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RESEARCH ARTICLE

Lung function changes in mice sensitized to ammonium hexachloroplatinate

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Abstract

Occupational exposure to halogenated platinum salts can trigger the development of asthma. The risk to the general population that may result from the use of platinum in catalytic converters and its emerging use as a diesel fuel additive is unclear. To investigate pulmonary responses to platinum, we developed a mouse model of platinum hypersensitivity. Mice were sensitized through application of ammonium hexachloroplatinate (AHCP) to the shaved back on days 0, 5 and 19, and to each ear on days 10, 11 and 12. On days 24 and 29, mice were challenged by oropharyngeal aspiration with AHCP in saline. Before and immediately after challenge, pulmonary responses were assessed using whole body plethysmography (WBP). A dose-dependent increase in immediate responses was observed in AHCP-sensitized and challenged mice. On days 26 and 31, changes in ventilatory responses to methacholine (Mch) aerosol were assessed by WBP; dose-dependent increases in Mch responsiveness occurred in sensitized mice. Lymph node cell counts indicate a proliferative response in lymph nodes draining the sites of application. Bronchoalveolar lavage fluid harvested from sensitized mice contained an average of 5% eosinophils compared to less than 0.5% in non-sensitized mice ($p < 0.05$); significant increases in total serum immunoglobulin E were observed for all sensitized mice. Although a second airway challenge on day 29 affected some results, only one airway challenge was needed to observe changes in lung function.

Keywords

Ammonium hexachloroplatinate, occupational asthma, platinum, pulmonary hyperresponsiveness, respiratory hypersensitivity, whole-body plethysmography

History

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Introduction

In developed countries, occupational asthma is the most common work-related lung disease (ALA, 2008; CDC, 2012; Petsonk, 2002; Reid & Reid, 2013). Asthma is accompanied by narrowing of the airways leading to bouts of chest tightness, wheezing, coughing and shortness of breath. Up to 15% of incident asthma cases in the United States are job-related (ALA, 2008; Balmes et al., 2003; Blanc & Toren, 1999). People with a family history of allergies are more likely to develop occupational asthma, but non-atopics can also develop the disease (Maestrelli et al., 2009; Pearce et al., 1999). Occupational asthma can be caused by exposure to high concentrations of irritants (e.g. hydrochloric acid, sulfur dioxide, ammonia). In this case, asthma symptoms appear immediately after first exposure (i.e. there is no latency period). In other instances, susceptible workers develop asthma as a consequence of a type I hypersensitivity reaction that occurs after repeated work-related exposure to a substance (e.g. isocyanates, metals). Long-term exposure to allergy-inducing substances can exacerbate symptoms and

lead to lifelong asthma. Symptoms usually diminish during time away from work (i.e. evenings, weekends, vacations). However, symptoms rebound upon return to work unless exposure to the asthma-inducing trigger is prevented.

Numerous case reports and occupational studies demonstrate that exposure to halogenated platinum salts (e.g. ammonium hexachloroplatinate (AHCP)) can cause occupational asthma in susceptible individuals (reviewed in IPCS, 2012; WHO, 2000). Exposed workers develop a type I hypersensitivity reaction accompanied by conjunctivitis, rhinorrhea, sneezing, cough and urticaria. Reports by Hughes (1980) and Roberts (1951) suggest that 50% of exposed workers could develop immediate type respiratory hypersensitivity reactions as a consequence of exposure to halogenated platinum salts during the manufacturing of catalysts. Merget et al. (2000) reported that 11% of workers exposed to 52.9 ± 19.7 ng soluble Pt/m³ in a German catalyst production plant developed positive skin prick tests over a 5-year period. Once platinum sensitization occurs, symptoms tend to worsen with continued exposure (Levene, 1971); in some cases, symptoms persist even when platinum exposure ceases (Merget et al., 1994).

Despite the well-documented health hazard posed by halogenated platinum salts, only limited laboratory-based research has been conducted to characterize the effects of exposure. Topical treatment of mice with halogenated

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platinum salts results in responses consistent with sensitization (Ban et al., 2010; Basketter et al., 1999; Dearman et al., 1998, 2013; Williams et al., 2014). For example, although they did not evaluate changes in lung function, Dearman et al. (1998) demonstrated that dermal exposure of mice to halogenated platinum salts resulted in an increase in Th2 cytokines (e.g. IL4 and IL-10) along with an increase in the Th1 cytokine IFN γ . These findings were supported by a second report in which mice were sensitized to sodium hexachloroplatinate by a combination of intradermal injections and intranasal instillation (Ban et al., 2010). In this study, platinum sensitization elicited a response characterized by elevated Th2 cytokines in concanavalin A-treated lymph node cell cultures, lung eosinophilia and IgE production (Ban et al., 2010). While this study provided new data on physiological changes associated with platinum sensitization, Ban et al. (2010) did not investigate potential changes in lung function occurring when mice dermally sensitized to platinum are subsequently challenged via pulmonary exposure. Most recently, topical administration of three different halogenated platinum salts resulted in lymph node cell proliferation and a positive result in a variation of the local lymph node assay (LLNA) (Williams et al., 2014). These and other studies (Schuppe et al., 1992, 1997a,b) provide evidence of dermal sensitization to halogenated platinum salts, but they do not provide information on sensitization by the inhalation route or changes in lung function following respiratory challenge with platinum.

The widespread use of platinum in automobile catalytic converters and its limited use as a fuel additive may result in release of airborne platinum into the environment (Dubiella-Jackowska et al., 2009; Jelles et al., 2001; Scott et al., 2005; Shafer et al., 2006). Consequently, inhalation exposure to platinum may occur in the general population. However, only two studies have investigated the sensitizing effects of inhaled halogenated platinum salts (Biagini et al., 1983, 1986). Cynomolgous monkeys were exposed to aerosolized platinum alone (Biagini et al., 1983) or concurrently with ozone (Biagini et al., 1986), leading to increased bronchial hyperreactivity to both platinum and non-specific stimuli (i.e. methacholine (Mch)) (Biagini et al., 1983, 1986). Interestingly, the concentration of platinum and Mch required to elicit a pulmonary response was significantly reduced when co-administered with ozone (Biagini et al., 1986). This result suggests that simultaneous exposure to halogenated platinum salts and an airway-damaging irritant may promote the development of allergic sensitization.

While there is clear evidence from human case reports that halogenated platinum salts are potent sensitizers, the data may be confounded by reports of cross-reactivity to different platinum compounds and other platinum group elements (Cristaudo et al., 2005; Hartmann & Lipp, 2003; Markman et al., 2003; Murdoch & Pepys, 1985, 1986, 1987; Santucci et al., 2000). Furthermore, while the results of animal-based studies demonstrate that sensitization can occur following either inhalation (Biagini et al., 1983, Biagini et al., 1986) or dermal exposures (Basketter et al., 1999; Dearman et al., 1998, 2013; Williams et al., 2014), the ability of halogenated platinum salts to trigger allergic respiratory responses following dermal sensitization has not been investigated.

Here, we developed a mouse model of platinum hypersensitivity with the capability to assess changes in lung function.

Materials and methods

Animals

Female BALB/c mice, 8–9 weeks old at study initiation, were used for these studies (Charles River Laboratories, Wilmington, MA). All mice were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-approved facility that provided constant environmental conditions with an ambient temperature of $21.5^{\circ}\text{C} \pm 1.5^{\circ}\text{C}$, relative humidity of $55\% \pm 5\%$, and a 12 h light/dark cycle. Mice were group-housed based on treatment groups in polycarbonate cages with hardwood chip bedding (NEPCO, Warrensburg, NY) and were provided a balanced diet mouse chow (5POO Prolab RMH3000, PMI Nutrition International, Richmond, IN) and water *ad libitum*. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of NHEERL, US EPA.

Chemicals

Test substances used for these studies, including ammonium hexachloroplatinate (AHCP) and acetyl- β -methacholine chloride (Mch), were purchased from Sigma Aldrich (St. Louis, MO). AHCP was prepared in dimethyl sulfoxide (DMSO; Wilmington, DE) for dermal dosing or pyrogen-free 0.9% sodium chloride, injection, USP (Hospira, Inc, Lake Forest, IL) for delivery to the airways.

Sensitization and challenge protocol

BALB/c mice were dosed topically with 1% AHCP (in DMSO) on the shaved back (100 μL) on experimental days 0, 5 and 15 and on the ears (25 $\mu\text{L}/\text{ear}$) on experimental days 10, 11 and 12, according to the exposure protocol described in Figure 1. Control mice received vehicle only. Mice were challenged with AHCP (10, 31 or 100 μg prepared in saline) through the airways by oropharyngeal aspiration (OPA; 50 μL inoculum) on experimental days 24 and 29. As shown previously, this procedure delivers material directly to the lungs (Foster et al., 2001). Anesthetized mice (3.5% isoflurane in oxygen) were suspended by their frontal incisors, and the tongue was gently grasped and pulled to one side with forceps to allow dosing to the oropharynx. After test article placement, the nose was briefly and gently occluded to encourage the mouse to inhale through its mouth. Immediate responses to AHCP were measured using whole-body plethysmography (WBP; Buxco Electronics, Troy, NY) immediately after OPA delivery. On experimental days 26 and 31, respiratory responses to the non-specific bronchoconstrictor Mch were also measured using WBP.

In vivo pulmonary responses

Antigen-specific pulmonary responses were assessed immediately after OPA delivery on experimental days 24 and 29 in unrestrained, unanesthetized mice using WBP. Mice were placed individually into a 12 chamber WBP device. Using expiration time (T_e), relaxation time (RT), and peak

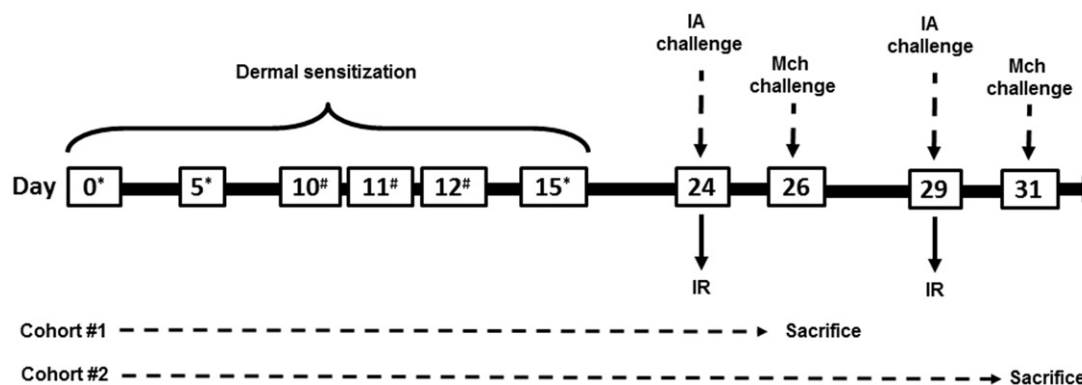


Figure 1. Exposure regimen. BALB/c mice were dosed with test article/vehicle topically on the shaved back on experimental days 0, 5 and 15 (*). Test article/vehicle was applied to the dorsum of both ears on experimental days 10, 11 and 12 (#). Blood was collected by submandibular bleed on experimental day 19. Mice were challenged by oropharyngeal aspiration (OPA) with AHCP on experimental days 24 and 29. Immediate responses (IR) were measured by whole-body plethysmography. On experimental days 26 and 31, pulmonary responses to aerosolized methacholine (Mch) were measured by WBP.

expiratory and inspiratory flows (PEF, PIF), the Biosystem software automatically calculates enhanced pause (Penh) every 12 seconds during the data collection period (Biosystem XA, version 2.5; Buxco Electronics, Sharon, CT). As previously described, Penh serves as a quantifiable indicator of pulmonary hyperresponsiveness (Farraj *et al.*, 2007; Hamelmann *et al.*, 1997). Average baseline measurements were collected for 10 min prior to OPA delivery of AHCP. Immediately following OPA delivery of AHCP, measurements were collected for 60 min and were used to determine Penh. Average Penh values were calculated for each animal over the entire measurement period (50 measurements for the 10 min baseline period and 300 measurements for the 60 min post-instillation period). Average values for each animal were used subsequently to calculate the average Penh for each dose group (3 independent studies, 6–9 mice/group). Group average Penh values were expressed as $\pm$ standard error of the mean.

Respiratory responsiveness to increasing concentrations of aerosolized Mch was measured by WBP 48 hours after OPA delivery of AHCP (i.e. experimental days 26 and 31). After measuring baseline parameters for 10 min, an aerosol of saline or 6.25, 12.5, or 25 mg Mch/mL of saline was nebulized through an inlet of each plethysmograph at a constant and identical flow rate (2 mL/s) using the Buxco aerosol delivery system. Response to saline and MCh were measured for 10 min (3 min aerosolization period and 7 min afterward). Average values for each animal were used subsequently to calculate the average Penh for each dose group (3 independent studies, 6–9 mice/group) as described above. Average baseline Penh values were then subtracted from each group. Group average Penh values were expressed as $\pm$ standard error of the mean.

Blood collection and euthanasia

Blood was collected on experimental day 19 by submandibular bleed. At study termination (either day 26 or day 31), mice were administered with Euthosol (Virbac AH, Inc., Fort Worth, TX) 1 hour after Mch challenge and then exsanguinated by cardiac puncture. Blood was collected in serum

collection tubes (BD Falcon, San Jose, CA) and processed to serum according to the manufacturer's instructions. Serum was stored at -80°C .

Lymph node cell collection

Immediately following euthanasia, the intrapulmonary, tracheobronchial and auricular lymph nodes were excised using aseptic technique and placed in room temperature RPMI 1640 containing 25 mM HEPES, 2.05 mM L-glutamine (Invitrogen, Grand Island, NY), 10% fetal bovine serum (Hyclone, Logan, UT), and 2% penicillin/streptomycin (Cellgro, Manassas, VA). Lymph nodes were mechanically disaggregated using a disposable plastic pestle and passed through a $100\mu\text{m}$ Celltrics filter (Partec, Munster, Germany) into a sterile 15 mL collection tube. Pooled lymph node cells were pelleted by centrifugation ($300 \times g$ for 7 min. at room temperature) and resuspended in 1 mL complete RPMI 1640. Cells were counted using a Coulter Counter (Beckman Coulter, Brea, CA), and viability was determined by trypan blue dye exclusion.

Bronchoalveolar lavage fluid (BALF) recovery and processing

After euthanasia, the trachea was exposed and cannulated, and the left lung was clamped off. The right lung was washed three times with 0.6 mL room temperature Ca^{++} , Mg^{++} and phenol red-free HBSS (Life Technologies, Bethesda, MD). Approximately 85% of the instilled volume was recovered in all treatment groups. The second two washes were pooled. All washes were temporarily stored on ice and then centrifuged ($360 \times g$ for 15 min at 4°C) to pellet BALF cells. The supernatant from the first wash was used for lactate dehydrogenase (LDH) and total protein measurements. The cell pellets from all three washes were pooled and resuspended in 1 mL Ca^{++} , Mg^{++} and phenol red-free HBSS for additional processing.

BALF cell counts and differentials

Total BALF cell numbers were determined using a Coulter Counter (Coulter, Hialeah, FL), and viability was assessed by

trypan blue dye exclusion. Cells (200 μ L of suspension) were cytocentrifuged (Shandon Southern Instruments, Sewickley, PA) onto duplicate slides for 10 min at 200 rpm. After drying, slides were stained with Wright Giemsa Stain Pack (Fisher Scientific, Suwanee, GA) using a Hema-tek 2000 (Miles Inc., Elkhart, IN). Differential cell categorization and percentages were determined by counting 200 total cells per slide.

Total serum IgE detection

Total serum IgE was determined using a commercial ELISA-based colormetric assay kit according to the manufacturer's instructions (BD Pharmingen, San Diego, CA). Briefly, 96-well flat-bottom Costar microtiter plates (Corning Life Sciences, Tewksbury, MA) were coated with 100 μ L/well of anti-mouse IgE antibody (BD Pharmingen) in coating buffer (i.e. phosphate-buffered saline (PBS)) and incubated overnight at 4 °C. After washing (with 0.5% Tween-20), the plates were incubated with blocking buffer (i.e. 1% bovine serum albumin (Sigma Aldrich) in coating buffer) at room temperature for 1 hour (Calbiochem, LaJolla, CA). Serum samples (1:10 dilution non-sensitized mice, 1:60 and 1:90 sensitized mice) were then added to the wells and allowed to incubate at room temperature for 1 hour. After washing, biotinylated rat anti-mouse IgE (BD Pharmingen) detection antibody was added to the wells and allowed to incubate at room temperature for 1 hour. After washing, 1.5 μ g/mL streptavidin peroxidase (Zymed, Grand Island, NY) was added followed by incubation at 25 °C for 1 hour. After washing, tetramethylbenzidine (TMB) substrate (Dako Corp., Glostrup, Denmark) was added. After development, the reaction was terminated by the addition of H₂SO₄ (Fisher Scientific, Pittsburgh, PA). Optical density was determined at 450 nm using a SpectraMax 340pc plate reader (Molecular Devices, Sunnyvale, CA). Mean IgE concentrations were determined using Softmax Pro software (Molecular Devices).

Total protein and LDH detection

LDH activity and total protein content of BALF were assessed using commercially available kits (Thermo Fisher Diagnostics, Waltham, MA) adapted for use with the Konelab 30 clinical chemistry analyzer (Thermo Clinical Lab Systems, Espoo, Finland) as described previously (Selgrade et al., 2006).

Statistical analysis

Statistical significance was defined as $p < 0.05$ as evaluated by one-way analysis of variance (ANOVA) and Bonferroni's multiple comparison test.

Results

Pulmonary responses to AHCP

As previously demonstrated, topical administration of AHCP leads to proliferation of lymphocytes in the lymph nodes draining the site of application (Williams et al., 2014). While this finding suggests that mice can be sensitized to halogenated platinum salts by the dermal route, it was not known if platinum delivered to the respiratory tract would trigger respiratory responses in dermally sensitized mice.

To address this question, BALB/c mice dermally sensitized to AHCP were challenged by OPA instillation of AHCP (Figure 1). Consistent with previous findings (Basketter et al., 1999; Dearman et al., 1998, 2013; Williams et al., 2014), challenge with AHCP (31 and 100 μ g) resulted in a dose-dependent increase in the number of lymph node cells present in the auricular lymph nodes (ALN) (Figure 2A; $p < 0.05$). The number of lymph node cells present in ALN significantly increased following a second challenge with 10, 31 or 100 μ g AHCP (Figure 2A; $p < 0.05$). Lymph node cell numbers were not increased in lung-associated (i.e. intrapulmonary and tracheobronchial (mediastinal)) lymph nodes (LLN) following a single OPA challenge on experimental day 24 (Figure 2B). However, the number of lymph node cells present in LLN dose-dependently increased following the second OPA AHCP challenge in sensitized mice (Figure 2B; $p < 0.05$).

Respiratory responses were assessed by whole-body plethysmography immediately after OPA instillation of AHCP. Average baseline Penh values were consistent across groups (at ~ 0.5 Penh; Figure 3A and C). An antigen-specific immediate response (IR) was evident in AHCP-sensitized mice following the first OPA challenge on day 24 with 31 and 100 μ g AHCP (Figure 3A; $p < 0.05$). Although to a lesser magnitude, average Penh values were also significantly elevated in non-sensitized mice challenged with 100 μ g AHCP on day 24 (Figure 3A; $p < 0.05$). Immediate responses were similar in mice receiving a second OPA challenge with AHCP on day 29 (Figure 3C).

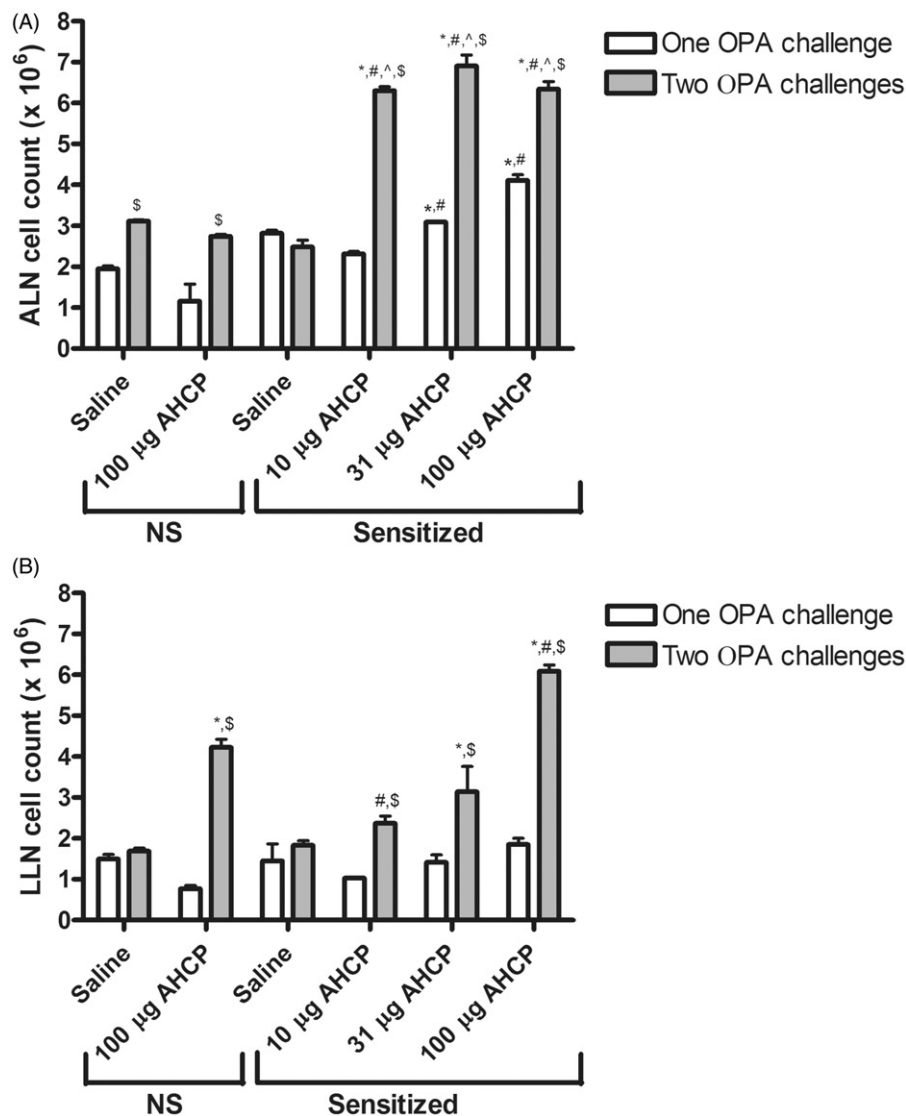
Methacholine (Mch), a cholinergic agonist, is widely used to study non-specific bronchial hyperreactivity. Two days after assessing the antigen-specific IR (i.e. on experimental days 26 and 31), we measured respiratory responsiveness to increasing doses of Mch aerosol. For sensitized mice challenged once or twice with 100 μ g AHCP, a statistically significant increase in Penh was observed with 25 mg/mL inhaled Mch when compared to non-sensitized mice challenged with 100 μ g AHCP (Figure 3B and D; $p < 0.05$). Average Penh values were not significantly increased for non-sensitized mice challenged with saline or AHCP, or for platinum-sensitized mice challenged with saline (Figure 3B and D).

Inflammatory cells infiltrate the airways of mice exposed to AHCP

Inflammatory cell infiltration of the airways often occurs following introduction of foreign material into the lungs. BALF total cell counts in mice challenged once or twice with AHCP were significantly increased compared to mice challenged with saline (Figure 4A and B; $p < 0.05$).

The cellular composition of BALF harvested from AHCP-sensitized mice was also impacted by challenge with AHCP. Specifically, assessment of differential inflammatory cell infiltration of the airways revealed that the proportion of eosinophils found in BALF, a marker of allergic inflammation (Akuthota et al., 2011), increased from $< 0.5\%$ of the total BALF cells in non-sensitized mice challenged with 100 μ g AHCP to $\sim 5\%$ in platinum-sensitized mice challenged once or twice with 100 μ g AHCP (i.e. on experimental days 24 and 31) (Figure 5A and C; $p < 0.05$). The proportion of

Figure 2. Assessment of lymph node cell proliferation by direct cell counts. Cells harvested from auricular lymph nodes (ALN) (A) and intrapulmonary/tracheobronchial (mediastinal) lymph nodes (LLN) (B) were counted using a Coulter counter. Data shown are $\pm$ SD ($n = 6$). * $p < 0.05$ compared to non-sensitized (NS) mice challenged with saline (ANOVA). # $p < 0.05$ compared to NS mice challenged with 100 μ g AHCP (ANOVA). ^ $p < 0.05$ compared to sensitized mice challenged with saline (ANOVA). \$ $p < 0.05$ compared to one OPA challenge (ANOVA).



neutrophils found in BALF was significantly elevated in both non-sensitized and platinum-sensitized mice challenged with 100 μ g AHCP on experimental days 24 and 31 (Figure 5B and D; $p < 0.05$). There were no statistically significant differences in the proportions of lymphocytes or macrophages in any experimental group (data not shown).

Biochemical markers of inflammation in response to AHCP

Total protein and LDH levels were measured in cell-free supernatants of BALF to assess inflammation and toxicity in our mouse model. Elevated levels of protein in BALF indicate increased permeability of the lung epithelium, which is a measure of pulmonary edema. Total protein levels in the BALF of mice challenged with 100 μ g AHCP on experimental day 24 were significantly increased compared to mice challenged with saline (Figure 6A; $p < 0.05$). There were no significant changes in total protein levels in any dose group following the second OPA challenge on day 29 (Figure 6C).

Changes in BALF LDH levels are often used as an indicator of non-specific cellular damage. There were no statistically significant differences in LDH levels across groups of mice (Figure 6B and D).

Total serum IgE levels are elevated in platinum-sensitized mice

Increased serum IgE levels are one of the key indicators of immediate type hypersensitivity (Burton & Oettgen, 2011). Total serum IgE levels were significantly elevated in platinum-sensitized mice compared to non-sensitized mice when measured on experimental day 19 (Figure 7A; $p < 0.05$). The magnitude of this elevation was even greater on experimental day 26 (Figure 7B; $p < 0.05$). Although these mice had been challenged with a range of AHCP concentrations, there were no dose-dependent differences in total serum IgE levels among sensitized and challenged mice (Figure 7B). Between experimental days 26 and 31, total serum IgE in sensitized mice challenged with saline decreased from about

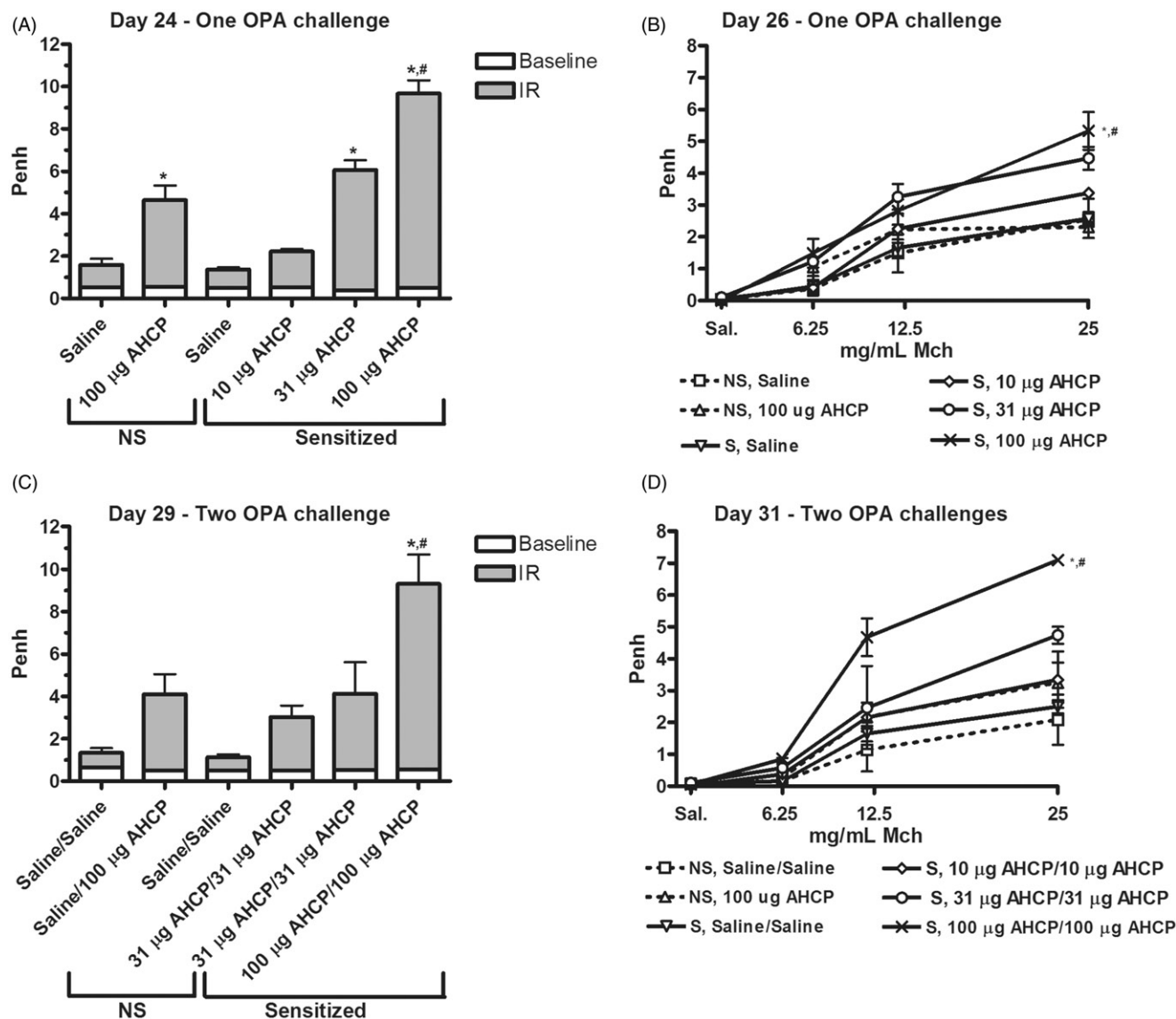


Figure 3. Pulmonary physiology. Pulmonary responses were measured using whole-body plethysmography. Platinum-sensitized mice exhibit an immediate response (IR) on experimental days 24 (A) and 29 (C). Platinum-sensitized mice exhibit a dose-dependent increase in methacholine (Mch) responsiveness on experimental days 26 (B) and 31 (D). For all panels, data shown were pooled from 3 independent studies $\pm$ SEM (each study with 3–9 mice/group). * $p < 0.05$ compared to non-sensitized (NS) mice challenged with saline (ANOVA). # $p < 0.05$ compared to non-sensitized mice challenged with 100 μ g AHCP (ANOVA).

20 000 pg/mL (Figure 7B) to about 7000 pg/mL (Figure 7C). However, in sensitized mice challenged twice with 100 μ g AHCP, serum IgE levels remained at about 20 000 pg/mL, and a dose-dependent increase in total serum IgE levels was observed with AHCP challenges at increasing doses (Figure 7C; $p < 0.05$).

Discussion

There is uncertainty as to which specific forms of platinum pose the greatest sensitization hazard. It has been suggested that the sensitization potential increases along with the number of chlorines coordinated with platinum (Cleare et al., 1976; Linnett & Hughes, 1999; Murdoch & Pepys, 1984a,b, 1985, 1986; Schuppe et al., 1992, 1997a). However, irrespective of the number of chlorines, AHCP, ATPC and CDDP induced a comparable degree of lymph node cell proliferation at similar concentrations in an LLNA study

(Williams et al., 2014). These data suggest that all three halogenated platinum salts are similar in their ability to sensitize mice via the dermal route. However, it remained to be seen whether or not an allergic response could be triggered by targeted airway delivery of platinum in these “sensitized” animals, and the utility of LLNA potency values for respiratory sensitizers is unknown (Basketter & Kimber, 2011). Consequently, studies that include measures of respiratory parameters are necessary to fully understand the (relevance and) functional consequences of dermal exposure to halogenated platinum salts and other respiratory allergens. In this study, we demonstrated that mice dermally sensitized with AHCP (1) exhibit an IR and experience eosinophilic infiltration of the lung following targeted delivery of AHCP to the respiratory tract, (2) are responsive to non-specific stimuli (i.e. Mch), and (3) have significantly elevated levels of total serum IgE. Taken together, these data indicate that dermal exposure to AHCP induces

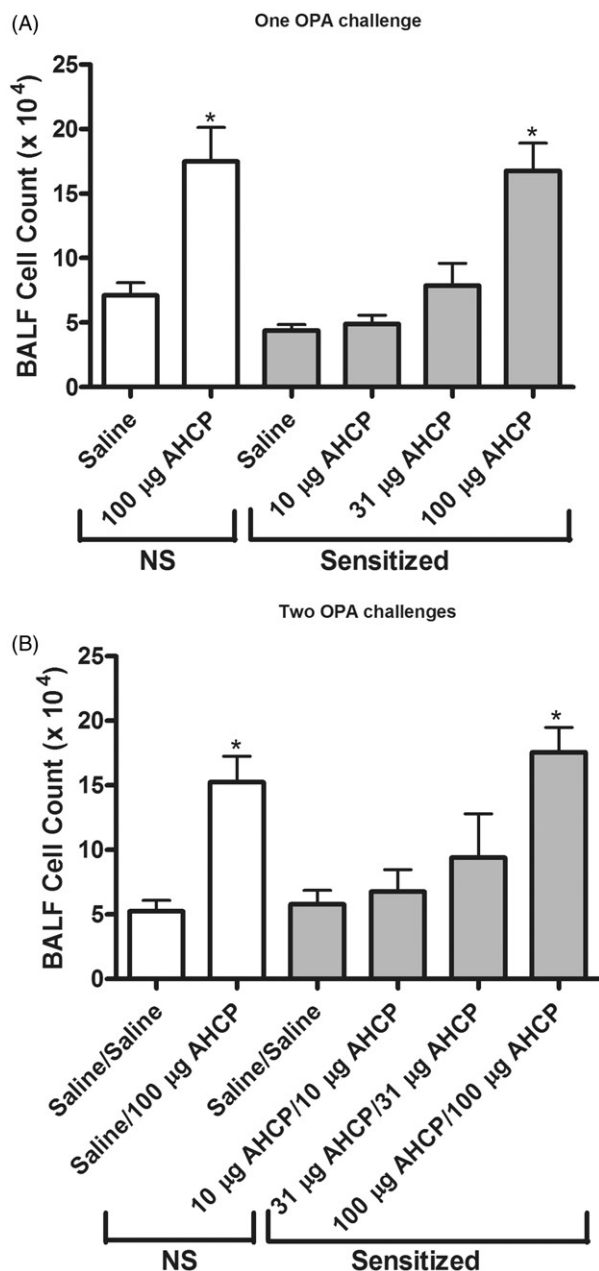


Figure 4. Lung instillation of AHCP results in cellular infiltration into BALF. BALF was collected from non-sensitized and platinum-sensitized mice on experimental days 26 (A) and 31 (B). Collected cells were enumerated using a Coulter counter. Data shown are pooled from 3 independent studies $\pm$ SEM (each study with 6–9 mice/group). * $p < 0.05$ compared to non-sensitized (NS) mice challenged with saline (ANOVA).

immunological changes underpinning the development of immediate type hypersensitivity and that a single respiratory challenge is sufficient to trigger pulmonary responses in dermally sensitized mice.

Sensitization precedes the development of allergic reactions. First, an individual must become sensitized through exposure to the antigen, ultimately producing IgE antibodies against it. Traditionally, workplace exposures leading to sensitization were believed to be predominantly via the respiratory tract (IPCS, 1991). And, two studies conducted in primates confirmed this route of sensitization (Biagini *et al.*, 1983, 1986). However, stringent workplace exposure controls have not been sufficient to prevent all cases of sensitization

(Baker *et al.*, 1990; Bolm-Audorff *et al.*, 1992; Brooks *et al.*, 1990; Linnett & Hughes, 1999; Merget *et al.*, 1988, 2000), suggesting that other exposure routes may also lead to platinum hypersensitivity.

It has been demonstrated that sensitization through the skin can lead to respiratory responses in laboratory animals and people (Arts *et al.*, 1998; Farraj *et al.*, 2006, 2007; Satoh *et al.*, 1995; Zhang *et al.*, 2002, 2004, 2006). However, this is not universally the case. Farraj (2007) reported discordance between total serum IgE levels, the Th2 cytokine profile induced by dermal application of isocyanates, and changes in respiratory function (Farraj *et al.*, 2007). An important conclusion drawn by Farraj *et al.* (2007) is that Th2 cytokine secretion from lymph nodes draining the skin are not always a reliable predictor of respiratory hypersensitivity. This assertion is supported by other published studies where Th2 cytokine profiles are not universally predictive of respiratory sensitizers (Ku *et al.*, 2008; Tarkowski *et al.*, 2007; Traidl *et al.*, 1999; Ulrich *et al.*, 2001; Vanoirbeek *et al.*, 2004, 2006). Together, these reports illustrate the need to confirm allergen-induced cytokine secretion with an evaluation of changes in lung function as well as additional indicators of respiratory hypersensitivity (Murphy *et al.*, 2012).

In this study, mice were sensitized by skin exposure and later subjected to intra-airway challenge with increasing concentrations of AHCP. Compared to non-sensitized mice, dramatically elevated Penh values were observed in AHCP-sensitized mice challenged with 100 µg AHCP. Importantly, the magnitude of response in sensitized mice challenged with AHCP was statistically significantly greater than that observed when non-sensitized mice were challenged with platinum. Furthermore, AHCP-sensitized mice were responsive to the non-specific stimulus Mch. The use of Penh has been the subject of debate (Fedulov & Kobzik, 2011; Hantos *et al.*, 2011), specifically when this variable is mistakenly equated with airway resistance (Kirschvink *et al.*, 2005). Although the association of Penh with lung injury has yet to be fully explained, there is a substantial amount of empirical data to support its use as a biomarker of disease (Allerton *et al.*, 2013; Bates *et al.*, 2004; Farraj *et al.*, 2007; Gavett *et al.*, 2003; Ghio *et al.*, 2014; Hoffman *et al.*, 1999; Kawikova *et al.*, 2004; Midoro-Horiuti *et al.*, 2010; Pauluhn, 2015; Raemdonck *et al.*, 2012; Sun *et al.*, 2014; Ward *et al.*, 2009, 2014). Notwithstanding, airway hyperresponsiveness data collected during this study should ultimately be confirmed with specific measures of airway resistance and compliance.

Instillation of AHCP resulted in a marked influx of neutrophils into the lungs. Accumulation of neutrophils in the airways has been reported in mouse models of allergic airway diseases, and neutrophil numbers have also been shown to correlate with the severity of airway obstruction in patients (Park *et al.*, 2006). Irrespective of the sensitization state of the mice, instillation of AHCP resulted in elevated neutrophils in BALF, suggesting a non-specific inflammatory response. This conclusion is also supported by detection of elevated protein levels in BALF and elevated Penh values in non-sensitized mice following a single challenge with AHCP.

In direct support of a hypersensitivity response, only BALF harvested from platinum sensitized mice contained significantly elevated numbers of eosinophils, indicating that the

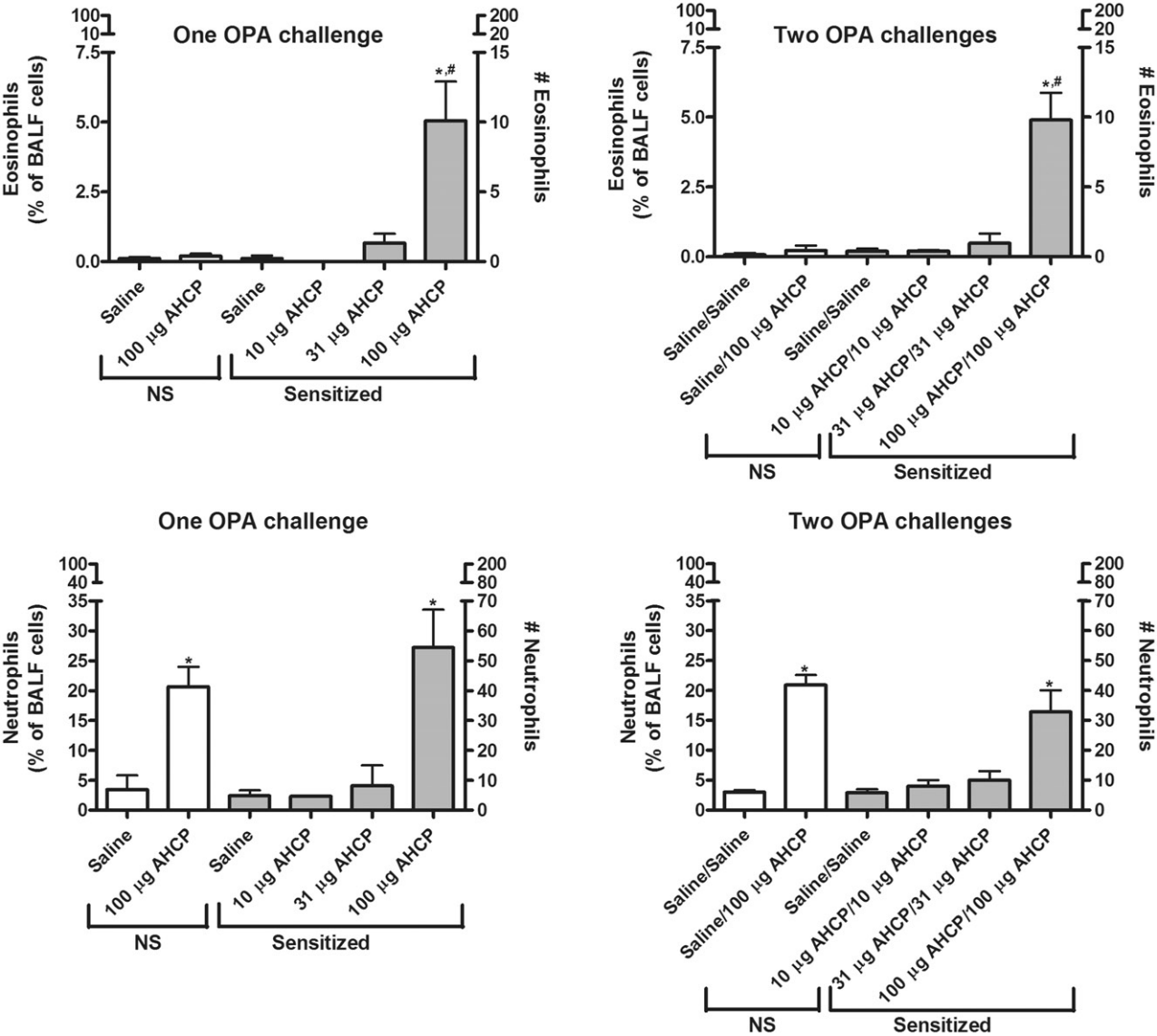


Figure 5. Lung instillation of AHCP results in differential inflammatory cell infiltration into BALF. (A & C) Eosinophils selectively infiltrate the BALF of platinum-sensitized mice following one or two OPA challenges. (B & D) Neutrophils infiltrated the BALF of non-sensitized and sensitized mice challenged with AHCP. For all panels, data shown were pooled from 3 independent experiments $\pm$ SEM (each study with $n = 3-6$ mice/group). Data shown for all panels from 200 cells. * $p < 0.05$ compared to non-sensitized (NS) mice challenged with saline (ANOVA). # $p < 0.05$ compared to non-sensitized mice challenged with 100 µg AHCP (ANOVA).

response was immune-mediated. Eosinophils play a significant role in asthma pathogenesis by releasing mediators and contributing to airway inflammation (Fahy, 2009). While eosinophilic infiltration of the lung can be very pronounced in the case of house dust mite allergy (Johnson et al., 2004), the percentage of eosinophils in the BALF was relatively low for AHCP. However, the degree of eosinophilic infiltration induced by AHCP was statistically significant compared to non-sensitized mice challenged with AHCP and highly reproducible. BALF was collected 48 hours after a single antigen provocation and differences in cell type may have been greater at other time points. Additionally, these data are in agreement with the degree of eosinophil infiltration reported by Ban (2010) when mice were sensitized (intradermal) and challenged (intranasal installation) up to five times with sodium hexachloroplatinate.

IgE antibodies play a pivotal role in allergic reactions. The immune environment that favors the switching from

IgG to production of IgE antibodies requires differentiation of naïve T cells to a Th2 phenotype. Cytokines (i.e. IL-4 and IL-13) and co-stimulatory signals (i.e. CD40) produced by Th2 lymphocytes induce B cells to produce IgE antibodies (Poulsen & Hummelshoj, 2007). Topical exposure to AHCP has been demonstrated to produce a Th2 cytokine profile (Dearman et al., 1998); however, levels of total or antigen-specific IgE were not measured in this study. Here, we show robustly increased total serum IgE levels in response to topical exposure to AHCP. Although total serum IgE levels were at their highest in all sensitized mice, regardless of the challenge dose of AHCP on experimental day 26, after mice received a second airway challenge with AHCP, a dose-responsive increase in IgE was observed. It should be noted that we did not evaluate antigen-specific IgE antibodies and that IgE levels can increase in response to parasitic infection (Murphy et al., 2012). However, the cumulative power of our combined dataset (i.e. IgE levels

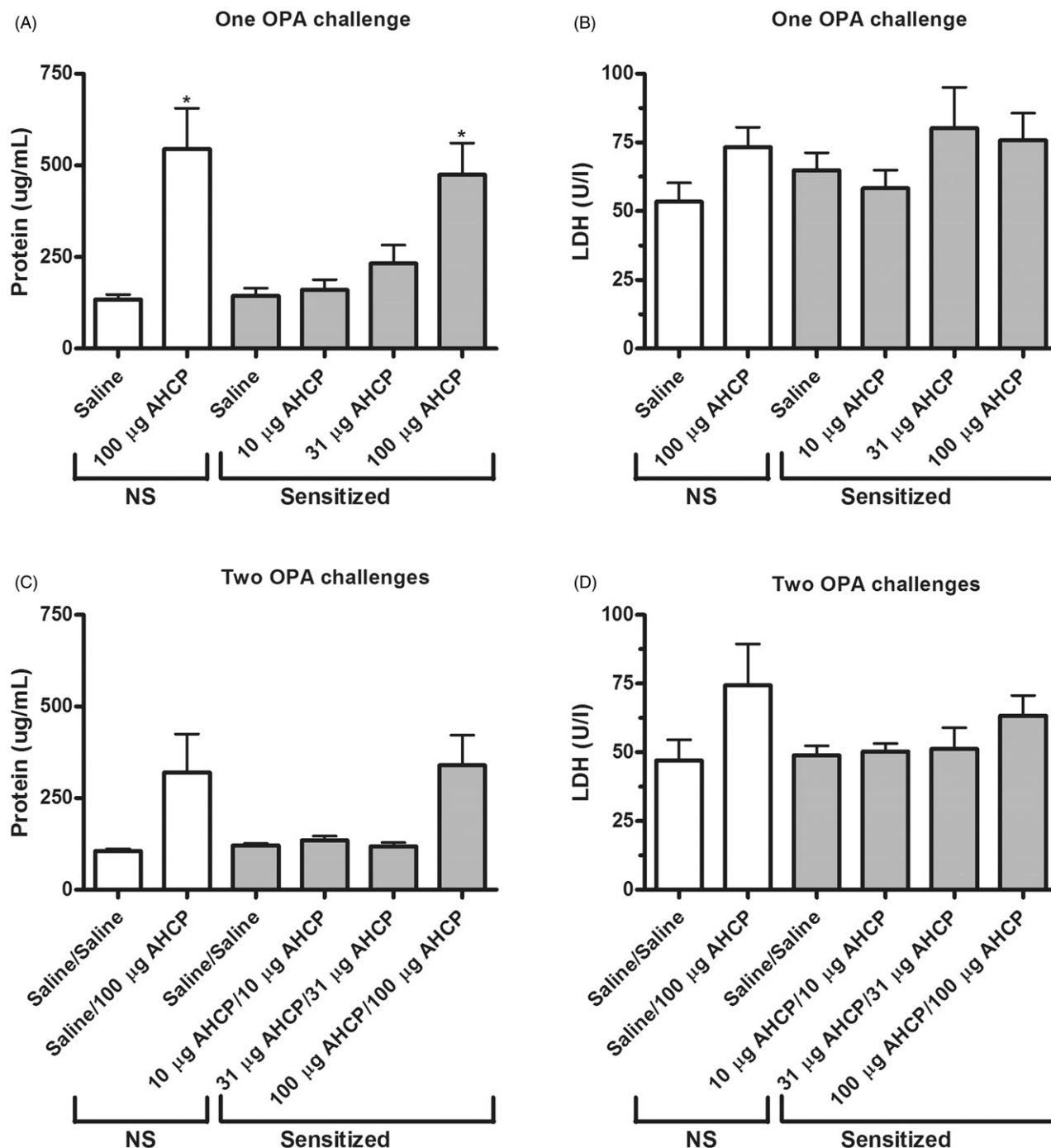


Figure 6. Protein levels are elevated in BALF collected from AHCP-challenged mice. (A & C) Protein levels in BALF were determined by Coomassie blue. (B & D) Lactate dehydrogenase levels were determined using Thermo LD-L Kit reagent and Thermo Trace Data-Trol controls. Data shown from both panels were pooled from 3 independent experiments $\pm$ SEM (each study with 3–6 mice/group). * p < 0.05 compared to non-sensitized (NS) challenged with saline (ANOVA).

were only increased in animals sensitized to platinum and, with subsequent challenge, the eosinophil number correlated with platinum exposure it seems unlikely that parasitic infection would cause this pattern of response) suggests that the observed effects are the result of allergic sensitization to platinum.

In some cases, it may be necessary to prime the lungs of mice previously sensitized to an allergen by the dermal route through targeted airway exposure (Matheson *et al.*, 2001, 2005; Scheerens *et al.*, 1999; Selgrade *et al.*, 2006). In the present study, dermal exposure and a single

respiratory challenge with AHCP increased the number of lymph node cells in the auricular lymph nodes, but the number of lymph node cells harvested from lung-associated lymph nodes did not increase until AHCP had been delivered to the lungs for a second time. Thus, including a second targeted airway exposure to AHCP impacted lymph node cell counts. However, as indicated by measures of airway response, eosinophil number, and IgE levels, priming the lung was not essential to the development and manifestation of a hypersensitivity response to AHCP in our model.

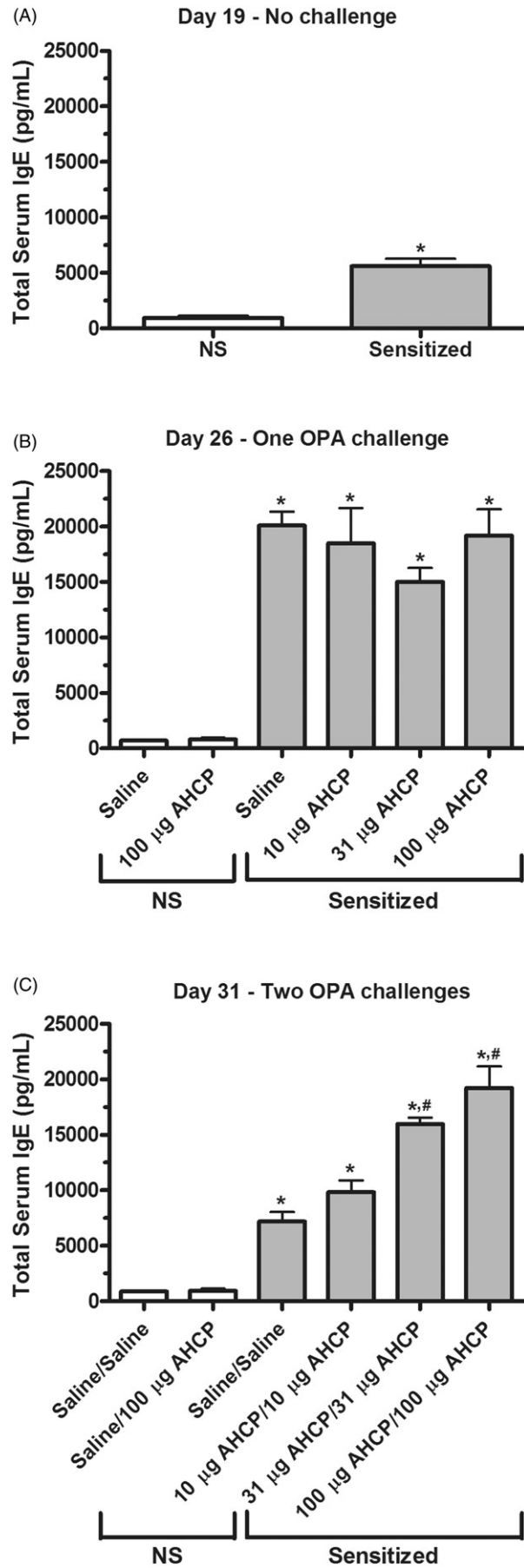


Figure 7. Total serum IgE levels were significantly elevated in mice sensitized to AHCP. Total serum IgE levels were determined by ELISA on experimental days 19 (A), 26 (B) and 31 (C). Data shown were pooled from 3 independent studies $\pm$ SEM (each study with 3–6 mice/group). * $p < 0.05$ compared to non-sensitized (NS) mice challenged with saline (ANOVA). # $p < 0.05$ compared to sensitized mice challenged with saline/saline (ANOVA).

Conclusions

Herein, we developed the first murine model of halogenated platinum salt sensitization with the capacity to assess respiratory responses. Using this model, we demonstrate, for the first time, that, in topically sensitized mice, a single airway challenge with AHCP is capable of inducing dose-dependent changes in pulmonary function. This model enables future investigations of the (1) variable allergenic potential of different halogenated platinum compounds, (2) potential for cross-reactivity among platinum compounds, (3) ability of diesel exhaust to act as an adjuvant, and (4) effect of exposing sensitized mice to real-world diesel exhaust containing platinum. It could also be extended to assess other metal-containing fuel additives. Findings from future studies using this model may have important implications for human health risk assessment.

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Declaration of interest

This article has been reviewed by the U.S. Environmental Protection Agency and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency or of the US Federal Government, nor does the mention of trade names or commercial products constitute endorsement or recommendations for use of those products. The authors report no financial or other conflicts of interest. The authors alone are responsible for the content and writing of this article.

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