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Authors

Proskocil, Becky J
Bruun, Donald A
Garg, Jasmine A
et al.

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ORIGINAL RESEARCH

The Influence of Sensitization on Mechanisms of Organophosphorus Pesticide–Induced Airway Hyperreactivity

Becky J. Proskocil[1], Donald A. Bruun[2], Jasmine A. Garg[1], Chloe C. Villagomez[1], David B. Jacoby[1], Pamela J. Lein[2], and Allison D. Fryer[1]

[1]Pulmonary and Critical Care Medicine, Oregon Health and Science University, Portland, Oregon; and [2]Department of Molecular Biosciences, University of California School of Veterinary Medicine, Davis, California

ORCID IDs: 0000-0002-7803-6447 (B.J.P.); 0000-0003-2195-9512 (D.B.J.); 0000-0001-7665-7584 (P.J.L.); 0000-0003-1712-9831 (A.D.F.).

Abstract

We previously demonstrated that antigen sensitization increases vulnerability to airway hyperreactivity induced by the organophosphorus pesticide (OP) parathion. Sensitization also changes the mechanism of parathion-induced airway hyperreactivity to one that is dependent on IL-5. To determine whether this effect can be generalized to other OPs, and to other classes of pesticides, we measured airway responsiveness to vagal stimulation or intravenous acetylcholine in nonsensitized and ovalbumin-sensitized guinea pigs 24 hours after a single subcutaneous injection of the OPs diazinon or chlorpyrifos, or the pyrethroid permethrin. Sensitization exacerbated the effects of chlorpyrifos on bronchoconstriction in response to vagal stimulation or intravenous acetylcholine. Pretreatment with function-blocking IL-5 antibody prevented chlorpyrifos-induced airway hyperreactivity in sensitized, but not in nonsensitized, guinea pigs. In sensitized guinea pigs, blocking IL-5 decreased eosinophil activation, as measured by decreased eosinophil major basic protein in the trachea. In contrast, sensitization did not alter diazinon-induced airway hyperreactivity, and permethrin did not cause airway hyperreactivity in either nonsensitized or sensitized guinea pigs. None of the pesticides affected inflammatory cells in the bronchoalveolar lavage fluid or blood. We have previously shown that three different OPs cause airway hyperreactivity via loss of neuronal M2 muscarinic receptor function. Similar to parathion, but unlike diazinon, the mechanism of chlorpyrifos-induced airway hyperreactivity is changed by sensitization. Thus, OP-induced airway hyperreactivity is dependent on sensitization status and on the OP used, which may influence therapeutic approaches.

Keywords: airway hyperreactivity; eosinophils; organophosphorus pesticides; permethrin; sensitization

Clinical Relevance

A low subcutaneous dose of an organophosphorus pesticide (OP) causes airway hyperreactivity that is dependent on the specific OP and on atopic phenotype. Understanding the mechanism(s) of OP toxicity in different populations will be critical to predicting the physiological response to OP exposure and to designing better therapeutic interventions.

The canonical mechanism of acute neurotoxicity after organophosphorus pesticide (OP) exposure is the irreversible inhibition of acetylcholinesterase (AChE), which is the enzyme that hydrolyzes the neurotransmitter, acetylcholine (ACh). ACh plays an integral role in controlling respiration via both the central (1) and peripheral nervous systems. OP poisoning can cause respiratory failure through central cholinergic disruption and acute bronchoconstriction and mucus

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plugging via overstimulation of peripheral nerves (2). In the lungs, ACh released from postganglionic parasympathetic nerves activates M3 muscarinic receptors on airway smooth muscle to initiate bronchoconstriction and on submucosal glands to stimulate mucus secretion. ACh also binds to inhibitory presynaptic M2 muscarinic receptors on parasympathetic nerves to decrease further release of ACh, thereby limiting bronchoconstriction (3).

Humans are chronically exposed to OPs, primarily via absorption through the skin and ingestion (4). Low concentrations of OPs are detected on fruits and vegetables, and OP metabolites are routinely detected in urine samples from adults and children living in the United States (5). During the early 1980s through the late 1990s, OPs were used extensively in homes and in agriculture, and during this same period of time the incidence of asthma increased, particularly in children (6). Consistent with this, numerous studies have linked exposure to OPs, including childhood exposures (7), to the development of respiratory symptoms and respiratory diseases.

Because of occupational exposure to OPs (8), farmers and other agricultural workers are the subject of many epidemiological studies investigating a link between OP usage and respiratory diseases. The primary route of exposure for agricultural workers is through the skin (9). Although agricultural workers show no symptoms of cholinergic toxicity, they are clearly absorbing OPs, as their blood AChE and butyrylcholinesterase activity were diminished (10, 11). In these workers, OP exposure correlated with an increase in cough (11), wheeze (11, 12), and bronchitis (13), and a decrease in lung function (10, 11).

Using an experimental animal model, we demonstrated that OPs can cause airway hyperreactivity at doses lower than those needed to inhibit AChE activity. A single subcutaneous injection of the OPs, parathion, diazinon, or chlorpyrifos, causes airway hyperreactivity in guinea pigs 24 hours later by inhibiting M2 receptor function on parasympathetic nerves, independent of AChE inhibition (14–16). These receptors normally inhibit ACh release; thus, M2 loss increases ACh release, resulting in increased vagally induced bronchoconstriction. These experimental

observations are consistent with clinical studies that have shown an association between the loss of M2 receptor function and asthma (17).

Eosinophilia and eosinophil products are linked to M2 muscarinic receptor dysfunction (18, 19), and both allergy and asthma are often associated with eosinophilia (20). A large subpopulation of the United States is sensitized to allergens (atopic) (21). Although agricultural workers are more often nonatopic than individuals working in other sectors or living in urban areas (22), occupational exposure to OPs is associated with increased allergic asthma in agricultural workers (23, 24). In nonsensitized guinea pigs, parathion-induced airway hyperreactivity and M2 muscarinic receptor dysfunction is not mediated by eosinophils (14), but instead by TNF-α (15). In guinea pigs sensitized to ovalbumin, a model of atopy, the mechanism of parathion-induced airway hyperreactivity, is changed to be mediated by IL-5 (14). IL-5 is a key factor required for recruitment and survival of eosinophils (25). Because of this switch in the mechanism of airway hyperreactivity with sensitization, and because a large percentage of the human population is allergic, determining whether atopy changes the mechanism of OP-induced airway hyperreactivity is important for determining the appropriate therapy for OP-induced airway hyperresponsiveness in allergic versus nonallergic individuals.

Increasingly, it is recognized that, although most OPs are potent inhibitors of AChE, they exhibit unique toxicological profiles even at doses that inhibit AChE similarly (for review, see Refs. 26, 27). We had previously shown that the OP, parathion, which is banned in many countries, including the United States, because of its potent toxicity, as well as the OPs, chlorpyrifos and diazinon, which are still routinely used in the United States and Europe (28), cause airway hyperreactivity via neuronal M2 receptor dysfunction in nonsensitized guinea pigs (16). The goal of this study was to determine whether chlorpyrifos and diazinon are similar to parathion in potentiating airway hyperreactivity in sensitized guinea pigs, and, if so, whether the mechanism changes to one that is IL-5 dependent. Although we previously

found that the non-OP, permethrin, did not cause airway hyperreactivity in nonsensitized guinea pigs (16), we also determined whether atopy modulates airway responsiveness after exposure to permethrin.

Materials and Methods

Animals
Pathogen-free female Hartley guinea pigs (150–200 g; 300–375 g; Charles River Laboratories, Wilmington, MA) were treated humanely, in accordance with standards established by the U.S. Animal Welfare Act and National Institutes of Health guidelines. All protocols were approved by the Animal Care and Use Committee at Oregon Health and Science University (Portland, OR).

Ovalbumin Sensitization
Guinea pigs were injected intraperitoneally with 4.2 mg ovalbumin (Sigma Aldrich Co., St. Louis, MO) on Days 1, 3, and 5 (29). OP exposure and physiological measurements were performed 21 days after the last injection.

Pesticides
Diazinon (*O,O*-diethyl-*O*-[2-isopropyl-4-methyl-6-pyridimyl] phosphorothioate; 99.5% purity), chlorpyrifos (*O,O*-diethyl [*O*-3,5,6-trichloro-2-pyridyl] phosphorothioate; 99.5% purity), and permethrin (3-phenoxybenzyl(1RS)-*cis*, trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate; 47.6% *cis* and 50.4% *trans* purity) were purchased from ChemService Inc. (West Chester, PA). Chlorpyrifos was initially resuspended in ethanol at 0.4 mg/μl. Pesticides were resuspended in peanut oil before subcutaneous injection into the subscapular region (16). Control animals received equal volumes (300 μl) of the vehicle. A subset of animals received 240 μg/kg antibody to IL-5 (AbIL5) intraperitoneally (TRFK5; BD Pharmingen, San Jose, CA) 3 days before OP treatment.

Measurement of Pulmonary Inflation Pressure and Bradycardia
Physiological experiments were performed 24 hours after pesticide injection as previously described (30). Briefly, guinea pigs were anesthetized (1.9 mg/kg urethane, intraperitoneal;

Sigma-Aldrich), paralyzed (10 μg/kg/min succinylcholine, intravenous; Sigma-Aldrich), ventilated (1 ml/100 g body weight and 100 breaths/min), and cannulated to monitor blood pressure and heart rate and for intravenous access. Both vagus nerves were electrically stimulated at 10 V, 2–25 Hz, 0.2-millisecond pulse duration, 5-second train duration at 1-minute intervals. ACh was administered intravenously at 1, 3, and 10 μg/kg. Bronchoconstrictions were measured as an increase in pulmonary inflation pressure (mm H_2O) over baseline ventilator pressure.

Bronchoalveolar Lavage and Blood

Total leukocytes were counted in bronchoalveolar lavage (BAL) fluid and lysed arterial blood. Differential counts were obtained from BAL cytospins or blood smears stained with Hemacolor (EMD Chemicals, Inc., Darmstadt, Germany).

AChE Assay

PBS-perfused brain and heparinized blood were collected to measure AChE activity using the Ellman assay (31), as previously described (16).

Histology

Protocols to detect eosinophils with chromotrope 2R (Sigma-Aldrich), nerves with an antibody to protein gene product (PGP) 9.5 (AbD Serotec, Raleigh, NC), and major basic protein (MBP) with an antibody (generously provided by G. J. Gleich, University of Utah, Salt Lake City, UT) in guinea pig lung have been previously published (14, 32, 33). Eosinophils were quantified in five to six airways per animal, as previously described (14). The area of MBP staining was quantified by setting a threshold using Metamorph 7.0 software (Molecular Devices, Sunnyvale, CA). MBP staining intensity in nerve bundles (5–10 per animal) was quantified using the same threshold setting for every image. MBP staining or intensity was normalized to the total area of tissue analyzed.

Statistical Analysis

Bronchoconstriction and bradycardia were analyzed by two-way ANOVA with repeated measures. AChE, BAL, blood, baseline animal measurements, and histology were analyzed within nonsensitized or sensitized groups by one-way ANOVA with a Bonferroni *post hoc* test, whereas controls in nonsensitized and sensitized groups were compared using a two-way *t* test. Data are expressed as the mean (±SEM). A *P* value of 0.05 or less was considered statistically significant.

Results

The OP, Chlorpyrifos, Causes Airway Hyperreactivity in Both Nonsensitized and Sensitized Guinea Pigs, and This Effect Is IL-5 Mediated in Sensitized Animals

Baseline pulmonary inflation pressure, heart rate, and blood pressure were not different between chlorpyrifos-treated and vehicle (ethanol/peanut oil) control animals, and sensitization did not influence these physiological parameters in either treatment group (*see* Table E1 in the online supplement). At the time of airway physiology measurements, on average, the sensitized animals that received 70 mg/kg chlorpyrifos weighed slightly, but significantly, less than the respective controls (Table E1). This was not due to a significant weight loss after chlorpyrifos injection (controls gained 6.6 ± 2.5 g overnight, whereas chlorpyrifos-treated animals gained 3.1 ± 2.9 g overnight). By chance, sensitized guinea pigs randomly selected for vehicle injections weighed significantly more than those selected for chlorpyrifos injection (vehicle-injected animals, 396.1 ± 10.6 g versus 70 mg/kg chlorpyrifos-injected animals 351.9 ± 7.1 g). There were no other changes in baseline measurements (Table E1).

Electrical stimulation of the vagus nerves caused a frequency-dependent increase in bronchoconstriction (Figures 1A, 1B, 1E, and 1F), and increasing concentrations of ACh (intravenous) caused a dose-dependent increase in bronchoconstriction (Figures 1C, 1D, 1G, and 1H). In sensitized guinea pigs that received the ethanol/peanut oil vehicle, bronchoconstrictions in response to vagal stimulation (Figure 1A versus Figure 1B) and to intravenous ACh (Figure 1C versus Figure 1D) were significantly higher than those in nonsensitized animals that received the same vehicle. This increase in airway responsiveness with ovalbumin sensitization has not been previously observed (14, 34). To determine whether the increase in airway responsiveness with sensitization was due to the ethanol/peanut oil vehicle, lung function was measured in sensitized animals that received no vehicle. Airway physiology measurements in sensitized guinea pigs that did not receive vehicle (*n* = 4) were similar to sensitized guinea pigs that received vehicle (bronchoconstriction at 25 Hz was 350 ± 44.3 mm H_2O in sensitized animals not injected with vehicle versus 337 ± 22.9 mm H_2O in sensitized animals injected with ethanol/peanut oil vehicle), demonstrating that it was not the vehicle, but rather sensitization, that caused a slight, but significant, increase in airway responsiveness.

All doses of chlorpyrifos (0.7, 7, and 70 mg/kg) significantly increased vagally and ACh-induced airway hyperreactivity in nonsensitized guinea pigs (Figures 1A and 1C). Chlorpyrifos at 70 mg/kg significantly increased vagally and ACh-induced bronchoconstrictions in sensitized guinea pigs, and this effect was significantly greater in sensitized than in nonsensitized guinea pigs injected with the same dose of chlorpyrifos, even taking into consideration the increase in airway responsiveness in sensitized animals (Figures 1B and 1D). However, the lower doses of chlorpyrifos had no effect on vagally and ACh-induced airway hyperreactivity in sensitized animals. Vagally induced and ACh-induced bradycardia were not affected by either sensitization or by chlorpyrifos treatment (Figures E1A–E1D). Blood AChE activity was only inhibited in sensitized animals treated with the highest dose of chlorpyrifos (Figure 1I), and brain AChE activity was not affected in any of the treatment groups (Figure 1J).

To determine whether the mechanism of chlorpyrifos-induced airway hyperreactivity changed as a function of atopic status, both nonsensitized and sensitized guinea pigs were injected intraperitoneally with AbIL5 3 days before injection with chlorpyrifos. Pretreatment with AbIL5 prevented chlorpyrifos-induced airway hyperreactivity in sensitized animals (Figure 1F), but not in nonsensitized animals (Figure 1E). In nonsensitized animals, AbIL5 alone slightly, but significantly, potentiated vagally induced bronchoconstriction (compare *open circles* in Figures 1A and 1E; bronchoconstriction
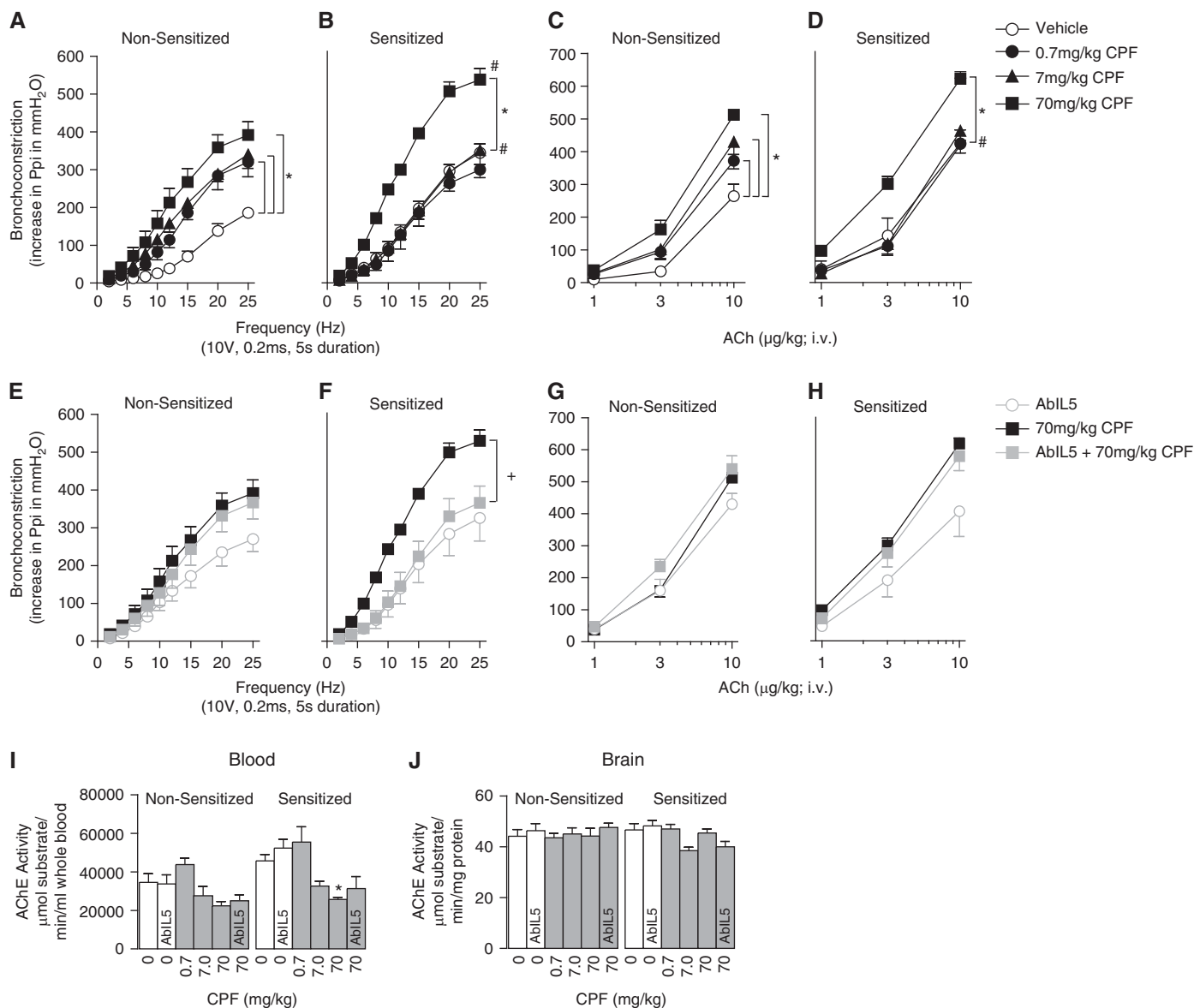
Figure 1. Airway physiology and acetylcholinesterase (AChE) activity in nonsensitized and sensitized guinea pigs treated with the organophosphorus pesticide (OP) chlorpyrifos (CPF) in the presence or absence of antibody to IL-5 (AbIL5). Vagally induced (*A*, *B*, *E*, and *F*) and acetylcholine (ACh)-induced (*C*, *D*, *G*, and *H*) bronchoconstrictions were measured in nonsensitized (*A*, *C*, *E*, and *G*) and sensitized (*B*, *D*, *F*, and *H*) animals 24 hours after subcutaneous injection of 0.7–70 mg/kg CPF. A subset of animals was injected intraperitoneally with AbIL5 3 days before exposure to CPF at 70 mg/kg subcutaneous (*E–H*). Data from animals injected with CPF at 70 mg/kg shown in *E–H* are the same data shown in *A–D*. AChE activity was measured in blood (*I*) and brain (*J*) collected from guinea pigs immediately after physiological studies were concluded. Data are presented as mean ± SEM. *$P < 0.05$ compared with respective controls, #$P < 0.05$ compared with equivalent treatment in nonsensitized animals, +$P < 0.05$ compared with sensitized/70 m/kg CPF. i.v., intravenous; Ppi, pulmonary inflation pressure.

at 25 Hz was 186 ± 13.1 mm H_2O in nonsensitized control animals versus 270 ± 33.4 mm H_2O in nonsensitized animals treated with AbIL5; $P < 0.01$). AbIL5 alone or in combination with 70 mg/kg chlorpyrifos had no effect on vagally mediated bradycardia (Figures E1E and E1F). Potentiation of ACh-induced bronchoconstriction by chlorpyrifos was

not prevented by AbIL5 in nonsensitized or sensitized guinea pigs (Figures 1G and 1H). Similarly, AbIL5 had no effect on ACh-induced bradycardia in sensitized animals (Figures E1G and E1H). AbIL5 treatment did not affect AChE activity in the blood or brain tissue of control animals or animals treated with chlorpyrifos (Figures 1I and 1J).

Sensitization Does Not Alter Airway Hyperreactivity Caused by the OP, Diazinon

In sensitized control animals, baseline heart rate was significantly decreased compared with nonsensitized control guinea pigs; however, sensitization had no effect on pulmonary inflation pressure and blood pressure (Table E1). Diazinon treatment
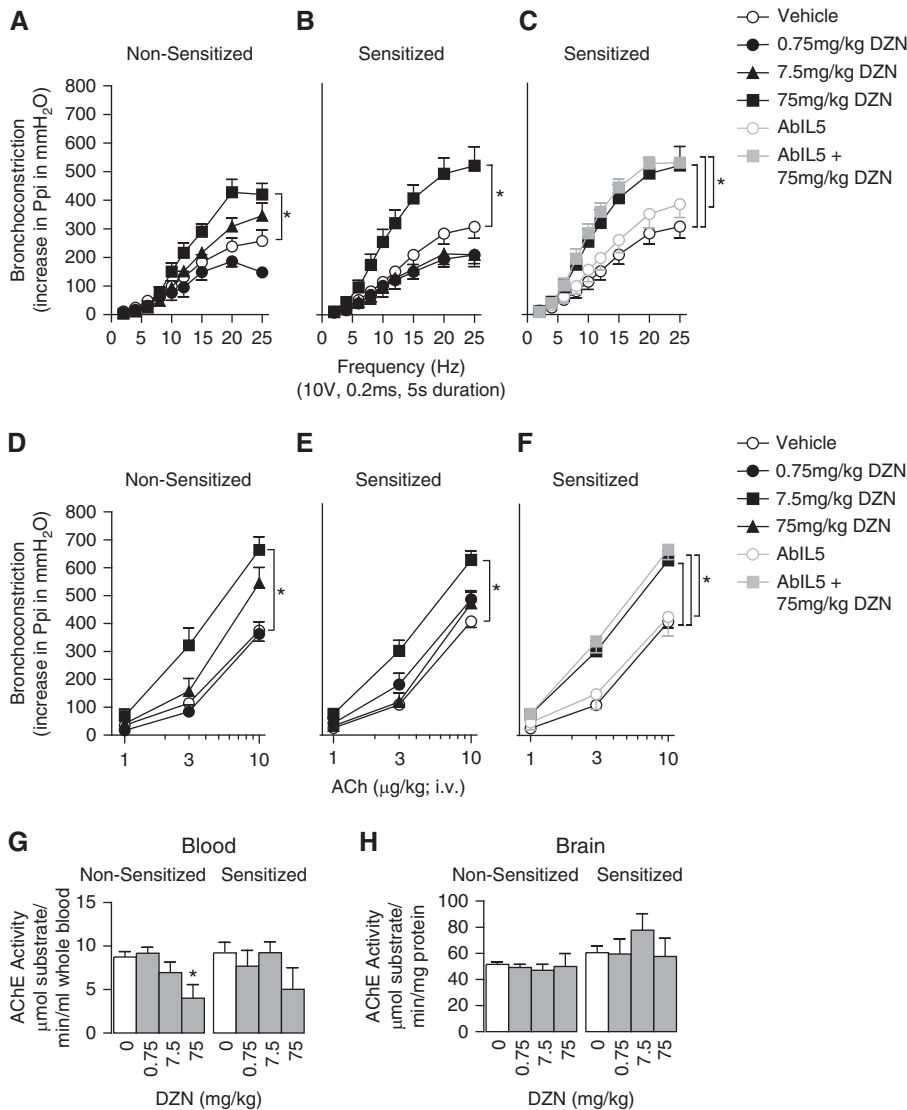
Figure 2. Airway physiology and AChE activity measurements in nonsensitized and sensitized guinea pigs treated with the OP diazinon (DZN). Vagally induced (*A–C*) and ACh-induced (*D–F*) bronchoconstrictions were measured in nonsensitized (*A* and *D*) and sensitized (*B, C, E,* and *F*) animals 24 hours after subcutaneous injection of DZN. Additional sensitized animals were injected intraperitoneally with AbIL5 3 days before DZN was administered subcutaneously at 75 mg/kg (*C* and *F*). AChE activity was measured in blood (*G*) and brain (*H*). Data are presented as mean ± SEM. *$P < 0.05$ compared with respective controls.

had no effect on these baseline measurements compared with vehicle (peanut oil) controls of the same atopic status (Table E1). Vagally induced bronchoconstrictions (Figures 2A and 2B) and ACh-induced bronchoconstrictions (Figures 2D and 2E) were not different between nonsensitized and sensitized vehicle control guinea pigs. In both nonsensitized (Figures 2A and 2D) and sensitized (Figures 2B and 2E) guinea pigs, 75 mg/kg diazinon

significantly potentiated vagally induced (Figures 2A and 2B) and ACh-induced (Figures 2D and 2E) bronchoconstrictions above vehicle controls of the same atopic status; however, ovalbumin sensitization did not further potentiate diazinon-induced airway hyperreactivity in response to either vagal stimulation or intravenous ACh. Although sensitization did not potentiate diazinon-induced airway hyperreactivity, additional experiments were performed

with sensitized animals to determine whether IL-5 mediated diazinon-induced airway hyperreactivity in sensitized animals. Pretreatment with AbIL5 did not prevent diazinon-induced airway hyperreactivity in sensitized animals (Figure 2C). In addition, potentiation of ACh-induced bronchoconstriction by 75 mg/kg diazinon was also not prevented by pretreatment with AbIL5 (Figure 2F). A lower dose of 0.75 mg/kg diazinon significantly increased ACh-induced bronchoconstrictions in sensitized animals compared with sensitized controls (Figure 2E). Vagally and ACh-induced bradycardia was not affected by any dose of diazinon in either nonsensitized or sensitized animals (Figure E2). AChE activity was measured in the blood and brain tissue (Figures 2G and 2H). AChE activity in the blood of nonsensitized animals was significantly inhibited only in the animals that received 75 mg/kg diazinon (Figure 2G), whereas brain AChE activity was unaffected in any treatment group (Figure 2H).

The Pyrethroid Permethrin Did Not Cause Airway Hyperreactivity in Either Nonsensitized or Sensitized Guinea Pigs

The same nonsensitized and sensitized control baseline and physiological measurements were used for the diazinon and permethrin experiments, because these animals were tested concurrently and the same vehicle (peanut oil) was used for pesticide injection. Permethrin treatment had no effect on baseline pulmonary inflation pressure, heart rate, or blood pressure in nonsensitized or sensitized guinea pigs (Table E1). At the time airway physiology measurements were performed, nonsensitized guinea pigs treated with 150 mg/kg permethrin weighed significantly more than nonsensitized controls (Table E1). Permethrin (15 and 150 mg/kg) did not increase vagally or ACh-induced bronchoconstriction in either nonsensitized or sensitized guinea pigs relative to vehicle controls of the same atopic status (Figures 3A–3D). Permethrin at 150 mg/kg significantly attenuated vagally induced bradycardia in sensitized guinea pigs (Figure E3B), but had no effect on vagally induced bradycardia in nonsensitized guinea pigs (Figure E3A) or on ACh-induced bradycardia in either nonsensitized or sensitized guinea pigs (Figures E3C
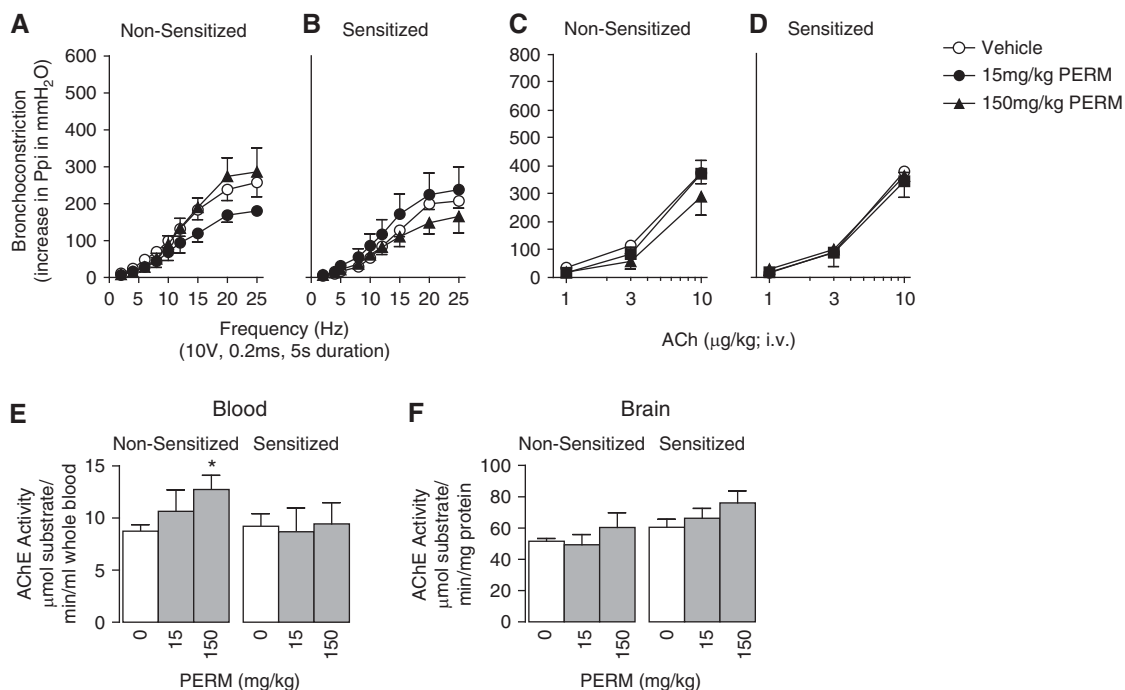
Figure 3. Airway physiology and AChE activity measurements in nonsensitized and sensitized guinea pigs treated with the pyrethroid permethrin (PERM). Vagally induced (*A* and *B*) and ACh-induced (*C* and *D*) bronchoconstrictions were measured in nonsensitized (*A* and *C*) and sensitized (*B* and *D*) animals 24 hours after subcutaneous injection of PERM. AChE activity was measured in blood (*E*) and brain (*F*). Data are presented as mean ± SEM. *$P < 0.05$.

and E3D). Permethrin at 150 mg/kg significantly increased AChE activity in the blood of nonsensitized guinea pigs (Figure 3F), but this increase in blood AChE activity did not correlate with a change in lung or cardiac physiology. Permethrin had no other effects on blood or brain AChE activity (Figures 3E and 3F).

AbIL5 Decreased Eosinophils and Eosinophil Activation in the Blood and Trachea of Sensitized Guinea Pigs

To determine whether sensitization increased chlorpyrifos-induced airway hyperreactivity via increased inflammation, BAL and blood samples were collected after measurements of airway physiology to obtain leukocyte cell counts. BAL macrophages and lymphocytes (Figures E4B and E4C, respectively) were significantly increased in sensitized control animals compared with nonsensitized control animals, whereas the total number of cells, neutrophils, and eosinophils in BAL fluid were not affected (Figures E4A, E4D, and E4E). The number of BAL neutrophils was significantly increased with 7 mg/kg chlorpyrifos in nonsensitized animals (Figure E4D). In nonsensitized animals that

received both 70 mg/kg chlorpyrifos and AbIL5, the total number of BAL cells increased (Figure E4A), in parallel with a significant increase in the number of macrophages (Figure E4B) and a nonsignificant increase in the number of lymphocytes (Figure E4C). In the blood, neither sensitization status nor chlorpyrifos had any effect on total cell counts or on differential cell counts of monocytes, lymphocytes, neutrophils, or eosinophils (Figures E4F–E4J). In sensitized animals, AbIL5 significantly decreased the number of blood eosinophils in both control animals and animals treated with 70 mg/kg chlorpyrifos (Figure E4J). Blood and BAL total cell counts and differential cell counts of macrophages/monocytes, lymphocytes, neutrophils, and eosinophils were not different between nonsensitized and sensitized animals, and were not altered by any dose of diazinon or permethrin (Table E2). Pretreatment with AbIL5 in sensitized animals that received 75 mg/kg diazinon significantly decreased the total number of cells and macrophages in the BAL fluid compared with sensitized controls (Table E2). AbIL5 significantly decreased the number of blood eosinophils and increased

the number of blood lymphocytes in sensitized animals that received 75 mg/kg diazinon (Table E2).

Because AbIL5 prevented the potentiation of vagally induced bronchoconstriction in sensitized animals treated with chlorpyrifos, but neither chlorpyrifos nor AbIL5 affected the number of eosinophils in the BAL fluid, we next determined whether chlorpyrifos affected either the number of eosinophils or the amount of MBP deposited around airway nerves in sensitized guinea pigs, and whether these parameters were altered by pretreatment with AbIL5. The number of eosinophils within 8 μm of airway nerves (Figure 4A) and the intensity of MBP staining around airway nerve bundles (Figure 4B) was not increased by chlorpyrifos treatment, and was not decreased by AbIL5 treatment in either control or chlorpyrifos-treated animals. Therefore, we next determined whether global changes in MBP distribution or the amount of MBP correlated with treatment-related changes in airway physiology. MBP quantification included both intracellular and extracellular MBP. The area of MBP staining in whole lung sections did not
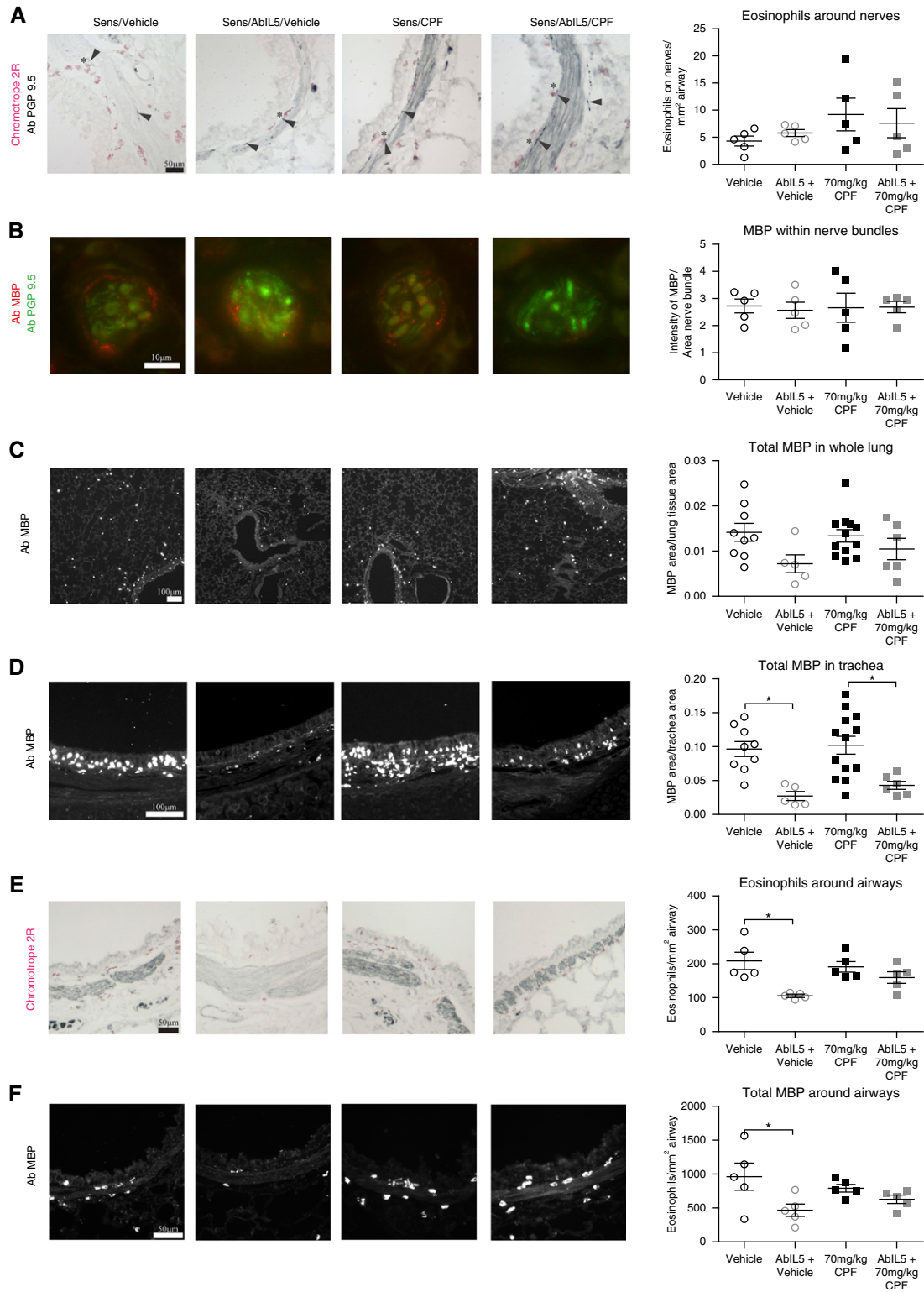
Figure 4. Quantification of the number of eosinophils and major basic protein (MBP) deposition in the lungs of sensitized guinea pigs exposed to 70 mg/kg CPF with or without pretreatment with AbIL5. All images are representative of those used for data collection. (*A*) The number of eosinophils (*red*) within 8 μm (*) of an airway nerve (protein gene product [PGP] 9.5, *dark gray*, indicated by *arrowheads*) were counted and normalized to the area of airway measured, which included the submucosal region under the basal lamina through to the connective tissue adjoining the alveoli, which includes airway smooth muscle. (*B*) The intensity of MBP immunoreactivity (*red*) was measured in airway nerve bundles labeled with PGP 9.5 (*green*). MBP intensity was normalized to area of nerve bundle. (*C*) The area of MBP staining was quantified in whole lung sections and normalized to total lung area analyzed (excluding luminal space). (*D*)

change with either chlorpyrifos or AbIL5 treatment (Figure 4C). In the trachea, the amount of MBP staining was not increased by chlorpyrifos treatment, but AbIL5 significantly decreased MBP staining in both control and chlorpyrifos-treated animals (Figure 4D). To determine whether the difference observed in the trachea was reflective of differences in the lower airways, the number of eosinophils and the area of MBP staining were quantified in lower airways. AbIL5 decreased eosinophils and MBP staining around airways in control animals, but not in chlorpyrifos-treated animals (Figures 4E and 4F, respectively).

Discussion

The goal of this study was to determine whether previous observations that sensitization shifted the dose–response relationship and altered the mechanism of parathion-induced airway hyperreactivity (14) could be generalized to other phosphorothioate OPs and other classes of pesticides—specifically, pyrethroids. The rationale for this study was that previous analyses have shown that different OPs exhibit different toxicological profiles at doses that elicit similar or no changes in AChE inhibition (26, 27, 35). Pesticides were delivered subcutaneously, because this method is thought to mimic dermal application with respect to the toxicokinetics of lipophilic compounds, such as OPs (16), and studies show that a primary route of human exposure to OPs is via the skin (9). Here, we observed that chlorpyrifos potentiated vagally and ACh-induced bronchoconstriction in nonsensitized guinea pigs independent of inhibition of AChE activity and independent of IL-5, a cytokine important for eosinophil maturation and recruitment (25). Sensitization exacerbated chlorpyrifos-induced airway hyperreactivity and changed the mechanism by which chlorpyrifos causes airway hyperreactivity to one that is dependent on IL-5. AbIL5 prevented chlorpyrifos-induced potentiation of bronchoconstriction in response to vagal stimulation, but not

to intravenous ACh, indicating that chlorpyrifos causes airway hyperreactivity via effects on the parasympathetic nerves, and not on airway smooth muscle. These findings are similar to those observed in nonsensitized and sensitized guinea pigs treated with parathion (14).

The highest dose of diazinon significantly increased both vagally and ACh-mediated bronchoconstriction in both nonsensitized and sensitized guinea pigs equally. Thus, unlike parathion and chlorpyrifos, sensitization may not change the mechanism by which diazinon causes airway hyperreactivity. This conclusion is corroborated by the observation that pretreating sensitized animals with AbIL5 did not prevent diazinon-induced airway hyperreactivity. The lowest dose of diazinon (0.75 mg/kg) did not cause airway hyperreactivity in nonsensitized guinea pigs, which is inconsistent with what we previously reported (16). One potential explanation for the discrepant dose–response relationship between studies is that male guinea pigs were used in the previous study (16), whereas female guinea pigs were used in this study. There are sex differences in the incidence of asthma (36, 37) and, similarly, there are sex differences in susceptibility to pesticides (38, 39).

Permethrin is a synthetic neurotoxin that prolongs sodium channel conductance, effectively interrupting normal nerve relays (40). We have previously shown that permethrin treatment does not increase vagally or ACh-mediated bronchoconstrictions in nonsensitized guinea pigs (16), and we now show that permethrin also does not increase vagally or ACh-mediated bronchoconstrictions in sensitized guinea pigs. However, in the heart, 150 mg/kg permethrin significantly reduced vagally mediated bradycardia in sensitized animals, but not in nonsensitized animals. The role of sensitization in modulating this permethrin effect in the heart is not clear; however, pyrethroid insecticides have been shown to disrupt normal cardiac conduction by interrupting sodium channel activation (41). Permethrin has also been shown to be immunotoxic (42); however, permethrin did not cause an inflammatory response in the BAL fluid or

blood. Thus, permethrin, at the doses we used, does not appear to have deleterious effects on lung function and inflammation in either nonsensitized or sensitized guinea pigs; however, it may affect cardiac function in sensitized animals.

Like parathion (14), but unlike diazinon and permethrin, sensitization potentiated the effect of chlorpyrifos, so the role of IL-5 and eosinophils was measured in chlorpyrifos-treated guinea pigs. Blocking IL-5 prevented chlorpyrifos-induced airway hyperreactivity in sensitized, but not in nonsensitized, animals. IL-5 is a key cytokine in the regulation of eosinophil recruitment and maturation (25). Sensitization recruits eosinophils to airway nerves (34), and, upon subsequent antigen challenge, eosinophils release MBP (43), which binds to and antagonizes M2 muscarinic receptors on parasympathetic nerves in the airways, resulting in airway hyperreactivity (18, 30). In this study, chlorpyrifos treatment, apparently did not act as a "challenge" to stimulate eosinophil release of MBP, because MBP deposition in the airways was not increased. In addition, heparin, which binds to and neutralizes MBP and restores airway reactivity in antigen-challenged guinea pigs (30), did not reduce vagally induced bronchoconstriction in sensitized chlorpyrifos-treated animals ($n = 3$; data not shown). Combined with the histology data, this suggests that MBP is not the mediator of chlorpyrifos-induced airway hyperreactivity in sensitized animals. Furthermore, like parathion (14), chlorpyrifos did not increase eosinophils in lung tissue, BAL fluid, or blood. As expected, AbIL5 reduced eosinophils and MBP staining in lungs of sensitized control animals; however, AbIL5 did not decrease eosinophils or MBP staining in lungs of sensitized animals that received 70 mg/kg chlorpyrifos. Only in the trachea did AbIL5 reduce MBP staining in chlorpyrifos-treated, sensitized animals. How MBP deposition in trachea impacts lower-lung function is not known. Because the histology data indicate that chlorpyrifos did not increase eosinophils or MBP staining in lung, whereas chlorpyrifos-induced airway hyperreactivity was prevented by AbIL5, it

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Figure 4. (Continued). The area of MBP immunoreactivity was quantified in epithelium and underlying connective tissue in the trachea and then normalized to the total area analyzed. (*E*) The number of eosinophils (*red*) located between the basement membrane and either the cartilage or alveoli were quantified and normalized to the total area analyzed. (*F*) The area of MBP immunoreactivity was quantified as in *E*. Data are presented as mean ± SEM. *$P < 0.05$.

is suggested that chlorpyrifos does not recruit eosinophils or increase MBP release, but instead may affect resident eosinophils to release unidentified mediators of airway hyperreactivity other than MBP. Alternatively, AbIL5 may prevent chlorpyrifos-induced airway hyperreactivity independent of eosinophils. IL-5 receptors are present on other inflammatory cells (44, 45) and airway smooth muscle (46), and, thus, prevention of chlorpyrifos-induced airway hyperreactivity by AbIL5 could be due to effects on cells other than eosinophils.

Although the OPs are delivered systemically, not all systems innervated by parasympathetic nerves are affected. M2 muscarinic receptors are also located on parasympathetic nerves that supply the heart (47). Neither chlorpyrifos nor diazinon had any effect on vagally or ACh-induced bradycardia (14). M2 and M3 muscarinic receptors are differentially regulated in different tissues (48, 49). Similar to what we observed with parathion (14), data presented here suggest that lung parasympathetic nerves are more sensitive to OPs than cardiac nerves.

We present data in guinea pigs that show that a single, subcutaneous exposure to a low dose of OP causes airway hyperreactivity 24 hours later, and that these effects varied depending upon the specific OP used and the atopic phenotype of the animal. Multiple epidemiological studies have shown a positive association of pesticide exposure with deleterious effects on human health, particularly in farmers who are routinely exposed to OPs (8) and in children who are more susceptible to OP exposure because of metabolic and behavioral differences that influence their exposures (50). Cumulatively, the data presented here underscore the importance of understanding the mechanism(s) of pesticide exposure, particularly in different human populations. Understanding OP effects and mechanism(s) of toxicity will be critical to predicting physiological response to OP exposure and to designing better therapeutic interventions. ■

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