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Airway exposure to hypochlorite prior to ovalbumin induces airway hyperreactivity without evidence for allergic sensitization

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ABSTRACT

Background: Some epidemiologic studies have indicated that attendance to chlorinated swimming pools is associated with airway hyperreactivity (AHR), allergies and asthma.

Aim: To investigate the effects of sodium hypochlorite (NaClO), the main pool disinfectant, on allergic sensitization and airway inflammation in mice.

Methods: In a first series of experiments, mice were sensitized to ovalbumin (OVA), followed by OVA aerosols with or without prior nasal instillation of NaClO (3 ppm active chlorine). In a second series, naïve mice received 1–7 nasal instillations of OVA, 10 min after instillations of NaClO or water. After 1, 3, 5 and 7 exposures airway reactivity to methacholine, cellular inflammation in bronchoalveolar lavage (BAL), serum OVA-specific IgEs and lung Th2 cytokines were measured.

Results: In the first mouse model, airway allergy parameters were not significantly altered upon NaClO administration. However in the second model, NaClO exposure prior to OVA did induce AHR, already after 1 combined application. Combined NaClO + OVA exposure did not lead to an influx of inflammatory cells in BAL fluid or production of anti-OVA IgEs. No AHR developed when OVA was heat-denatured, pre-chlorinated, or replaced by bovine serum albumin or lipopolysaccharide.

Conclusion: Nasal instillation of NaClO prior to OVA induces AHR without allergic sensitization. This response is OVA-specific.

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1. Introduction

Airway allergy is one of the most prevalent disorders in the Western population. Its prevalence has risen over the last decades reaching epidemic proportion (Woolcock and Peat, 1997; Upton et al., 2000). The exact reasons for this increase in prevalence are still unclear. Besides the hygiene hypothesis (Svanes et al., 1999),

explanations have included an increasing exposure to environmental factors that affect the process of sensitization to inhaled allergens (Davies et al., 1998; Venn et al., 2001; D'Amato, 2002). Among these environmental factors exposure to chlorine-based irritants, especially swimming pool disinfection by-products, has been evoked (Weisel et al., 2009). Most swimming pools worldwide are disinfected by some chlorination method, either using chlorine gas or sodium hypochlorite (NaClO). Chlorine (Cl₂) rapidly reacts in water to form hydrochloric acid (HCl) and hypochlorous acid (HOCl) with its reactive anion hypochlorite (ClO⁻). Although the issue remains controversial (Weisel et al., 2009), various epidemiological observations indicate that exposure to swimming pool related chlorination by-products (such as trichloramine) may be linked to respiratory problems. Thus, among competitive swimmers, 36–79% were found to show airway hyperresponsiveness (AHR) (Zwick et al., 1990; Potts, 1996; Helenius et al., 1998; Langdeau et al., 2000), 74% reported disabling rhinitis (Bougault et al., 2010) and 33% had proven asthma (Helenius et al., 1998; Langdeau et al., 2000). Epidemiological and clinical studies also indicate that occupational exposure to a swimming pool environment may cause respiratory symptoms and occupational asthma (Thickett et al., 2002;

Abbreviations: AHR, airway hyperreactivity; AUC, area under the curve; BAL, bronchoalveolar lavage; BSA, bovine serum albumin; ClO⁻, hypochlorite; dH₂O, distilled water; LPS, lipopolysaccharide; NaClO, sodium hypochlorite; OVA, ovalbumin.

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Jacobs et al., 2007). Bernard et al. conducted several large-scale epidemiological studies and reported an increased risk of developing asthma, airway inflammation, hay fever and allergic rhinitis (Bernard et al., 2006, 2008, 2009) in adolescents in association to chlorinated swimming pool attendance, both indoor and outdoor. Kohlhammer et al. (2006) described an association between swimming pool attendance and the occurrence of hay fever, and Zwick et al. (1990) showed a higher prevalence of sensitizations to inhalation allergens in competitive swimmers compared to controls. The higher prevalence of rhinitis and asthma in cleaners has also been linked, among other possible causes, to the daily use of bleach (which is an aqueous solution of NaClO) (Zock et al., 2009).

Several mechanisms could be responsible for the observed associations between chlorination products and airway allergy. Firstly, chlorination products may induce epithelial hyperpermeability leading to an increased amount of allergen having access to the subepithelial dendritic cells and thereby amplifying sensitization in atopic subjects (Bernard, 2007). Allergens have also been shown to be very important targets for oxidants released when Cl_2 or NaClO are added to water; e.g. ClO^- is able to react with proteins, which could result in either breaking tolerance towards self-proteins or increasing immunogenicity creating new epitopes or revealing cryptic epitopes through protein unfolding (Olszowska et al., 1989; Marcinkiewicz et al., 1991; Westman and Harris, 2004). For other allergenic proteins however, such as Fel d 1 and Der p 1, the opposite could be observed; these proteins can be denatured by chlorine-based oxidants and in this way lose their immunogenicity (Chen and Eggleston, 2001; Matsui et al., 2003).

To our knowledge, little or no experimental research has been conducted to establish whether exposure to chlorine-based chemicals affect the pathogenesis of asthma. We, therefore, first investigated in a validated mouse model of asthma, whether exposure to low concentrations of hypochlorite would increase the response of ovalbumin (OVA)-sensitized mice to challenges with OVA. Having found that this did not occur, we then investigated whether exposure to low concentrations of hypochlorite would facilitate the sensitization to OVA. Although we did not observe any such “adjuvant effect” of hypochlorite, we discovered a marked effect of combined hypochlorite and OVA administration on non-specific airway responsiveness.

2. Materials and methods

All experimental procedures were approved by the local ethics committee for animal experiments.

2.1. Experimental protocols

Male BALB/c mice (5–6 weeks) were obtained from Harlan (Horst, The Netherlands). They were housed under conventional pathogen free conditions.

In the first series of experiments (protocol 1, Fig. 1A), we used a mouse model of OVA-induced experimental allergic asthma as described previously (Hellings et al., 2001, 2003). Mice ($n=8-10/\text{group}$) were sensitized by 7 i.p. injections of 10 μg OVA (grade V, Sigma, Saint Louis, USA) in 0.5 ml saline on alternate days (days 1–13). After an allergen-free interval of 19 days mice were exposed daily for 5 min to nebulized saline (groups I and II) or OVA 1% in saline (groups III and IV) during 8 consecutive days (days 33–40). 10 min prior to the challenge, mice received nasal instillations with either 50 μl of NaClO (Loda[®], eau de javel 10[°], 1 in 10,000 dilution = 0.0003% of active chlorine = 3 ppm) in distilled water (dH_2O) (groups II and IV; Fig. 1A) or 50 μl of dH_2O alone (groups I and III; Fig. 1A). Endpoints were assessed 24 h after the last challenge.

In a second series of experiments (protocol 2, Fig. 1B) naïve mice ($n=6/\text{group}$) received 7 nasal instillations with 50 μl of OVA 1% in saline on alternate days as described before (Huvenne et al., 2010). The control groups received 50 μl of saline. 10 min prior to the OVA-instillation, the mice were nasally exposed to either 50 μl of NaClO in dH_2O (3 ppm active chlorine) or 50 μl of dH_2O alone (Fig. 1B). Endpoints were assessed 48 h after 1, 3, 5 and 7 nasal co-exposures.

In subsequent experiments naïve mice ($n=5-8$) were exposed to 1 nasal instillation of 50 μl NaClO (3 ppm active chlorine) or dH_2O prior to either 50 μl bovine serum albumin (BSA, Sigma, Saint Louis, USA; 1% in saline) or 50 μl lipopolysaccharide (LPS from *Escherichia Coli*; Sigma, Saint Louis, USA; 15 $\mu\text{g}/\text{ml}$) endotoxin. The

dose of LPS used corresponded with the amount present in the OVA-batch as measured using the Limulus Amebocyte Lysate (LAL) assay. Mice were sacrificed 48 h later for analyses.

In a final series of experiments, pre-chlorinated OVA was used. Pre-chlorination was done by adding 3 μg of free chlorine (NaClO) to 0.01 g OVA in saline. Secondly heat-denatured OVA was used. OVA was denatured by heating a 1% solution in saline to 100 $^\circ\text{C}$ during 1 h (Joo and Kato, 2006). Naïve mice received a nasal instillation of 50 μl of normal or denatured OVA (1% in saline) 10 min after exposure to 50 μl of NaClO (3 ppm active chlorine) or dH_2O ; or received 2 consecutive instillations of 50 μl of pre-chlorinated OVA. 48 h later, airway responsiveness was measured, mice were sacrificed and endpoints were assessed.

All experimental procedures were approved by the local Ethical Committee for Animal Experiments.

2.2. Airway resistance measurements and responses to methacholine

Airway reactivity was measured using a forced oscillation technique with the Flexivent system (FlexiVent, SCIREQ, Montreal, Canada; Vanoirbeek et al., 2010). Mice were anaesthetized by i.p. injection of pentobarbital (70 mg/kg, Nembutal[®], Sanofi Santé Animale, CEVA, Brussels, Belgium). The trachea was tracheotomized, an 18-gauge metal needle was inserted and connected to the Flexivent device. Mice were quasi-sinusoidally ventilated with a tidal volume of 10 ml/kg at a frequency of 150 breaths/min and a positive end-expiratory pressure of 2 cm H_2O to achieve a mean lung volume close to that during spontaneous breathing. After measurement of a baseline, each mouse was challenged with methacholine aerosol, generated with an in-line nebulizer and administered directly through the ventilator for 5 s, with increasing concentrations of a methacholine aerosol (0, 0.625, 1.25, 2.5, 5, and 10 mg/ml). R was measured with a “snapshot” protocol each 20 s for 2 min. The mean of these six values was used for each methacholine concentration. For each mouse, R was plotted against methacholine concentration (from 0 to 10 mg/ml) and the area under the curve (AUC) was calculated (Vanoirbeek et al., 2010).

2.3. Measurement of OVA-specific IgE levels

After methacholine challenge, blood was sampled from the retro-orbital vascular plexus of the anaesthetized mice. The blood was centrifuged and serum was stored at $-20\text{ }^\circ\text{C}$ until analysis. For the measurement of OVA-specific IgEs, 96 well plates were coated overnight with rat anti-mouse IgE (10 μg) in 100 μl PBS. The remaining binding sites were blocked with gelatin 0.2% and plates were incubated with 100 μl of serum. After washing, the following substances were sequentially added, incubated and washed: OVA (1 $\mu\text{g}/100\text{ }\mu\text{l}$, Sigma), peroxidase-labeled rabbit anti-OVA IgG (240 ng/100 μl , Rockland, Gilbertsville, PA, USA) and buffer containing tetramethylbenzidine dihydrochloride (1 $\mu\text{l}/100\text{ }\mu\text{l}$, ACROS, New Jersey, NJ, USA) and H_2O_2 (1 $\mu\text{l}/100\text{ }\mu\text{l}$). Then, H_2SO_4 was added and the optical density was measured at 450 nm.

2.4. Analysis of bronchoalveolar lavage fluid

The bronchoalveolar tree was lavaged four times with phosphate buffered saline (PBS) supplemented with 5% of BSA. The first bronchoalveolar lavage (BAL) was performed with 0.7 ml of BSA/PBS 5%, centrifuged (400 $\times$ g, 5 min) and the supernatant was stored at $-20\text{ }^\circ\text{C}$ until analysis. The cellular pellet was added to the subsequent three lavages, each one performed with 1 ml BSA/PBS 5%. The BAL fluid was again centrifuged (400 $\times$ g, 5 min) and the cellular pellet resuspended in 200 μl of saline. 10 μl of cell suspension was added to 90 μl of Türk's solution (Merck diagnostic, Darmstadt, Germany) and total number of cells was counted in a Bürker–Türk chamber. For differential cell counts, cytopspins were prepared from the cell suspensions and they were stained according to the May–Grünwald–Giemsa method and 300 cells were differentiated into macrophages, lymphocytes, neutrophils and eosinophils.

2.5. Histologic analysis

After performing BAL, the right inferior lung lobe was removed for histological examination and fixed overnight in buffered formalin. To obtain nasal tissue, the palatal mucosa was stripped and the palatal bone was broken. The mucosa present on both sides of the septum and the lateral walls was removed and fixed in buffered formalin. After dehydration and embedding in paraffin, 5 μm sections were stained with hematoxylin and eosin (H&E) to demonstrate epithelial integrity and inflammatory infiltrates.

2.6. Measurement of Th2 related cytokines, chemokines and growth factors in homogenized lung tissue

After performing BAL, the left lung was removed and homogenized with 500 μl of BSA/PBS 5%. The homogenate was centrifuged and levels of cytokines, chemokine and growth factor were measured in the supernatant. The pellet was dried en weighed and measured concentrations were corrected for the lung dry weight. Concentrations of IL-4, IL-5, IL-13, IL-10, RANTES and GM-CSF were measured using

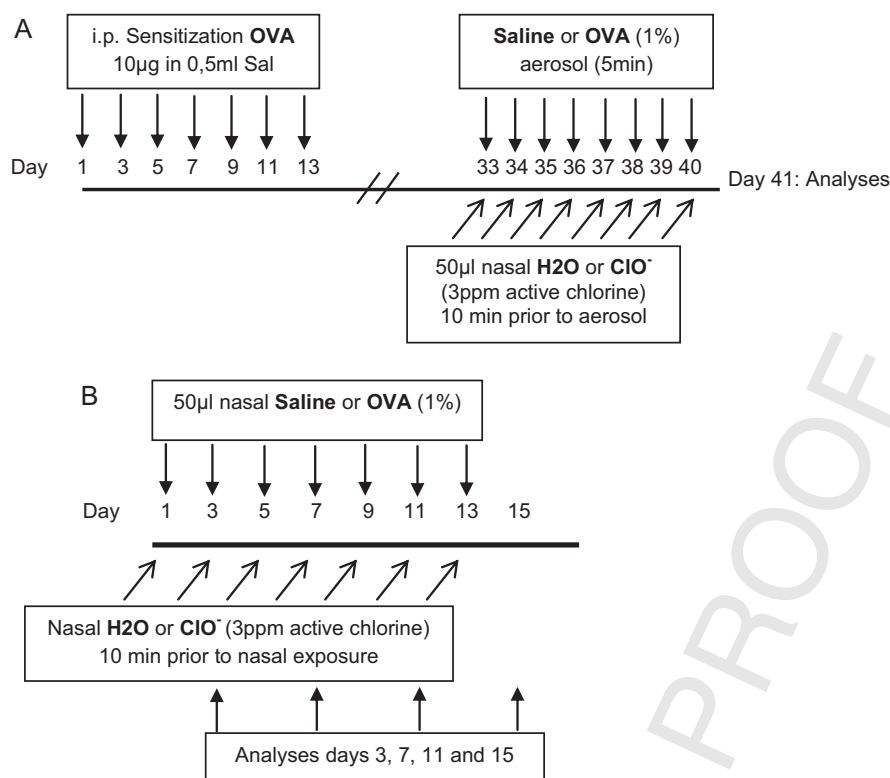


Fig. 1. Experimental protocols. (A) Mouse model of experimental OVA-induced airway allergy. (B) Mouse model of inhalation tolerance to OVA. Analyses include airway reactivity measurements in response to methacholine, systemic specific OVA-IgE detection, BAL inflammatory cell count, lung tissue cytokines measurements. Abbreviations: H₂O = deionized water, Sal = saline, OVA = ovalbumin, ClO⁻ = hypochlorite.

Cytometric Bead Array Flex (BD Biosciences, Erembodegem, Belgium) according to the manufacturers' specifications.

2.7. Data analyses

All data are presented as means with standard deviation and were analyzed using one-way ANOVA followed by a Dunnett post-hoc test (Graph Pad Prism 4.01, Graphpad Software Inc, San Diego, USA). Comparisons between two variables were calculated using a Mann–Whitney test. A level of $p < 0.05$ (two tailed) was considered to be significant.

3. Results

3.1. Effects of repeated nasal exposure to NaClO on allergic responses in OVA-sensitized mice

In the first series of experiments (Fig. 1A) mice were sensitized by 7 i.p. injections of OVA on alternate days and then daily exposed to either nebulized saline or OVA for 8 days. As expected, 24 h after the last challenge, OVA-mice showed bronchial eosinophilia, bronchial Th2 cytokine production, AHR and elevated specific anti-OVA IgE titers in serum as previously published (Hellings et al., 2001). Overall, nasal NaClO applications prior to the OVA-aerosol had no major impact on the allergic response, as evaluated by measuring inflammatory cells in BAL (Fig. 2C) or serum levels of OVA-specific IgE (Fig. 2D). Only the AHR in response to methacholine was slightly increased in the group that were exposed to NaClO prior to the OVA challenges (Fig. 2A and B). Nasal applications of NaClO did not result in increased levels of IL-4, IL-5, IL-13 and IFN- γ in homogenized lung tissue of OVA mice (data not shown).

3.2. Effects of repeated nasal exposure to NaClO on allergic responses to OVA in naïve mice

In the second series of experiments, naïve mice that received 7 times a nasal instillations of NaClO prior to OVA (Fig. 1B)

showed a significantly increased response to incremental doses of methacholine compared to the control groups ($p < 0.01$) (Fig. 3A and B). However, total BAL cell counts did not differ (not shown) and the numbers or distribution of inflammatory cells in BAL, did not differ either (Fig. 3C). This absence of inflammatory cells was confirmed by histological analysis of lung tissue of exposed mice: no inflammatory infiltrate was detected around the airways or in the lung tissue. However, scarce granulocytes were detected in the subepithelial space of nasal tissue, only in mice having received both NaClO and OVA (Fig. 3D) cells. OVA-specific IgE was near the detection limit in all samples, without differences between the group instilled with OVA (1.04 ± 0.66 EU/ml serum) and the group instilled with NaClO prior to OVA (0.27 ± 0.27 EU/ml serum) (graphs not shown). Concentrations of relevant cytokines and chemokines were measured in lung homogenates of exposed mice. IL-4 and IL-5 were not detectable in homogenized lung tissue from the four groups, and no differences were found in concentrations of IL-13, IL-10 or GM-CSF between the group exposed to NaClO and OVA and the group exposed to OVA alone: IL-13 (1.22 pg/mg tissue ± 2.08 H₂O/OVA group, 0.47 pg/mg tissue ± 0.27 ClO/OVA group), IL-10 (3.32 pg/mg tissue ± 1.52 H₂O/OVA group, 3.00 pg/mg tissue ± 2.39 ClO/OVA group), RANTES (478.50 pg/mg tissue ± 222.5 H₂O/OVA group, 474.1 pg/mg tissue ± 176.5 ClO/OVA group), GM-CSF (1.11 pg/mg tissue ± 0.41 H₂O/OVA group, 0.96 pg/mg tissue ± 0.38 ClO/OVA group) (graphs not shown).

3.3. Induction of airway responses by NaClO and OVA in naïve mice, in relation to number of exposures

At all time points, methacholine reactivity was higher in mice exposed to NaClO prior to OVA than in control mice or mice exposed to either OVA or NaClO alone; the increase in airway reactivity was already significant in mice that had received 1 combined

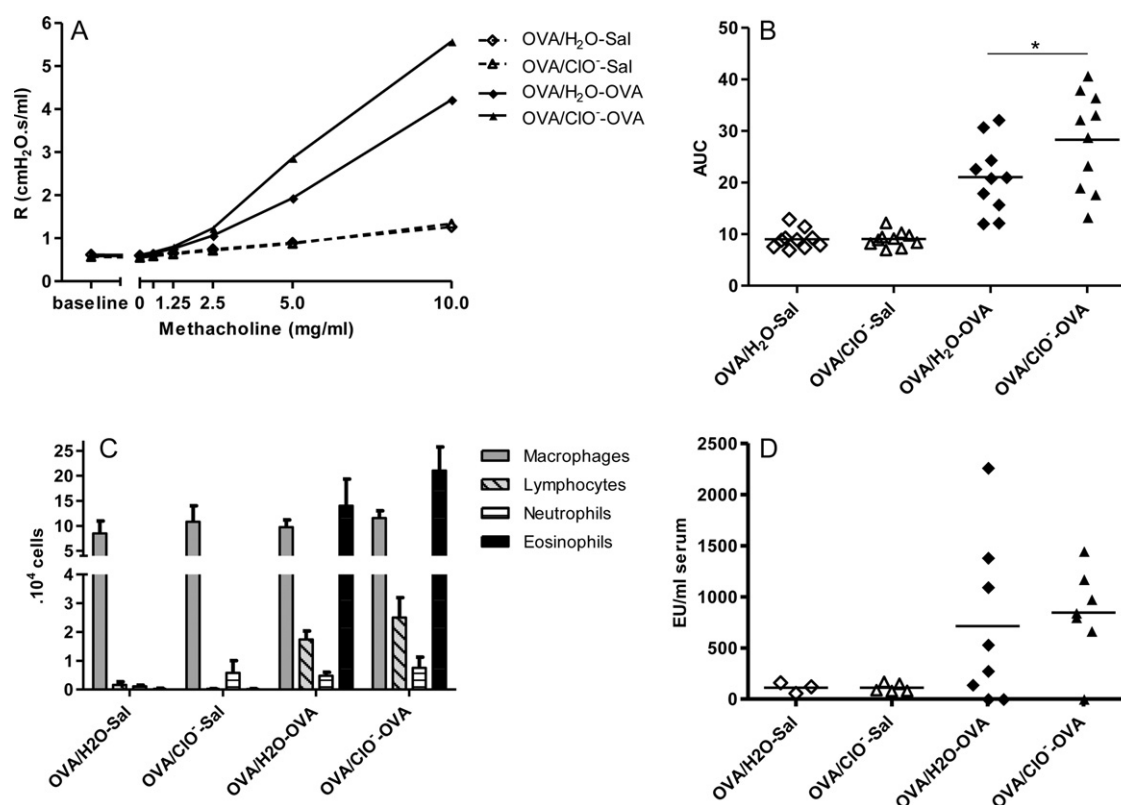


Fig. 2. Effects of repeated nasal exposure to NaClO on responses in OVA-sensitized mice. (A) Airway resistance (R) was measured in OVA-sensitized mice, 24 h after the last OVA-challenge, with or without prior nasal instillations with NaClO, in response to methacholine. Mean values of R are presented. Data shown are results of 2 independent experiments ($n = 10$). (B) Individual values of area under the curve (AUC) of R against concentration of methacholine between 0 and 10 mg/ml. Individual values and group means are depicted. Data shown are results of 2 independent experiments ($n = 10$). * $p < 0.05$. (C) Total and differential cell counts in bronchoalveolar lavage (BAL) 24 h after the last OVA-challenge, with or without prior nasal instillations with NaClO. Bars represent means $\pm$ SD. Data shown are results of 2 independent experiments ($n = 10$). (D) Levels of anti-OVA specific IgE in the serum were measured with sandwich ELISA technique 24 h after the last OVA-challenge. Individual values and group means are depicted ($n = 8$). Abbreviations: H₂O = deionized water, Sal = saline, OVA = ovalbumin, ClO⁻ = hypochlorite.

NaClO + OVA treatment, and it persisted at the same level after 3, 5 and 7 combined applications (Fig. 4A and B).

3.4. Additional experiments

To assess the specificity of OVA in relation to the NaClO induced AHR, mice received 1 nasal instillation of OVA, BSA or LPS after a single NaClO-exposure. Airway reactivity measurements showed that only the group that received NaClO prior to OVA developed AHR, and not the groups in which NaClO was given prior to BSA or LPS (Fig. 5A and B).

Mice that received 2 consecutive nasal instillations of *in vitro* pre-chlorinated OVA did not develop AHR compared to mice that received separate instillations of NaClO and then OVA ($p < 0.01$, Fig. 5C and D). Mice that received one instillation of heat-denatured OVA after NaClO, did not show AHR compared to mice that received an application of NaClO and followed by intact OVA ($p < 0.01$, Fig. 5C and D).

4. Discussion

In this study, we first demonstrated in a well-established mouse model of experimental allergy that nasal exposure to NaClO – given at a relevant concentration – has no detectable influence on pre-existing allergic airway inflammation. Previously, it has been shown that introduction of certain environmental or microbial agents in the challenge phase of similar allergy models can aggravate the allergic airway inflammation (Hellings et al., 2006; Ban et al., 2006). Moreover, there is evidence in the literature that atopic

subjects have a more pronounced reaction to chlorine provocations compared to healthy controls (Shusterman et al., 2003, 2004) and allergic responses are more pronounced when an allergen challenge is accompanied with exposure to diesel exhaust particles (DEP) (Diaz-Sanchez et al., 1997). Therefore, we had hypothesized that nasal instillations with NaClO in the challenge phase of a mouse model of experimental respiratory allergy would affect the functional and immunological outcomes. Although we found a borderline significance in AHR for the NaClO-exposed mice, the trends towards higher BAL eosinophil count and higher concentrations of specific anti-OVA IgE did not reach significance, despite n -value of 8–10. Neither did we detect a significant change in the concentrations of Th2 related cytokines in the lungs. These findings cannot be explained by the fact that inflammatory parameters had already reached their maximum levels, since comparable results were found when the challenge phase of the protocol was shortened (4 OVA-challenges instead of 8) and thus inflammatory features toned down (not shown).

Having failed to demonstrate an adverse effect of NaClO on the magnitude of the response to an allergic challenge, we decided to investigate whether exposure to NaClO would act as an adjuvant, i.e. by interfering with the sensitization process. So, in a further series of experiments, we hypothesized that nasal exposure to NaClO would be able to break inhalation tolerance to OVA. Repeated respiratory exposure to OVA is known to induce a so called “inhalation tolerance” in rodents, caused by IL-10 producing dendritic cells leading to T-cell anergy (Holt et al., 1981; Akbari et al., 2001). It has been shown that this inhalation tolerance can be broken by simultaneous exposure to certain environmental agents, such as ozone

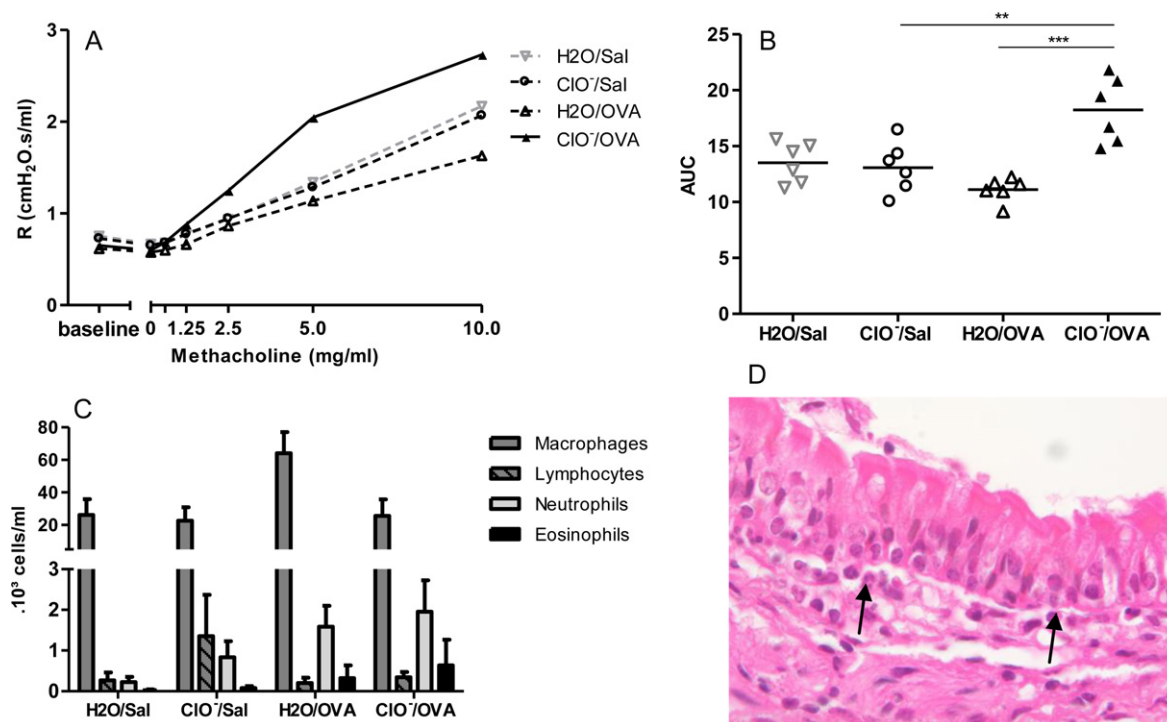


Fig. 3. Effects of repeated nasal exposure to NaClO on sensitization to OVA in naïve mice. (A) Airway resistance (*R*) was measured in naïve mice, 48 h after 7 nasal OVA-applications, with or without prior nasal instillations with NaClO. Mean values of *R* are presented (*n* = 5). (B) Individual values of AUC of *R* against concentration of methacholine between 0 and 10 mg/ml. Individual values and group means are depicted (*n* = 5). **p* < 0.01 and ****p* < 0.0001. (C) Total and differential cell counts in BAL, 48 h after the last nasal OVA-exposure, with or without prior nasal instillations with NaClO. Bars represent means ± SD (*n* = 5). (D) Hematoxylin and eosin (H&E) staining of paraffin section of resected nasal tissue, pre-elevated 48 h after the 7th combined instillation of NaClO and OVA. Arrows indicate granulocytes present in the subepithelial space.

(Schelegle et al., 2003), DEP (Bleck et al., 2006), tobacco smoke (Trimble et al., 2009) and microbial endotoxins (Huvenne et al., 2010). Since regular attendance to chlorinated swimming pools has been associated with a higher risk of developing asthma, AHR (Bernard et al., 2006, 2008) and a higher sensitization risk (Zwick et al., 1990), we tested the hypothesis that hypochlorite would elicit a similar effect on allergen sensitization.

Initially, we used a complete protocol involving 7 exposures to OVA with or without prior NaClO over a period of 13 days. This led to induction of AHR in the combined NaClO + OVA group compared to the relevant control groups (vehicle only, OVA only, NaClO only) and we thought that this fitted our hypothesis that NaClO exposure had broken tolerance to the allergen. This was plausible in view of the observation that Westman and Harris

(2004) showed that chlorination of rat serum albumin is able to break immunologic tolerance to the antigen, and experiments from Prokopowicz et al. (2010) that chlorination of OVA enhances the allergen T-cell response and the uptake by antigen-presenting cells. However, it appeared clearly that mice that had been co-exposed to NaClO and OVA did not show any bronchial inflammation or production of Th2 related cytokines in the airways. There was also no evidence for synthesis of specific anti-OVA IgEs, suggesting that the animals had not even become sensitized to the protein. No upregulation of GM-CSF, a growth factor that has been shown to be involved in the breaking of inhalation tolerance (Stampfli et al., 1998) was detected, nor did we detect a difference in IL-10 levels present in the lungs of co-exposed mice.

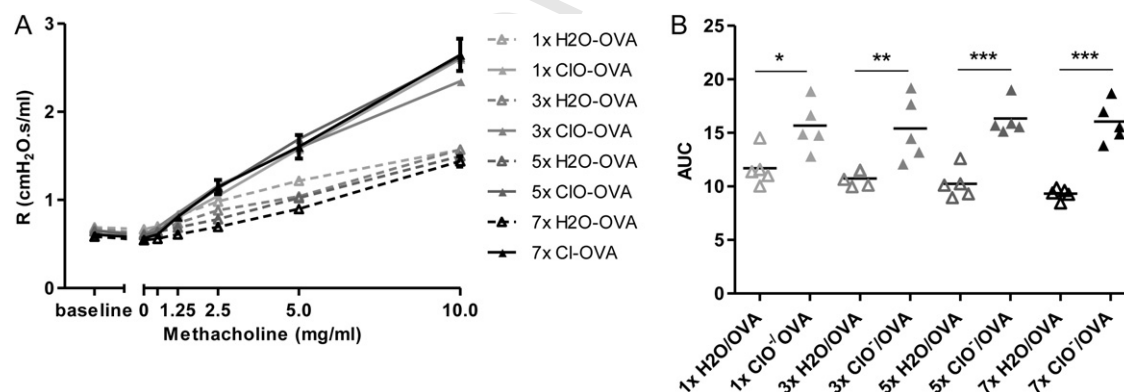


Fig. 4. Effects of nasal exposure to NaClO on airway responses to OVA in relation to number of exposures. (A) Airway resistance (*R*) was measured in naïve mice, 48 h after 1, 3, 5 and 7 nasal OVA-applications, with or without prior nasal instillations with NaClO. Mean values of *R* are presented (*n* = 5). Data depicted at 7 × H₂O/OVA and 7 × ClO₂/OVA represent different mouse experiments then represented in Fig. 3. (B) Individual values of area AUC of *R* against concentration of methacholine between 0 and 10 mg/ml. Individual values and group means are depicted (*n* = 5). **p* < 0.05, ***p* < 0.01, and ****p* < 0.0001.

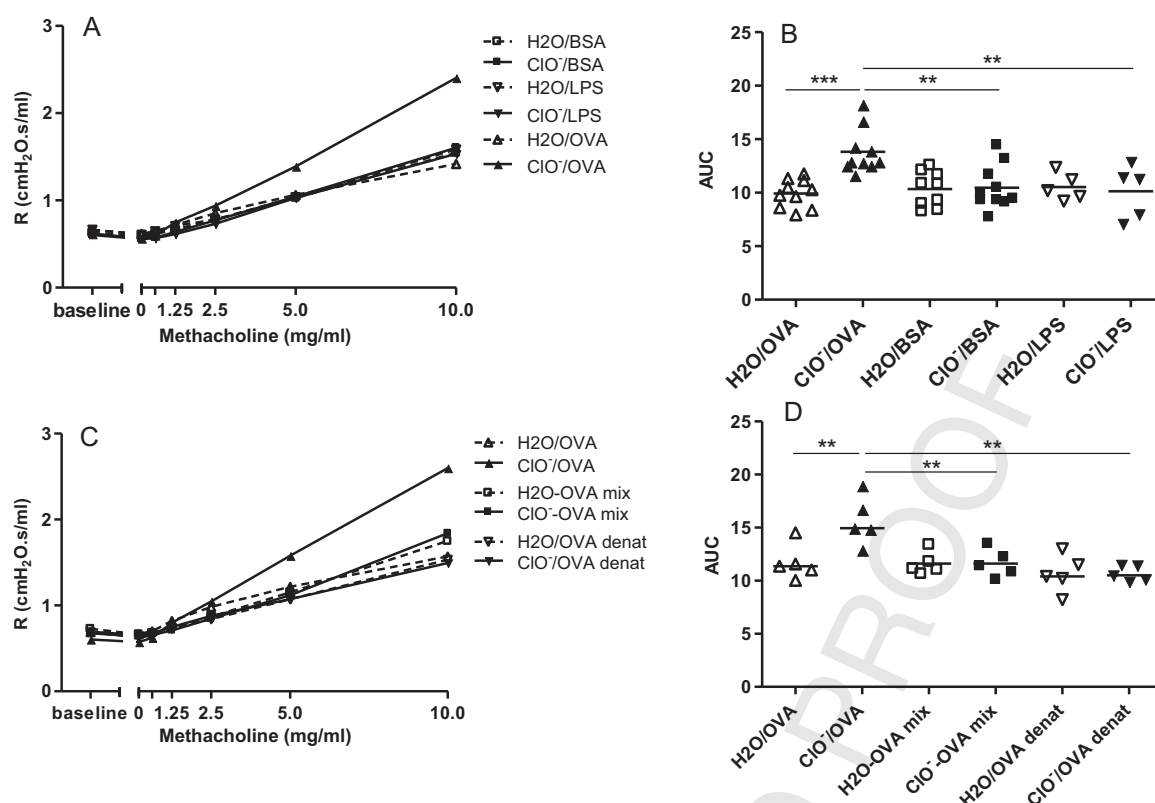


Fig. 5. Additional experiments to investigate the role of intact OVA-structure on the airway responses induced by NaClO and OVA in naïve mice. (A) Airway resistance (R) was measured in naïve mice, 48 h after a single nasal NaClO or dH₂O-application, followed by a nasal instillation with either OVA (10 mg/ml in saline), BSA (10 mg/ml in saline) or LPS (15 μ g/ml in saline). Mean values of R are presented ($n=5$). (B) Individual values and group means are depicted ($n=5$). Data depicted at H₂O/OVA and ClO⁻/OVA represent different mouse experiments then represented in Fig. 4. ** $p < 0.01$ and *** $p < 0.0001$. (C) Airway resistance (R) was measured in naïve mice, 48 h after combined exposures. The first pair of groups received a single nasal NaClO or dH₂O-application, followed by a nasal instillation with OVA (1%), the second pair of groups received two consecutive instillations with a mixed OVA–NaClO solution (0.5% OVA and ClO⁻ 1.5 ppm) or OVA (1%) alone, the pair of groups received a NaClO or dH₂O-application, prior to a nasal instillation with denaturated OVA (1%). Mean values of R are presented ($n=5$). (D) Individual values and group means are depicted ($n=5$). ** $p < 0.01$ and *** $p < 0.0001$.

In other words, we found no evidence for an immunological “adjuvant” effect of NaClO, although combined exposure to NaClO and OVA did induce AHR. This effect on bronchial reactivity independently from sensitization to the allergen and without signs of bronchial inflammation was confirmed in our time-response experiment. Indeed, we discovered that the AHR could already be induced by a single combined nasal exposure to NaClO and OVA, that it persisted for at least 48 h after the co-exposure and that it remained present after at least 7 applications. This response proved to be robust because it was obtained consistently in several independent experiments.

In additional experiments, we demonstrated that our findings could not be attributed to the LPS-contamination in the OVA and that they are specific for the OVA-protein, since AHR could not be induced when BSA was administered instead of OVA. Moreover, it is unlikely that this response to OVA simply results from an alteration of its structure by chlorination, since AHR did not develop when OVA had been pre-mixed with NaClO. On the other hand, it seems that an intact structure of OVA is required to produce the response, because no response was obtained with heat denaturated OVA.

At this stage, we have no mechanistic explanation for our findings, but the fact that the AHR is already present after 1 combined application, excludes changes in adaptive immunity which is a main player in the induction of allergic asthma. Since chlorination products are known airway irritants (Kuwabara et al., 2007), we hypothesize that they can activate chemoreceptors present in the airways. The most plausible receptors for detection of ClO⁻ are the

transient potential receptor (TRP) channels, present on the airway sensory nerves (Bessac et al., 2008). Although the main function of these chemosensory channels is to protect the airways from chemical challenges, it is thought that activation can lead to adverse effects in the airways like e.g. bronchoconstriction and neurogenic inflammation. Further experiments have to confirm this hypothesis, especially the combined effects of OVA and ClO⁻.

In conclusion, we demonstrated that respiratory exposure to relevant concentrations of NaClO does not significantly affect the development of experimental asthma in mice. However, when mice are co-exposed to NaClO and OVA, they develop AHR, independently from airway inflammation and already after 1 combined application. This response appears to be specific for the structure of OVA. The relevance of these experimental data for the observations of asthma and airway hyperresponsiveness in swimmers remains to be established.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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