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The respiratory allergen glutaraldehyde in the local lymph node assay: Sensitization by skin exposure, but not by inhalation

Jos J. van Triel^{a,*}, Bianca W.J. van Bree^{a,1}, David W. Roberts^b, Hans Muijsers^a, Evert Duistermaat^a,
Ruud A. Woutersen^{c,d}, C. Frieke Kuper^a

^a TNO Quality of Life, Department of Toxicology and Applied Pharmacology, Zeist, The Netherlands

^b Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Byrom Street, Liverpool L3 3AF, United Kingdom

^c TNO Quality of Life, Department of Research and Development, Zeist, The Netherlands

^d Division of Toxicology, Wageningen University, Wageningen, The Netherlands

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ABSTRACT

Previously, a selection of low molecular weight contact and respiratory allergens had tested positive in both a skin and a respiratory local lymph node assay (LLNA), but formaldehyde was negative for sensitization by inhalation. To investigate whether this was due to intrinsic properties of aldehyde sensitizers, the structurally related allergen glutaraldehyde (GA) was tested. BALB/c mice were exposed by inhalation to 6 or 18 ppm GA (respiratory LLNA), both generated as a vapor and as an aerosol. Other groups received 0.25% or 2.5% GA on the skin of the ears (skin LLNA). Lymphocyte proliferation and cytokine production were measured in the draining lymph nodes. GA was positive in the skin LLNA and its cytokine profile (IL-4: IFN- γ) skewed towards a Th2-type immune response with increasing dose. Inhalation exposure did not result in increased lymphocyte proliferation or increased cytokine levels, despite comparable tissue damage (irritation) in the skin and respiratory tract. We hypothesize that the highly reactive and hydrophilic GA oligomerizes in the protein-rich mucous layer of the respiratory tract, which impedes sensitization but still facilitates local irritation. Within the context of risk assessment in respiratory allergy, our results stress the importance of prevention of skin – besides inhalation – exposure to aldehydes like GA.

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1. Introduction

The skin and the respiratory tract are considered to be the two main routes of sensitization. Despite some similarities between the two routes, there are also important mechanistic differences in the currently understood etiology of allergic contact dermatitis – the most common skin allergic disease – and asthma and allergic rhinitis, the most common respiratory allergies. Occupational asthma (OA) accounts for 9–15% of asthma in adults and is the most reported occupation-related lung disease in many countries (Bakerly et al., 2008). Glutaraldehyde (GA) was found to be responsible for 6% of the occupational asthma cases in the West-Midlands, an industrialized part of the United Kingdom (Bakerly et

al., 2008). Elsewhere, also numerous cases of GA-induced occupational asthma have been described (Curran et al., 1996; Takigawa and Endo, 2006; Vyas et al., 2000).

In contrast to sensitization by skin exposure, no validated test methods are available to investigate a compound's potential to induce sensitization by inhalation. The murine local lymph node assay (LLNA) is a widely used and accepted method to test the potential of a compound to induce sensitization via skin application (EC, 2006; Kimber et al., 1994; Kimber and Weisenberger, 1989). Interestingly, all low molecular weight (LMW) respiratory allergens tested so far were positive in dermal sensitization assays (Basketter and Scholes, 1992; Kimber et al., 2007). Indeed, the skin is considered an effective route to sensitize the respiratory tract for LMW allergens in test animals (Arts and Kuper, 2003; Botham et al., 1989; Karol, 1986; Warbrick et al., 2002), and there is evidence that this is true in humans as well (Redlich and Herrick, 2008).

Arts et al. investigated a set of contact- and respiratory allergens in a dermal LLNA as well as in a respiratory LLNA (exposure via inhalation) to compare the sensitizing potential and potency between both routes (Arts et al., 2008; De Jong et al., 2009). Although the potency ranking differed somewhat between both exposure routes, all allergens tested positive via both routes with

Abbreviations: FA, Formaldehyde; GA, Glutaraldehyde; LLNA, Local lymph node assay; LMW, Low molecular weight; NALT, Nasal-associated lymphoid tissue.

* Corresponding author at: TNO Quality of Life, Department of Toxicology and Applied Pharmacology, Utrechtseweg 48, PO Box 360, 3700 AJ, The Netherlands. Tel.: +31 30 69 44 842; fax: +31 30 69 44 986.

E-mail address: jos.vantriel@tno.nl (J.J. van Triel).

¹ Present address: Maastricht University Medical Centre (MUMC), Department of Human Biology, Maastricht, The Netherlands.

the exception of formaldehyde (FA; GA was not included in the set of allergens): FA was positive in the skin LLNA, but negative in the respiratory LLNA. These negative results upon inhalation exposure might be caused by the FA concentration not being high enough, although slight irritation of the upper respiratory tract was observed. Alternatively, FA might actually be unable to sensitize by inhalation.

To investigate whether the negative results of FA vapor in the respiratory LLNA were due to some intrinsic properties of aldehyde sensitizers, we selected the structurally related chemical glutaraldehyde (GA). In contrast to FA, GA is a recognized respiratory allergen. In several animal models, GA was found to be considerably more potent than FA (Dearman et al., 1999; Hilton et al., 1998; Stern et al., 1989). Moreover, dermal application of FA induced a contact allergy-related, Th1-skewed cytokine profile in draining lymph nodes, whereas GA induced a Th1-skewed immune response at low doses and an asthma-related Th2-type cytokine response at high doses (Azadi et al., 2004; Dearman et al., 1999). In the current study, BALB/c mice were exposed by inhalation to 6 or 18 ppm GA – both generated as a vapor and as an aerosol – for 1.5 h/day, on 3 consecutive days. In comparison to exposure to a vapor, exposure to an aerosol may lead to higher local concentrations (even at equivalent overall concentrations) since the test substance impacts as droplets, leading (locally) to an increased number of molecules per antigen-presenting cell. In parallel to the inhalation exposures (respiratory LLNA), other groups were treated with a low (0.25%) or high (2.5%) dose of GA on the skin of the ears (standard dermal LLNA). The animals were sacrificed three days after the last exposure and cellular proliferation and cytokine expression were determined in the draining lymph nodes. In addition, the exposure sites were investigated histopathologically.

2. Materials and methods

2.1. Animals and maintenance

Male, 6–7-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 the experiment. Mice were housed in macrolon cages and maintained under conventional laboratory conditions in groups of six per cage. The animals received a standard pelleted rodent diet (RM3 (E) SQC pellets, Special Diet Service, Witham, UK) and unfluorinated tap water *ad libitum*. All animal procedures were approved by the Institute's Commission of Animal Welfare.

2.2. Materials

Glutaraldehyde (GA; 51% in H₂O, Grade I) and raffinated olive oil were obtained from Sigma–Aldrich (St Louis, MO, USA); acetone was purchased from Biosolve (Valkenswaard, The Netherlands).

2.3. Study design

Groups of six animals were exposed head/nose-only to target concentrations of 6 or 18 ppm GA, both generated as a vapor and as an aerosol, for 1.5 h/day on three consecutive days. To prevent evaporation of GA from the aerosol, the test compound was generated at (near) 100% relative humidity (RH), which implicated that the particles did not dry out and remained relatively large. The GA vapor was generated at approximately 50% RH. Negative inhalation controls were exposed to clean air for 1.5 h/day. In parallel to the inhalation exposures (respiratory LLNA), other groups were treated with GA on the skin (dermal LLNA). These animals (6 per group) received 25 µl/day of either 0.25% or 2.5% GA, dissolved in a 4:1 (v/v) mixture of acetone and olive oil (AOO) on the dorsum of each ear on three consecutive days, or AOO alone (negative skin control). Three days after the last exposure the animals were necropsied and the respiratory tract was collected for histopathological examination. Mandibular and auricular lymph nodes (LNs) were collected, LN cells were harvested, and DNA was labeled with [³H]-thymidine to determine proliferation. In addition, production of several cytokines by the cultured LN cells was measured using a flowcytometer.

The inhalation concentrations of 6 and 18 ppm and exposure duration were chosen based on data regarding respiratory irritation (RD₅₀ of 13.9 ppm reported by Werley et al., 1995), considering the 4h-LC₅₀ (rat) of 23.5–44.3 ppm (Ballantyne and Myers, 2001); the dermal doses of 0.25 and 2.5% were based on a skin LLNA, performed by Azadi et al. (2004).

2.4. Inhalation exposure, test atmosphere generation and analysis

To generate the GA vapor, a freshly prepared solution of 1% GA in water was delivered to a glass evaporator (DURAN® DIN 12591, Lenz Laborglas GmbH & Co. KG, Wertheim, Germany) by a motor-driven syringe pump (WPI type SP22i, World Precision Instruments, Sarasota, FL). The temperature in the evaporator was controlled by circulating heated water at 45 °C. The air flow to the evaporator was regulated by a mass flow controller (Bronkhorst Hi Tec, Ruurlo, The Netherlands) and humidified to approximately 50% RH.

GA aerosols were generated using a 2.4 MHz ultrasonic nebulizer (Model 241CT Nebulizer, Sonaer, New York). Freshly prepared solutions of 0.1% (for 6 ppm GA) or 0.3% GA (for 18 ppm GA) in water were delivered to the nebulizer by a peristaltic pump (Minipulse, Gilson, Villiers le Bel, France). The aerosol was moved from the nebulizer to the inhalation chamber by a flow of air (mass flow controlled and saturated with water vapor). Negative inhalation controls were exposed to clean air.

Concentrations of GA in the test atmosphere were determined by total carbon analyzer (vapor: Ratfisch RS55T, Munich, Germany; aerosol: Bernath Atomic, Werningen, Germany). To calibrate the analyzers, both were connected to the vapor generation equipment. Subsequently, three concentrations – including the target concentration – were generated, and GA concentrations were calculated based on the nominal concentration and the complete evaporation of the test compound. Average actual concentrations (± standard deviation) during exposure were determined to be 6.7 (± 0.3) and 18.9 (± 0.8) ppm for the vapor, and 6.6 (± 0.4) and 19.0 (± 0.8) ppm for the aerosol, respectively.

The mass median aerodynamic diameter (MMAD) and geometric standard deviation (gsd) of GA aerosols were determined using an aerodynamic particle sizer (APS; TSI Incorporated, Shoreview, MN, USA). MMAD and gsd were 9.10 µm and 1.47 in the 6 ppm atmosphere, and 9.07 µm and 1.46 in the 18 ppm GA atmosphere, respectively.

Mice were restrained in Battelle tubes, and each tube was then placed into head/nose-only inhalation units for exposure to the test atmosphere. The units had a volume of approximately 50 L. Air flows in the exposure units were at least 1 L/min per animal. Temperature was between 20.0 and 23.0 °C during exposure. Average RH was 44 (± 7)%, 51 (± 1)%, 93 (± 1)%, 93 (± 0)%, and 53 (± 2)% for the 6 and 18 ppm vapor, 6 and 18 ppm aerosol, and the negative inhalation control atmosphere, respectively.

2.5. Local effects, body and organ weights, and necropsy

All animals were observed at least once daily and their bodyweights were determined shortly before the first exposure and just prior to necropsy (five days after the first exposure). At necropsy, animals were anaesthetized with sodium pentobarbital, killed by exsanguination from the abdominal aorta, and examined for gross abnormalities. Mandibular and auricular LNs, the ears, nose, larynx, trachea, and lungs were excised. Lungs and lymph nodes were weighed. Subsequently, draining LNs were used for determination of proliferation and cytokine production.

2.6. Local lymph node activation

The pulmonary and dermal sensitizing potency of GA 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 Ock et al., 2002). Auricular and mandibular LNs were collected at necropsy in all animals. Previous experiments have demonstrated enlargement of auricular LNs upon topical exposure on the ear, and of mandibular LNs upon inhalation exposure to various contact and respiratory sensitizers (Arts et al., 2008). Since no macroscopic enlargement of other LNs was observed at necropsy, no additional LNs were collected.

The cells of left and right LNs were pooled for each animal in 5 ml PBS. Single-cell suspensions were prepared under aseptic conditions by pressing the LNs between 2 glass slides. Subsequently, the glass slides were rinsed with an additional 5 ml of RPMI-1640 + Glutamax medium with 5% FCS. Each single-cell suspension was collected and centrifuged for 10 min at 300 × g and 4 °C. The supernatant was removed and the pellet was resuspended in 800 µl supplemented medium (RPMI-1640 + Glutamax, supplemented with 10 U/ml penicillin and 100 µg/ml streptomycin with 10% FCS). Viable cell counts were performed with a Bürker–Türk counting chamber after mixing 10 µl cell suspension with 10 µl trypan blue. After counting, the concentration of the cell suspensions was adjusted to 7.5 × 10⁶ cells/ml. When necessary, cell suspensions of several animals within one group were pooled in order to obtain concentrations of 7.5 × 10⁶ cells/ml. These cell suspensions were further used for the determination of lymphocyte proliferation as well as for cytokine production after culturing with Concanavalin A (Con A).

For the lymphocyte proliferation assay, 200 µl of the cell suspensions (7.5 × 10⁶ cells/ml) were seeded in triplicate in round-bottomed 96-well microtiter plates (Corning #3799). To each well an aliquot of 10 µl [³H]-thymidine (3.7 kBq/µl) was added. The cells were incubated at 37 °C in a humidified 5% CO₂ atmosphere for 20–24 h. After incubation, cells were harvested on glass fiber filters using an automatic cell harvester (Harvester 96, Tomtec, Orange, CT). Scintillation liquid (betaplate-scint, Perkin-Elmer) was added to the filters and subsequently [³H]-thymidine incorporation was measured by liquid scintillation counting in a β-plate counter (1450 Microbeta Plus liquid scintillation counter, Wallac, Turku, Finland). For further calculations the median of the triplicates was used. Lymphocyte prolifer-

ation was determined by correcting the measured incorporation of [^3H]-thymidine for the total amount of cells counted. Mean [^3H]-thymidine incorporation was calculated per experimental group. Stimulation indices (SI) were then calculated by dividing the mean [^3H]-thymidine incorporation per experimental group by the mean [^3H]-thymidine incorporation of the (vehicle-treated or air-exposed) control group. Chemicals that are able to elicit a stimulation index of 3 or more are considered skin sensitizers in the dermal LLNA.

2.7. Cytokine profiling

Production of cytokines by activated LN cells was determined after culturing the isolated LN cells (2×10^6 cells/ml) with Con A ($5 \mu\text{g/ml}$) in round-bottomed 96-well microtiter plates (Corning #3799) for 24 h at 37°C . After 24 h the supernatants were collected and concentrations of GM-CSF, IFN- γ , IL-1 α , IL-2, IL-4, IL-5, IL-6, IL-10, IL-17, and TNF- α were measured using a Flow Cytomix Mouse Th1/Th2 10plex kit (Bender Med Systems, BMS820FF, Vienna, Austria). This Th1/Th2 10plex kit consists of a set of fluorescent beads which are coated with anti-cytokine antibodies. Multiple cytokines can be quantified simultaneously in each sample due to the fluorescent bead populations which can be distinguished by differences in internal dye intensity and bead size. The levels of cytokines were measured according to the manufacturer's protocol. In short, $25 \mu\text{l}$ of sample or one of the standard mixture dilutions (for the generation of a standard curve) was added to the wells of a flat-bottomed 96-well plate (NUNC, Roskilde, Denmark). Subsequently, bead mixture and biotin-conjugate mixture were added to each well and the plate was then incubated for 2 h at room temperature protected from light (on a shaker, 500 rpm). After the incubation, the plate was centrifuged for 5 min at $200 \times g$ and the pellets were washed twice with assay buffer. Next, Streptavidin-PE solution was added to each well and the plate was incubated for 1 h at room temperature protected from light (on a shaker, 500 rpm). Then, the plate was centrifuged again for 5 min at $200 \times g$. The pellets were washed twice with assay buffer and resuspended in assay buffer for the determination of cytokine levels. Cytokine levels were measured as fluorescence intensities using a flowcytometer (FACSCanto™ II, BD Biosciences, Erembodegem, Belgium). FlowCytomix Pro 2.2 Software (Bender Med Systems, BMS8400FF, Vienna, Austria) was used to calculate the amount of cytokines (pg/ml) in the supernatants, using the standard reference curve that was included in the assay. Cytokine production was expressed in pg per lymph node.

2.8. Airway histopathology

Neutral, phosphate-buffered 4% (v/v) formaldehyde was used to inflate and preserve the lungs and to preserve the nasal tissues, larynx and ears. Next, these organs were embedded in paraffin wax, and $5 \mu\text{m}$ 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 arytenoid projections. Each lung lobe was cut at one median sagittal level. Tissues of all animals were examined microscopically.

2.9. Statistical analysis

Bodyweights, absolute and relative organ weights, stimulation indices and cytokine production were analyzed by one-way analysis of variance (ANOVA), followed by a two-sided Dunnett's test. Stimulation indices and cytokine production were evaluated after logarithmic transformation. Groups exposed by inhalation were compared to the inhalation control group. Similarly, effects of the dermal treatment were evaluated relative to the skin control group. A p value <0.05 was considered statistically significant.

Table 1
Body and organ weights.

Group	Bodyweight (g)		Bodyweight gain (g)	Rel. weight lungs (g/kg bw)	Rel. weight cervical LN (g/kg bw)	Rel. weight auricular LN (g/kg bw)
	Day 0	Day 5				
Inhalation						
Neg. control	22.4 (0.9)	22.5 (1.1)	0.1 (0.4)	7.31 (0.21)	0.40 (0.24)	0.34 (0.07)
6 ppm vapor	20.1 (1.0)	20.1 (0.9)	0.0 (0.5)	7.93 (0.48)	0.40 (0.12)	0.37 (0.09)
6 ppm aerosol	19.4 (0.5)	20.2 (0.7)	0.8 (0.9)	7.73 (0.59)	0.49 (0.08)	0.33 (0.08)
18 ppm vapor	21.1 (1.4)	19.2 (2.5)	−1.9 (1.6)	8.07 (0.86)	0.49 (0.21)	0.34 (0.10)
18 ppm aerosol	20.9 (0.7)	19.3 (2.8)	−1.6 (2.8)	8.45 (0.90)	0.43 (0.16)	0.41 (0.11)
Skin						
Neg. control	20.0 (0.6)	20.3 (0.7)	0.3 (0.4)	7.65 (0.60)	0.33 (0.06)	0.30 (0.05)
0.25%	19.1 (0.9)	21.2 (0.8)	2.1 (1.0)**	7.40 (0.59)	0.35 (0.11)	0.47 (0.04)*
2.5%	21.2 (0.9)	21.5 (0.6)	0.3 (0.4)	7.71 (0.22)	0.76 (0.52)	0.85 (0.15)*

Means ($\pm$ SD) are expressed. Lung and lymph node (LN) weights were calculated relative to bodyweight at necropsy on day 5.

* $p < 0.05$, relative to negative skin control.

** $p < 0.01$, relative to negative skin control.

3. Results

3.1. Clinical signs, body and organ weights

Dermal administration of 2.5% GA caused blepharospasms on the first day of exposure, probably due to grooming, which were not observed after subsequent applications. In addition, erythema was observed at the application site on days 3–5 in the animals which received the high dermal dose. No abnormalities were observed in the groups exposed dermally to vehicle or to the low GA dose. Immediately after the first exposure, all animals exposed to GA by inhalation were lethargic and exhibited a decreased breathing frequency, which was more pronounced in animals exposed to the GA vapor. All animals recovered quickly and were less affected upon subsequent exposures. No clear dose-related differences were observed between groups which received the low versus the high GA concentration by inhalation.

Body-, lung-, and lymph node weights are presented in Table 1. The observed increase in bodyweight gain in the low dermal dose group was considered an incidental finding. Although no further statistical differences in bodyweight gain were observed between groups, most animals exposed to 18 ppm GA, either as a vapor or as an aerosol, demonstrated a slight bodyweight loss. Relative lung weights tended to be increased in all groups exposed to GA by inhalation, but statistical significance was not reached. In addition, inhalation of GA did not affect relative weight of cervical or auricular lymph nodes. Animals exposed to GA on the ears demonstrated a dose-dependent increase in relative weight of the auricular lymph nodes. No significant differences in relative weight of the cervical lymph nodes were observed in dermally treated animals, although weights seemed elevated in response to the high dermal dose (Table 1).

3.2. Histopathology

Inhalation exposure to GA induced histopathological lesions primarily in the nasal tissues. In only 2 of 6 animals exposed to the 18 ppm aerosol, lesions were also found in the larynx, and no histopathology was observed in the lungs (Table 2). The lesions in the nasal passages involved all types of epithelium, viz. squamous, ciliated and non-ciliated respiratory and olfactory epithelium. GA vapor affected the more proximal and middle part of the nasal passages (levels I–III; Fig. 1), whereas effects of the aerosol extended onto the distal part of the nasal tissue. In addition, GA induced polymorphonuclear cell infiltration in the vomeronasal organ (organ of Jacobson, level II) in all groups, and degeneration and necrosis in that organ at level III in the 18 ppm vapor and the 6 and 18 ppm aerosol groups. Overall, the GA-induced effects were consistent

Table 2
Histopathological lesions in the respiratory tract upon GA inhalation.

Incidence of changes					
Changes	Control	6 ppm vapor	6 ppm aerosol	18 ppm vapor	18 ppm aerosol
Nasal tissue	(6)	(6)	(6)	(6)	(6)
Level I					
Hyperplasia, squamous epithelium		4	2	4	4
Degeneration/necrosis, squamous epithelium					
Very slight		1			
Slight		2	1	3	
Moderate			3		1
Severe					1
Level II					
Squamous metaplasia, respiratory epithelium		1	1	3	5
Degeneration/necrosis, squamous epithelium				3	6
Degeneration/necrosis, respiratory epithelium				1	1
Degeneration/necrosis, olfactory epithelium			2		1
Polymorphonuclear cell infiltrate, vomeronasal organ		2	4	1	1
Level III					
Squamous metaplasia, respiratory epithelium				1	3
Hyperplasia, squamous epithelium			1	1	
Degeneration/necrosis, squamous epithelium				1	1
Degeneration/necrosis, olfactory epithelium					
Slight		1	2	3	1
Moderate					1
Degeneration/necrosis, vomeronasal organ			4	3	6
Level IV					
Squamous metaplasia/necrosis, respiratory epithelium and lymphoepithelium of NALT					2
Regeneration, respiratory epithelium and lymphoepithelium of NALT			6	3	4
Degeneration/necrosis, olfactory epithelium					1
Larynx	(6)	(6)	(6)	(6)	(6)
Hyperplasia, squamous epithelium					2
Lungs	(6)	(6)	(6)	(6)	(6)
No abnormalities detected	6	6	6	6	6

The number of animals/tissues examined is indicated in parentheses.

with distinct irritation, with 6 ppm vapor being least and 18 ppm aerosol most irritating.

The skin of the ears was affected dose-dependently by dermal application of GA. Thickening of the skin (acanthosis) was observed at the site of application in all animals exposed to the low dose and one animal of the high dose group. Severe dermatitis was found in 5 of 6 animals exposed to the high GA dose (Fig. 2). No skin lesions were observed in the animals exposed by inhalation.

3.3. Lymphocyte proliferation

Exposure to GA by inhalation did not induce macroscopic enlargement of respiratory tract draining lymph nodes. Since previous experiments have demonstrated activation of the mandibular LN upon inhalation of various contact and respiratory sensitizers (Arts et al., 2008), these lymph nodes were processed for analysis of lymphocyte proliferation. Inhalation of GA, either as a vapor or as an aerosol, did not induce lymphocyte proliferation in the mandibular LN. As expected, dermal application of GA did not affect proliferation in the mandibular LNs (Fig. 3). In auricular LNs however, highly significant dose-dependent stimulation of lymphocyte proliferation was observed in response to dermal exposure, with SI values up to 30 (Fig. 3). Inhalation of GA did not affect proliferation in auricular LNs.

3.4. Cytokine profiling

Inhalation exposure to GA did not induce significant increases in cytokine production in mandibular lymph nodes. IL-4 production, which was below the limit of detection in control animals, tended to be elevated in all GA-exposed cell cultures of both LNs (data not shown). These differences were not statistically significant, partly because lymph node cultures had to be pooled in most groups to attain sufficient cell numbers, and thus the number of data per

group was too low to allow statistical analysis. In the absence of an association with cellular proliferation, the relevance of the cytokine data is questionable.

Dermal application of GA induced a dose-dependent, highly significant elevation in production of IL-4 and IFN- γ in auricular lymph nodes. IFN- γ production was increased 7- and 47-fold, and IL-4 production was increased 11- and 140-fold in the 0.25% and 2.5% GA groups, respectively (Fig. 4). Thus, as judged by the IL-4/IFN- γ ratio, a Th2-dominant cytokine expression profile was observed in GA-exposed LN cultures. Other cytokines levels which were elevated in auricular lymph nodes upon dermal GA exposure included IL-2 (Th1-associated), IL-10 (Th2-associated), IL-17 (polyfunctional; more associated with Th2) and GM-CSF (associated with asthma and allergic sensitization of the respiratory tract) (data not shown).

4. Discussion

The sensitizing potential of glutaraldehyde (GA) – a recognized human skin and respiratory allergen – was examined in a local lymph node assay (LLNA) in BALB/c mice, exposed either by inhalation or via the skin.

In the skin LLNA, a dose-dependent increase in cellular proliferation was found in the draining lymph nodes, with stimulation indices (SI) of 14 and 33 in response to 0.25% and 2.5% GA, respectively (Fig. 3). Similar responses have also been found by others (Azadi et al., 2004; Dearman et al., 1999; Hilton et al., 1998) albeit at 3-fold to 20-fold higher GA concentrations. The dose of an allergen in the sensitization phase may play a pivotal role in Th1/Th2 differentiation of the immune response to various foreign substances (Mosmann and Sad, 1996). Azadi et al. (2004) reported a Th1-dominant cytokine expression profile in response to dermal sensitization with <0.75% GA, and a delayed-type allergic response upon subsequent dermal challenge. Sensitization and challenge with a higher dose (2.5% GA) produced a Th2-dominant cytokine

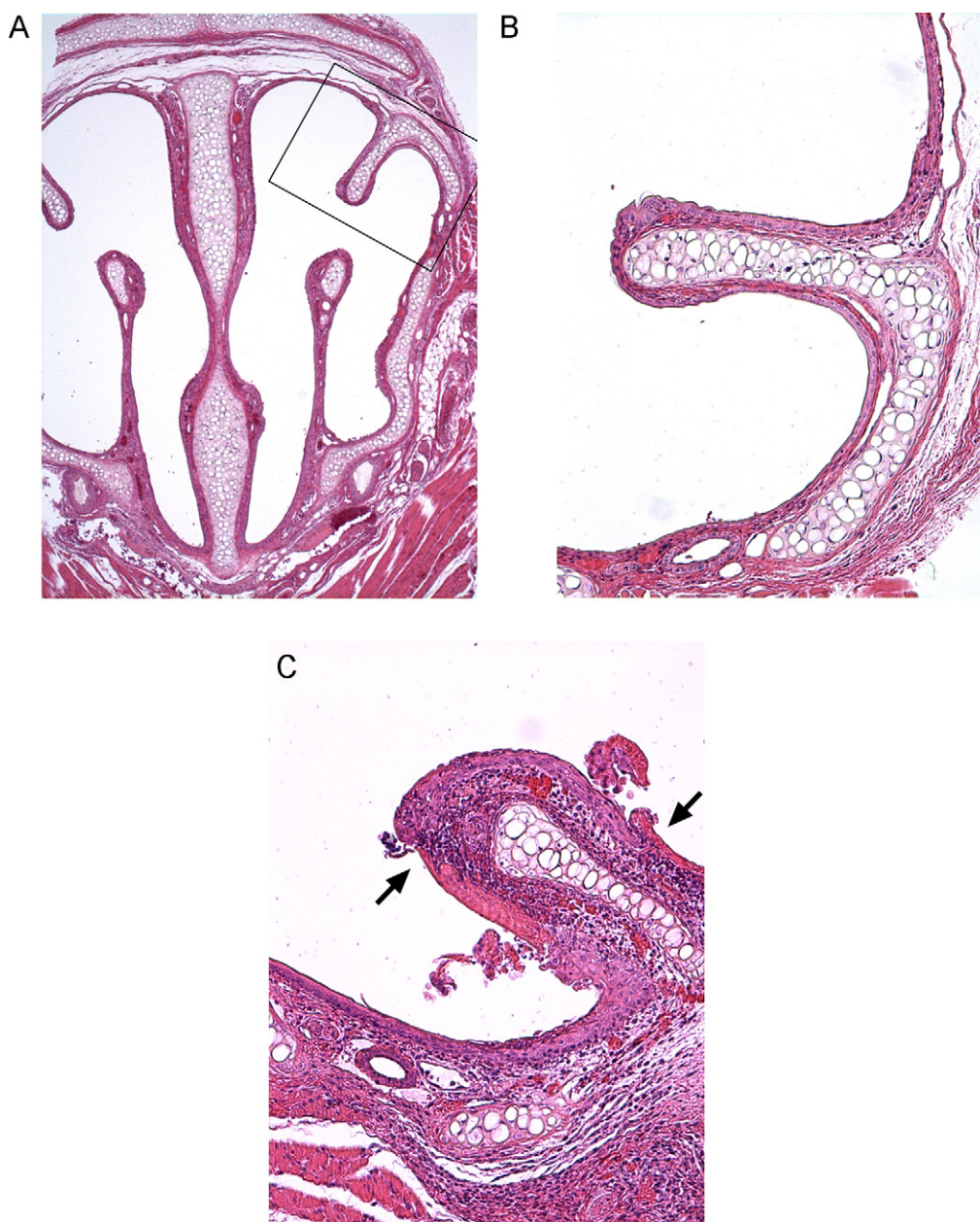


Fig. 1. Effects of GA vapor on the posterior part of the nasal passages at level I. (A) Unexposed animal, overview (50× magnification). (B) Unexposed animal, detail of nasal turbinate covered with squamous and unciliated respiratory epithelium (200× magnification). (C) Animal exposed to 18 ppm GA vapor, detail of nasal turbinate showing squamous cell metaplasia, hyperplasia and epithelial erosion (arrow), and polymorphonuclear cell infiltration (200× magnification). H&E-stained sections.

profile, IgE induction and an immediate-type hypersensitivity reaction upon challenge. We observed Th2-dominance already at the low dermal concentration (supporting the notion that our test model was quite sensitive) and the IL-4/IFN- γ ratio shifted even more towards a Th2-like cytokine expression profile with increasing dose (Fig. 4).

In contrast to the positive response in the skin LLNA, GA was negative in the respiratory LLNA. A previously performed respiratory LLNA was negative for formaldehyde (FA) – a structurally related aldehyde and a recognized contact allergen with suspected respiratory sensitizing potential (Arts et al., 2008). We initially hypothesized that this negative result was caused by FA vapor concentrations being too low, since the other contact allergens dinitrochlorobenzene (DNCB) and oxazolone were positive upon inhalation. Negative results upon GA inhalation in the present study could not have been caused by the concentration being too low: the

observed histopathological effects in the upper respiratory tract demonstrated distinct irritation. The histopathological findings in the nasal tissues in response to the high concentration GA vapor were comparable to the high dose effects of GA applied to the skin of the ears (Figs. 1C and 2B, respectively); the high concentration aerosol was slightly more toxic. Less pronounced irritant effects were also observed at the low exposure levels, with nasal tissues being slightly more affected than the skin of the ears. Indirect evidence of GA's inability to induce sensitization by inhalation could be derived from studies published by Werley et al. (1995). Guinea pigs, exposed to 13.9 ppm GA (1 h/day for 5 days) followed by repeated inhalation challenges with 4.4 ppm GA, did not exhibit GA-induced respiratory allergy, as measured by changes in breathing function and pattern upon challenge.

GA is expected to be a strong respiratory sensitizer, based on its chemical structure. It is considered a strong electrophile and

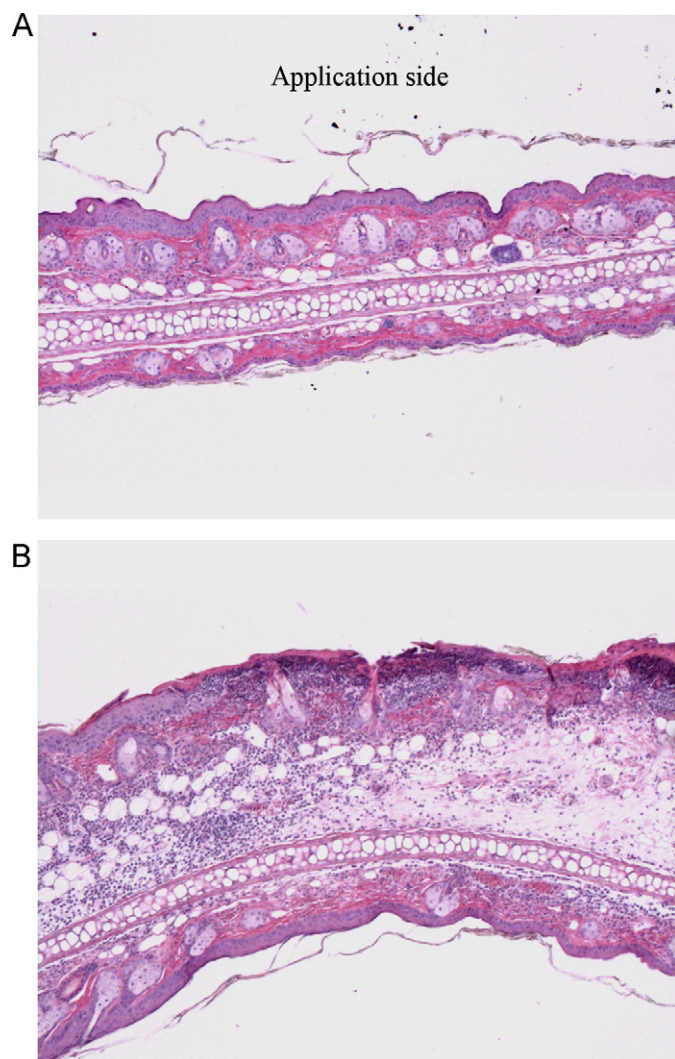


Fig. 2. Effects of dermal application of GA to the ears. (A) Animal exposed to the low GA dose, demonstrating thickening of the epidermis (acanthosis) at the site of application on the dorsal side of the ear (50 $\times$ magnification). (B) Animal exposed to the high GA dose, showing dermatitis, i.e. squamous cell hyperplasia and epithelial erosion (macroscopically observed encrustations), oedema, and polymorphonuclear cell infiltration at the application site. Acanthosis is also observed at the ventral side of the ear due to grooming (50 $\times$ magnification). H&E-stained sections.

has widespread use as a protein cross-linking agent, reacting by mechanisms such as aldol condensation and Michael-type addition (Migneault et al., 2004). Jarvis et al. (2005) calculated an asthmagenicity 'hazard odds ratio' of 7.3 (95% confidence interval of 3.3–16) for molecules containing 2 carbonyl groups like GA. Enoch et al. (2009) describe that Schiff base formation with proteins is likely to be the initial step in GA-sensitization. The authors propose a model for identification of potential respiratory sensitizers based on a chemicals' electrophilicity and protein cross-linking capability.

Based on the above observations, GA's inability to induce a positive lymph node response upon inhalation exposure is at first sight surprising. GA is a hydrophilic compound. Its calculated log *P* (octanol/water) value is -0.17 (Roberts et al., 2006), indicating that it will tend to partition more into water than into membranes. GA in aqueous solutions is known to exist as a dynamic equilibrium mixture of species including solvated GA monomer, GA hydrates, dimers and oligomers, and dehydro-dimers and -oligomers resulting from aldol condensations with elimination of water. The relative proportions of these species depend on the total concentration, the pH of the solution and time. In fresh solutions

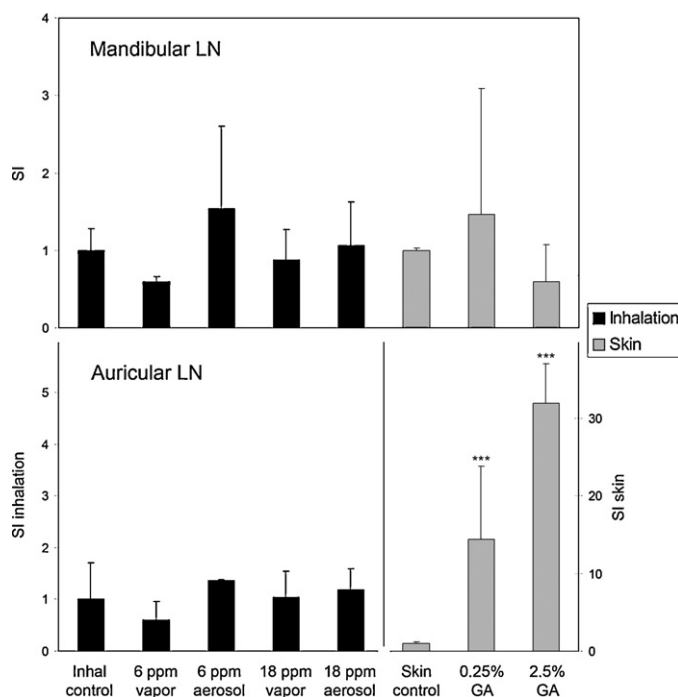


Fig. 3. Stimulation index (SI) in mandibular and auricular LNs upon inhalation or dermal exposure to GA. Groups of animals ($n=6$) were exposed by inhalation (in black) to 6 or 18 ppm GA generated either as a vapor or as an aerosol, or to air only (inhal control) on days 0, 1 and 2. Other groups were exposed to 0.25% or 2.5% GA, or vehicle only (skin control) on the skin of the ears (in gray). Mandibular and auricular lymph nodes were excised at necropsy on day 5 and proliferation was determined by [^{3} H]-thymidine incorporation, expressed as stimulation index (SI; mean $\pm$ SD) relative to the corresponding control group; *** $p < 0.001$, relative to skin control.

at neutral pH the GA monomer and its hydrates are believed to predominate. However, amino groups of proteins can promote the formation of the aldol dehydro-dimers and oligomers (Fig. 5A). We hypothesize that, when GA is absorbed from the airways, it dissolves in the mucous layer – which essentially is a viscous solution of lysine-rich proteins in water – where it reacts rapidly

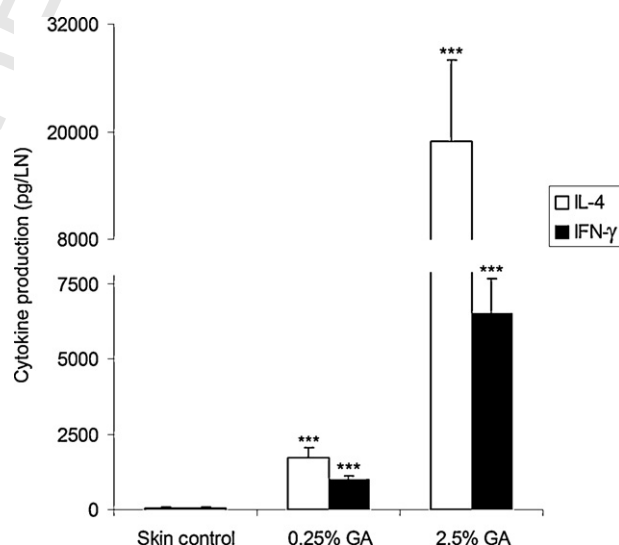


Fig. 4. IL-4 and IFN- γ production by auricular lymph node cells after dermal exposure to GA. Animals were exposed to 0.25% or 2.5% GA, or vehicle only (skin control) on the skin of the ears on days 0–2. Cytokine production was determined in supernatants of auricular lymph node cells, isolated at necropsy on day 5 and cultured with Con A. Data are expressed as pg per lymph node (mean $\pm$ SD); *** $p < 0.001$, relative to skin control.

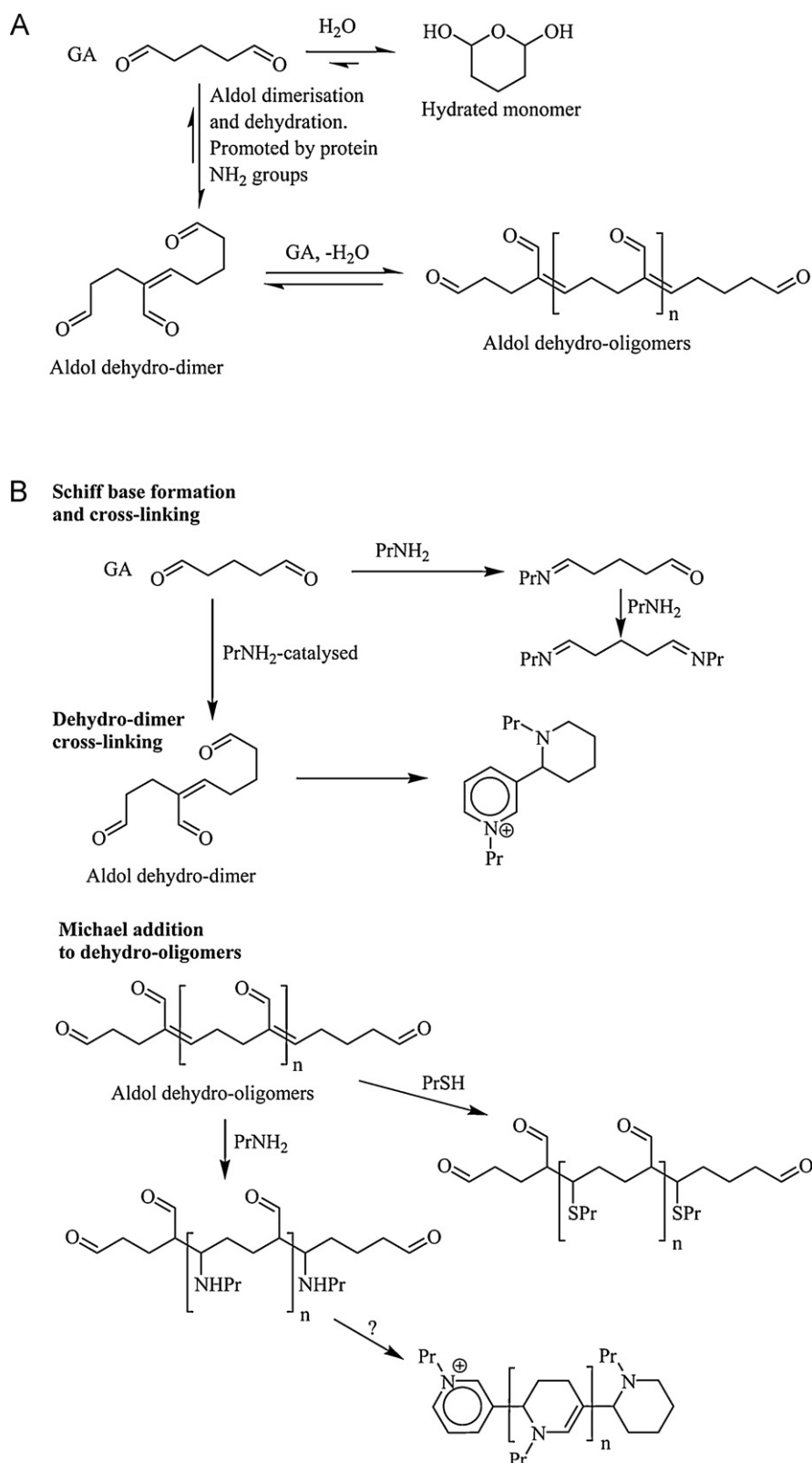


Fig. 5. The major chemical species present in aqueous solutions of GA. Pathways for their formation (A), and their reactions with proteins (Pr) (B).

with the dissolved proteins (Fig. 5B). This prevents GA from reaching the antigen presenting cells (APCs) in sufficient quantities to induce sensitization. Although the mucous layer may act as a barrier protecting against sensitization to GA, it would not be expected to protect against all sensitizers. In contrast to GA, other low molecular weight compounds like diisocyanates are capable of sen-

sitization by inhalation – even when generated as a vapor (Arts et al., 2008). It might be that these substances are less reactive and more lipophilic and thus are less impeded by the mucous layer. Formaldehyde, however, has solubility and reactivity characteristics rather similar to GA. Thus, the mucous lining of the airways may also protect against respiratory sensitization by FA. However,

this hypothesis should be substantiated by investigating a panel of compounds with GA-like characteristics (i.e. highly reactive and hydrophilic).

The skin does not have such an aqueous mucous lining. Once sensitization has occurred via the skin, re-exposure by inhalation may lead to a respiratory allergic response, which is triggered by the small proportion of GA that succeeds in crossing the protective mucous barrier. An interesting parallel with our present observations has recently been reported regarding sensitization via the skin: the human stratum corneum contains a layer of thiol-rich proteins, which apparently acts as a barrier against skin sensitization by dinitrothiocyanobenzene (DNTB). The SCN group of DNTB is highly reactive towards these stratum corneum thiol groups, with the result that insufficient DNTB reaches the epidermis to induce sensitization. Again, it is not a barrier against all sensitizers: the structurally related DNCB is able to pass through it and act as a strong sensitizer (Pickard et al., 2009).

The lack of DNTB-induced skin sensitization in the study by Pickard et al. (2009) could – at least partly – be explained by the absence of its skin-irritating properties. In the present study however, irritation in response to GA exposure was observed in the respiratory tract as well as (and to the same extent as) in the skin. Thus, although the mucous lining of the airways seemed to prevent sensitization upon inhalation, severe GA-induced local irritation still occurred. Possibly these irritant-induced effects result from toxic effects of the protein-GA reaction products or from slower reactions in epithelial membranes of precipitated higher oligomers.

For a number of respiratory allergens, animal studies have demonstrated that the skin is a potent route to prime the respiratory tract for an allergic response upon inhalation challenge. Our data with the recognized human skin and respiratory allergen glutaraldehyde support this: GA was strongly positive in the skin LLNA, but was negative in the respiratory LLNA. Thus, differences in incidence of GA-induced asthma might be explained not only by variable concentrations of GA in the atmosphere, but also – and maybe predominantly – by variations in dermal exposure. In any case, these results stress the importance of skin protection at the workplace to prevent GA-induced sensitization of the respiratory tract, and thus the development of GA-induced respiratory allergy.

Conflict of interest

All authors declare that there is no conflict of interest.

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