W. H., Van Och, F. M., Den Hartog Jager, C. F., Spiekstra, S. W., Slob, W., Vandebriel, R. J., and Van Loveren, H. (2002). Ranking of allergenic potency of rubber chemicals in a modified local lymph node assay. *Toxicol. Sci.* **66,** 226–232.

De Sousa, M. A., and Parrott, D. M. (1969). Induction and recall in contact sensivitity. Changes in skin and draining lymph nodes of intact and thymectomized mice. *J. Exp. Med.* **130,** 671–690.

EC. (2006). Regulation (EC) No. 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No. 793/93 and Commission Regulation (EC) No.

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1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. O.J. *Eur. Comm.* (L396), 1–849. (http://ec.europa.eu/environmental/chemicals/reach/reach_intro.html).

Frankild, S., Volund, A., Wahlberg, J. E., and Andersen, K. E. (2000). Comparison of the sensitivities of the Buehler test and the guinea pig maximization test for predictive testing of contact allergy. *Acta Derm. Venereol.* **80,** 256–262.

Gautrin, D., Desrosiers, M., and Castano, R. (2006). Occupational rhinitis. *Curr. Opin. Allergy Clin. Immunol.* **6,** 77–84.

Gerberick, G. F., Robinson, M. K., Ryan, C. A., Dearman, R. J., Kimber, I., Basketter, D. A., Wright, Z., and Marks, J. G. (2001). Contact allergenic potency: Correlation of human and local lymph node assay data. *Am. J. Cont. Derm.* **12,** 156–161.

Hansch, C., Leo, A., and Hoekman, D. (1995). In *Exploring QSAR—Hydrophobic, Electronic, and Steric Constants*. American Chemical Society, Washington, DC

Hilton, J., Dearman, R. J., Harvey, P., Evans, P., Basketter, D. A., and Kimber, I. (1998). Estimation of relative skin sensitizing potency using the local lymph node assay: A comparison of formaldehyde with glutaraldehyde. *Am. J. Contact. Derm.* **9,** 29–33.

Kimber, I., Agius, R., Basketter, D. A., Corsini, E., Cullinan, P., Dearman, R. J., Gimenez-Arnau, E., Greenwell, L., Hartung, T., Kuper, F., *et al.* (2007). Chemical respiratory allergy: Opportunities for hazard identification and characterisation. *ATLA* **35,** 243–265.

Kimber, I., Dearman, R. J., Scholes, E. W., and Basketter, D. A. (1994). The local lymph node assay: Developments and applications. *Toxicology* **93,** 13–31.

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.

Kimber, I., and Weisenberger, C. (1989). A murine local lymph node assay for the identification of contact allergens. Assay development and results of an initial validation study. *Arch. Toxicol.* **63,** 274–282.

Loveless, S. E., Ladics, G. S., Gerberick, G. F., Ryan, C. A., Basketter, D. A., Scholes, E. W., House, R. V., Hilton, J., Dearman, R. J., and Kimber, I. (1996). Further evaluation of the local lymph node assay in the final phase of an international collaborative trial. *Toxicology* **108,** 141–152.

Malo, J. L. (2005). Future advances in work-related asthma and the impact on occupational health. *Occup. Med.* **55,** 606–611.

Menné, T., and Calvin, G. (1993). Concentration threshold of non-occluded nickel exposure in nickel-sensitive individuals and controls with and without surfactant. *Cont. Derm.* **29,** 180–184.

Mommers, C., Arts, J., Schijf, M., and Kuper, F. (2006). Exposure is the proof of the pudding: Oxazolone is a potential respiratory allergen. *The Toxicologist (Suppl Tox. Sci.)* **90,** 76.

Parrott, D. M., and De Sousa, M. A. (1966). Changes in the thymus-dependent areas of lymph nodes after immunological stimulation. *Nature* **212,** 1316–1317.

Pauluhn, J., Eidmann, P., and Mohr, U. (2002). Respiratory hypersensitivity in guinea pigs sensitized to 1,6-hexamethylene diisocyanate (HDI): Comparison of results obtained with the monomer and homopolymers of HDI. *Toxicology* **171,** 147–160.

Schaper, M., and Brost, M. A. (1991). Respiratory effects of trimellitic anhydride aerosols in mice. *Arch. Toxicol.* **65,** 671–677.

Vandebriel, R. J., de Jong, W. H., Spiekstra, S. W., van Dijk, M., Fluitman, A., Garssen, J., and van Loveren, H. (2000). Assessment of preferential T-helper 1 or T-helper 2 induction by low molecular weight compounds using the local lymph node assay in conjunction with RT-PCR and ELISA for interferon-gamma and interleukin-4. *Toxicol. Appl. Pharmacol.* **162,** 77–85.

Van den Broeck, W., Derore, A., and Simoens, P. (2006). Anatomy and nomenclature of murine lymph nodes: Descriptive study and nomenclatory standardization in BALB/cAnNCrl mice. *J. Immunol. Methods* **312,** 12–19.

Van Loveren, H., Cockshott, A., Gebel, T., Gundert-Remy, U., De Jong, W. H., Matheson, J., McGarry, H., Musset, L., Selgrade, M. K., and Vickers, C. (2008). Skin sensitization in chemical risk assessment: Report of a WHO/IPCS international workshop focusing on dose-response assessment. *Regul. Toxicol. Pharmacol.* **50,** 155–162.

Van Och, F. M., Slob, W., de Jong, W. H., Vandebriel, R. J., and 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, F. M., van Loveren, H., de Jong, W. H., and Vandebriel, R. J. (2002). Cytokine production induced by low-molecular-weight chemicals as a function of the stimulation index in a modified local lymph node assay: An approach to discriminate contact sensitisers from respiratory sensitisers. *Toxicol. Appl. Pharmacol.* **184,** 46–56.

White, J. (2006). MDHS 25 revisited; development of MDHS 25/3, the determination of organic isocyanates in air. *Ann. Occup. Hyg.* **50,** 15–27.

White, S. I., Friedmann, P. S., Moss, C., and Simpson, J. M. (1986). The effect of altering area of application and dose per unit area on sensitization by DNCB. *Br. J. Dermatol.* **115,** 663–668.

Zissu, D., Binet, S., and Limasset, J.-C. (1998). Cutaneous sensitization to some polyisocyanate prepolymers in guinea pigs. *Contact Derm.* **39,** 248–251.

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