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Glyphosate-rich air samples induce IL-33, TSLP and generate IL-13 dependent airway inflammation

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ABSTRACT

Several low weight molecules have often been implicated in the induction of occupational asthma. Glyphosate, a small molecule herbicide, is widely used in the world. There is a controversy regarding a role of glyphosate in developing asthma and rhinitis among farmers, the mechanism of which is unexplored. The aim of this study was to explore the mechanisms of glyphosate induced pulmonary pathology by utilizing murine models and real environmental samples. C57BL/6, TLR4−/−, and IL-13−/− mice inhaled extracts of glyphosate-rich air samples collected on farms during spraying of herbicides or inhaled different doses of glyphosate and ovalbumin. The cellular response, humoral response, and lung function of exposed mice were evaluated. Exposure to glyphosate-rich air samples as well as glyphosate alone to the lungs increased: eosinophil and neutrophil counts, mast cell degranulation, and production of IL-33, TSLP, IL-13, and IL-5. In contrast, in vivo systemic IL-4 production was not increased. Co-administration of ovalbumin with glyphosate did not substantially change the inflammatory immune response. However, IL-13-deficiency resulted in diminished inflammatory response but did not have a significant effect on airway resistance upon methacholine challenge after 7 or 21 days of glyphosate exposure. Glyphosate-rich farm air samples as well as glyphosate alone were found to induce pulmonary IL-13-dependent inflammation and promote Th2 type cytokines, but not IL-4 for glyphosate alone. This study, for the first time, provides evidence for the mechanism of glyphosate-induced occupational lung disease.

1. Introduction

Many low-molecular weight chemicals including some pesticides and herbicides are capable of inducing occupational asthma (Henneberger et al., 2014). Glyphosate [(*N*-phosphonomethyl) glycine] is one of the most commonly used broad spectrum nonselective herbicides in the world. Approximately 83,000 t of it was applied to agricultural fields and approximately 3000 t was applied to the lawns and garden areas of homes in the United States (USA EPA, 2007).

Since glyphosate was brought to market in the 1970s as the active ingredient in the formulation of Roundup®, several animal studies have investigated the toxicity of glyphosate when administered by intravenous, oral, intraperitoneal, dermal, and ocular routes (Tai et al., 1990; Agriculture Canada, 1991; Cox, 1995). Its gastrointestinal toxicity to humans was also documented (Sawada et al., 1988; Talbot et al., 1991; Tominack et al., 1991; Menkes et al., 1991; Temple and Smith, 1992). According to the SAR (structure–activity relationships) model of Jarvis et al. (2005), the hazard index value of glyphosate is 0.6257, which evidently supports the hazardous nature of glyphosate and its possible role in inducing asthmatic symptoms. However, the inhalational effects of glyphosate particularly its effect on development of asthma was not entirely explored.

Because experimental asthma has been largely studied using various proteins as disease mediators, our understanding of asthma pathogenesis relies heavily on adaptive immune responses. The understanding of the induction of allergic pathology caused by small molecules like glyphosate is challenging due to a fundamentally distinct immune response that may not be

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obtainable like adaptive immune responses from environmental allergens of high molecular weights. Given that innate immune responses to stimuli are specific to the anatomic site involved, those animal studies that administered glyphosate to the airway would be best suited to provide insight into the pathogenesis of airway disease produced in agricultural workers. To our knowledge, the inhalational hazards of glyphosate have been studied experimentally by two groups (US EPA, 1982; Martinez et al., 1990). It was shown that glyphosate inhalation caused wheezing, reduced activity, and dark nasal discharge even at low exposure levels in rats. How these small molecules contribute to the development of these phenotypes remains a mystery. We hypothesized that exposure to air pollutant containing herbicides, endotoxin or other environmental contaminants induces airway inflammation by activation of the innate immune system through pattern recognizing innate pro-cytokines that contribute to the airway pathology. Here we report the exploration of the mechanism behind the airway inflammation caused by agricultural air samples containing glyphosate, endotoxin, and other environmental contaminants as well as reagent-grade glyphosate delivered at low and high doses in the presence or absence of exogenous antigen.

2. Materials and methods

2.1. Mice

C57BL/6 female (6–9 weeks) mice were purchased from Jackson Laboratory (Sacramento, CA). TLR4−/− mice (backcrossed 10 generations) were received from Cincinnati Children's Hospital Medical Center (CCHMC). Both strains were subsequently bred in house. Female mice of wild type and IL-13−/− BALB/c background were received from the laboratory of Dr. Fred Finkelman, CCHMC. Mice were housed in individually ventilated cages in a pathogen free facility at the Department of Environmental Health, University of Cincinnati (UC) following the UC Institutional Animal Care and Use Committee (IACUC) guidelines and all experiments were conducted following a UC IACUC – approved protocol.

2.2. Antibodies and reagents

We purchased the following antibodies for flow cytometry: Ly-6G (Gr-1) eFluor® 450 (RB6-8C5; Isotype Rat IgG2b) from eBioscience (San Diego, CA). CD16/CD32 (2.4G2; Isotype Rat IgG2b) and SiglecF-PE (E50-2440; Isotype Rat IgG2a) were purchased from BD PharMingen (San Jose, CA). A kit for measuring serum levels of MMCP1 was purchased from eBioscience.

2.3. Collection of farm air samples during summer pesticide spray seasons

Air samples were collected by three sets of total inhalable aerosol samplers (Button Inhalable Aerosol Sampler, SKC Inc., Eighty Four, PA) operated in parallel on three farms in Butler County, Ohio during summer glyphosate spray seasons. Samplers were installed at 1.5 m height at the edge of the field downwind from the spraying locations (sizes: approx. 5000–10,000 m^2). The sampling period was approximately 24 h starting from glyphosate spraying and air samples were collected at a flow rate of approximately 4 l/min on glass fiber filters. The filters from one set of samplers containing aerosolized glyphosate were eluted using PBS and the suspensions were filtered. A stock solution was prepared by pooling the samples collected from three farms (from now on referred as 'Real Env.') and used for intranasal treatment of mice. The filters from the other two sets of samplers were analyzed for glyphosate and endotoxin to estimate the levels of glyphosate and endotoxin in 'Real Env.' samples.

2.4. Analysis of glyphosate in filter extracts

Glyphosate residues from filters were extracted using KH_2PO_4 buffer/1 M NaOH in an automatic shaker followed by freeze drying. The freeze dried samples were dissolved with deionized water and filtered through 0.45 μM Millipore filter. Glyphosate levels in the suspensions were determined by Abraxis ELISA Kit at 450 nm. The average amount of glyphosate per filter was 17.33 μg, which correspond to average airborne concentration of 22.59 ng/m^3.

2.5. Analysis of endotoxin in filter extracts

Endotoxin in filter extracts were analyzed using the Limulus amebocyte lysate assay (Pyrochrome LAL; Associates of Cape Cod Inc., Falmouth, MA), as described previously (Adhikari et al., 2009, 2010). The samples were spiked with endotoxin standard of 0.50 EU/ml to assure that there was no inhibition or enhancement between the filter extracts and the reagents. The average amount of endotoxin per filter was 24.49 EU, which correspond to average airborne concentration of 4.87 EU/m^3.

2.6. Treatment of mice with farm-derived air samples, glyphosate and sensitization with OVA

PBS suspended farm air sample ('Real Env.'; estimated amount of glyphosate: 8.66 μg/ml) and reagent grade glyphosate (Sigma–Aldrich, St. Louis, MO) (100 ng, 1 μg or 100 μg) were delivered (in 30 μl) to the nose of anesthetized mice which were witnessed to aspirate the solution. Treatments were administered either: daily for 7 days or 3 times a week for 3 weeks. Same exposure schedule was followed for OVA alone (100 μg) and for OVA (100 μg) plus different dose of farm air sample and glyphosate. Mice were sacrificed 24 h after final airway treatment with sodium pentobarbital.

2.7. Histological analysis of lung

Formalin-fixed paraffin embedded lung sections (5 μm thick) were prepared for H&E and chloroacetate esterase (CAE) staining. The entire histological slide from each mouse was examined in blinded fashion and given a global categorical severity score based on infiltration of cells into parenchymal, peribronchial, and perivascular regions of lungs.

2.8. Immunohistochemical staining

To analyze IL-33 and TSLP expression in the lungs section, the following antibodies were used for immunostaining: mouse IL-33 (0.2 mg/ml, AF3626, R&D Systems, Minneapolis, MN); mouse TSLP biotinylated (0.2 mg/ml, BAF555, R&D Systems) and respective isotype controls (R&D Systems). IL-33 and TSLP antibody–antigen complex were detected using Cy3 donkey anti-goat IgG (1:10,000) (Invitrogen/Molecular probes, Grand Island, NY). Slides were counterstained with DAPI (Vector Labs, Burlingame, CA). Images were obtained using a Nikon A1R Si microscope.

2.9. Isolation of lung inflammatory cells

Lungs were perfused with PBS, removed, manually minced into 1–2 mm fragments and then placed in Hank's Balanced Salt Solution (Sigma–Aldrich) containing Liberase TL (50 μg/ml; Roche Diagnostics, Indianapolis, IN) and DNase I (0.5 mg/ml; Sigma–Aldrich). Tissue was digested at 37 °C in a CO_2 incubator for