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Muramic Acid, Endotoxin, 3-Hydroxy Fatty Acids, and Ergosterol Content Explain Monocyte and Epithelial Cell Inflammatory Responses to Agricultural Dusts

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MURAMIC ACID, ENDOTOXIN, 3-HYDROXY FATTY ACIDS, AND ERGOSTEROL CONTENT EXPLAIN MONOCYTE AND EPITHELIAL CELL INFLAMMATORY RESPONSES TO AGRICULTURAL DUSTS

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In agricultural and other environments, inhalation of airborne microorganisms is linked to respiratory disease development. Bacterial endotoxins, peptidoglycans, and fungi are potential causative agents, but relative microbial characterization and inflammatory comparisons amongst agricultural dusts are not well described. The aim of this study was to determine the distribution of microbial endotoxin, 3-hydroxy fatty acids (3-OHFA), muramic acid, and ergosterol and evaluate inflammatory responses in human monocytes and bronchial epithelial cells with various dust samples. Settled surface dust was obtained from five environments: swine facility, dairy barn, grain elevator, domestic home (no pets), and domestic home with dog. Endotoxin concentration was determined by recombinant factor C (rFC). 3-OHFA, muramic acid, and ergosterol were measured using gas chromatography–mass spectrometry. Dust-induced inflammatory cytokine secretion in human monocytes and bronchial epithelial cells was evaluated. Endotoxin-independent dust-induced inflammatory responses were evaluated. Endotoxin and 3-OHFA levels were highest in agricultural dusts. Muramic acid, endotoxin, 3-OHFA, and ergosterol were detected in dusts samples. Muramic acid was highest in animal farming dusts. Ergosterol was most significant in grain elevator dust. Agricultural dusts induced monocyte tumor necrosis factor (TNF) α, interleukin (IL)-6, IL-8, and epithelial cell IL-6 and IL-8 secretion. Monocyte and epithelial IL-6 and IL-8 secretion was not dependent on endotoxin. House dust(s) induced monocyte TNFα, IL-6, and IL-8 secretion. Swine facility dust generally produced elevated responses compared to other dusts. Agricultural dusts are complex with significant microbial component contribution. Large animal farming dust(s)-induced inflammation is not entirely dependent on endotoxin. Addition of muramic acid to endotoxin in large animal farming environment monitoring is warranted.

Inhalation of components of airborne microorganisms as found in organic dusts from various environmental settings, particularly agricultural environments, may lead to adverse moderate respiratory health effects including asthma, chronic bronchitis, chronic obstructive disease, and hypersensitivity pneumonitis (Von Essen et al., 1999). Microbial agents from

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gram-negative bacteria (endotoxins, lipopolysaccharide [LPS]), gram-positive bacteria (peptidoglycan, PGN), and fungi are among potential causative agents. Endotoxin is routinely measured and is linked to inflammatory outcomes, but there is disagreement as to the association of endotoxin and disease in exposed workers. Several studies attempted to establish dose-response relationships between endotoxins and respiratory diseases (Schwartz et al., 1995; Reynolds et al., 1996; Donham et al., 2000; Vogelzang et al., 1998; Milton et al., 1996 ; Park et al., 2001), but a universal dose-response relationship has not been developed since some studies reported high exposure without symptoms (Rask-Andersen et al., 1989) or low exposure with a possible dose-response relationship (Kateman et al., 1990).

PGN is a microbial agent also recognized to induce inflammatory responses. Muramic acid (chemical marker of PGN) was shown to correlate with inflammatory outcomes in exposed workers in European swine barns (Zhiping et al., 1996; Larsson et al., 1999). However, it has not been systematically studied in U.S. agricultural environments. Fungi (chemical marker is ergosterol) also exist in agricultural environments. The relative distribution and combination of these agents may explain disease manifestation.

In the midwestern United States, exposure to organic aerosols in grain elevators, swine confinement facilities, and dairy farms was associated with chronic obstructive pulmonary disease (Bailey et al., 2007). Our study focusing on organic dust from swine confinement facilities showed that non-endotoxin components in the dust mediate bronchial epithelial cell inflammatory responses (Romberger et al., 2002), and that this dust regulates the expression of the gram-positive ligand receptor Toll-like receptor-2 (TLR2; (Bailey et al., 2008). Although endotoxin induces inflammation in mononuclear phagocytes, the non-endotoxin components in the swine facility dust appear to be driving monocyte, macrophage, and dendritic cell innate immune inflammatory responses (Poole et al., 2007, 2008, 2009). Because the swine facility dust has high levels of muramic acid, it has been speculated that gram-positive microbial components are important in the development of swine facility dust-induced chronic respiratory diseases (Poole et al., 2008). However, distribution of muramic acid levels in other U.S. farming environments is not well known.

In the United States, there are currently no established occupational guidelines and controls for endotoxin exposure. However, among workers in swine and poultry production facilities the suggested recommended limits for endotoxin exposure are 614 EU/m^3 and 900 EU/m^3, respectively (Donham et al., 1995, 2000, 2002; Reynolds et al., 1996). The levels of muramic acid and ergosterol in various environmental settings (livestock, animal confinement facilities, grain elevators) have not been well determined. This information may be critical in establishing future guidelines and controls for reducing disease. As traditional culture methods to quantify microbial exposures have proven to be of limited use, non-culture methods and assessment for microbial constituents are recommended (Douwes et al., 2003). Applied gas chromatography–mass spectrometry (GC-MS) methods that provide high detection selectivity are one such way to characterize these complex dust samples (Sebastian et al., 2003).

This study aimed to (1) investigate dust samples from differing agricultural environments (swine confinement facility, dairy barn, grain elevator) as compared to domestic homes for their respective inflammatory effects in human monocytes and bronchial epithelial cells, and (2) measure endotoxin, 3-OHFA, muramic acid, and ergosterol utilizing a chemical-analytical approach. It was hypothesized that these dusts may differ in microbial community chemical composition and ability to induce cellular inflammatory responses.

METHODS

Chemicals and Equipment

All organic solvents were high-performance liquid chromatography (HPLC) grade. Acetone,

methyl *tert*-butyl ether, diethyl ether, and hexane were purchased from Fisher Scientific (Fair Lawn, NJ). Methanol and heptane were obtained from Mallinckrodt (Phillipsburg, NJ). Potassium hydroxide, ammonium hydroxide, paraffin oil, pentadecanol, and pyridine were purchased from Sigma Aldrich (St. Louis, MO). Bis(trimethylsilyl)-trifluoroacetamide (BSTFA) was purchased from Regis (Morton Grove, IL). Lyophilized C_{13} labeled algal cells were purchased from Isotec (Miamiburg, OH). Endotoxin-free water was obtained from Lonza (Basel, Switzerland). Strata-X and Strata-XC solid-phase cartridges were purchased from Phenomenex (Torrance, CA).

3-OHFA, muramic acid, and ergosterol were separately analyzed with a Waters Quattro Micro GC-MS/MS system operated in the electron ionization (EI) positive mode. The carrier gas was ultra-high-purity helium at a head column pressure of 69 kPa. Injections were made using an Agilent 7683B autosampler in the splitless mode onto a DB-5ms capillary column (30 mm × 0.25 mm, 0.25 μm film thickness; J&W Scientific, Santa Clara, CA).

Dust Sample Collection and Preparation

Settled surface dust was collected, using the Omega HEPA vacuum with a dust collection filter sock (Midwest Filtration Company, Cincinnati, OH), during the month of September 2008 from five different locations: floor of the operating facility of a swine confinement facility (housing approximately 400–600 hogs), floor of the feeding barn of a dairy farm (approximately 50 in the building sampled, 2500 total on the facility), floor of the storage facility of a grain elevator (feed grain/feed mill), domestic home without pets (pets never allowed during home ownership), and domestic home with pet, dog (~60-lb dog). House dust was collected in the carpeted living room. In each facility three 1-m^2 areas were sampled, vacuuming the whole area in 2 orthogonal passes. Dust samples in the Filter Sock were immediately placed in sterile Whirl-Pak bags (Nasco, Ft. Atkinson, WI). Samples were stored at –20°C.

For chemical marker analysis studies, dust samples (5 mg) were extracted with 10 ml of 0.05% TWEEN-20 by vortexing for 1 min followed by 1 h at 100 rpm on a rotary mixer at 20°C. The Tween extraction solution was then aliquoted for separate analysis of endotoxin (rFC), 3-OHFA (GC-MS/MS), muramic acid (GC-MS/MS), and ergosterol (GC-MS). Samples for mass spectrometry were not centrifuged before aliquoting. Individual aliquots were stored at –80°C, then lyophilized prior to sample digestion for mass spectrometry. Although Tween extraction is standard practice for mass spectrometry analysis, Tween is cellular toxic and thus not recommended for cell culture studies. All samples were analyzed in triplicate (i.e., three 5-mg extracts were used from each dust sample).

For cell culture studies, the dust samples were placed into solution and sterile filtered by a standard published procedure (Romberger et al., 2002). Briefly, 100 mg of each dust sample was placed in 1 ml Hanks balanced salt solution (HBSS) without calcium (Biofluids). The mixture was vortexed for 1 min and allowed to stand at room temperature for 1 h. The mixture was centrifuged at 1200 × g for 10 min, and the supernatant was recovered and centrifuged again. The final supernatant was filter (0.22 μm) sterilized. Individual aliquots were stored at –80°C prior to cell culture studies. HBSS extractions were also analyzed by rFC and mass spectrometry.

Endotoxin Bioassay

Analysis of endotoxin was performed using the recombinant factor C (rFC) assay developed by Lonza (Walkersville, MD) using a reference of endotoxin activity in endotoxin units (EU) relative to a standard reference LPS (*Escherichia coli* O55:B5). The activation of rFC is determined by the fluorescence generated by the enzymatic cleavage of a peptide–coumarin substrate. Endotoxin concentrations of samples were calculated according to the standard curve. Lower limit of quantification of this assay is 0.01 EU/ml. Both Tween and HBSS extractions were analyzed using the rFC

bioassay. Endotoxin was not detected in HBSS and Tween blanks.

3-OHFA Analysis

GC-MS/MS analysis was modified from previous studies (Saito et al., 2009; Reynolds et al., 2005). To prepare samples for GC-MS/MS analysis of 3-hydroxy fatty acids (3-OHFA; marker of LPS), lyophilized dust samples and standards (C_8–C_{10} and C_{12}–C_{18} at 0, 1, 2, 5, 20, 100, and 500 ng) were spiked with 25 ng C_{11} as a surrogate and digested in 0.5 ml methanolic HCl overnight at 85°C. Samples were diluted with 1 ml of Lonza pyrogen-free water and spiked with 10 µl of 100 µg/ml pentadecanol prior to solid-phase extraction (SPE). Strata-X (60 mg/3 ml) polymeric reverse-phase columns were conditioned with 1 ml diethyl ether and 1 ml water. Samples were loaded onto the column and pulled through dropwise with vacuum. Cartridges were dried under full vacuum for 20 min. 3-OHFA was eluted from the column with diethyl ether and the eluent was dried under a gentle stream of nitrogen. Dried samples were incubated with 50 µl BSTFA/1% trimethylchlorosilane (TMCS) and 5 µl pyridine at 85°C for 30 min to form trimethylsilyl derivatives. Following derivatization, cooled samples were diluted to 100 µl with heptane for GC-MS/MS analysis.

3-OHFA were separated by gas chromatography with an inlet temperature of 280°C and an oven temperature profile of 90 to 250°C at 5°C/min, 250 to 290°C at 20°C/min, and holding 290°C for 5 min. The GC–mass spectrometer interface temperature was 300°C. The mass spectrometer was operated in multiple reactions monitoring (MRM) mode with fragment ions generated with collision energy of 10eV. The two MRM transitions (quantitation and confirmation) monitored for each 3-OHFA in their respective retention time windows were as follows: C_8 m/z 231 > 189, 131, C_9 m/z 245 > 203, 131, C_{10} m/z 259.02 > 217, 131, C_{11} m/z 273 > 241, 131, C_{12} m/z 287 > 255, 131, C_{13} m/z 301 > 269, 131, C_{14} m/z 315 > 283, 131, C_{15} m/z 329 > 297, 131, C_{16} m/z 343 > 311, 131, C_{17} m/z 357 > 325, 131, and C_{18} m/z 371 > 339, 131. Results are presented in picomoles 3-OHFA per milligram dust or per milliliter HBSS extract. 3-OHFA were not detected in Tween and HBSS blanks.

Muramic Acid Analysis

To prepare samples for GC-MS/MS analysis of muramic acid (marker of PGN), lyophilized samples and standards (0, 2, 5, 10, 50, 100, or 500 ng) were digested in 1 ml methanolic HCl overnight at 100°C. A solution of C_{13} muramic acid as an isotope dilution internal standard was obtained by digestion of 4 mg C_{13}-labeled algal cells (99% C_{13}) as for the samples. Prior to SPE, 30 µl of the C_{13} muramic acid solution was spiked into each sample and standard as an internal standard. SPE of the sample was done with Strata-XC (60 mg/3 ml) strong cation exchange columns conditioned with 2 ml methanol and 2 ml aqueous 0.1% H_3PO_4. Samples were loaded to the column with vacuum assistance and then dried under full vacuum for 20 min. Muramic acid was eluted from the column with 5% NH_4OH in acetone. Ten microlieters of 100-µg/ml pentadecanol was added to the eluent and then dried under a stream of nitrogen. Dried samples were incubated with 50 µl BSTFA/1% TMCS and 5 µl pyridine at 85°C for 30 min to form trimethylsilyl derivatives. Following derivatization, cooled samples were diluted to 100 µl with heptane for GC-MS/MS analysis.

Muramic acid was analyzed with an oven temperature profile of 120 to 290°C at 20°C/min, holding at 280°C for 4 min. The inlet temperature was 260°C and the GC–mass spectrometer interface temperature was set at 300°C. The mass spectrometer was operated in MRM mode with fragment ions generated with collision energy of 6 eV. The two MRM transitions monitored for muramic acid were m/z 185 > 142 and 185 > 130 and m/z 190 > 145 for C_{13} muramic acid. Results are presented in nanograms muramic acid per milligram dust or per milliliter of HBSS extract. Muramic acid was not detected in Tween and HBSS blanks.

Ergosterol Analysis

To prepare samples for GC-MS analysis of erogsterol (marker of fungal biomass), lyophilized

samples and standards (0, 1, 4, 10, or 40 ng) were spiked with 10 ng D2-ergosterol as an internal standard and digested in 3 ml of 10% methanolic KOH for 90 min at 80°C. SPE was done with Strata-X (60 mg/3 ml) polymeric reverse-phase columns conditioned with 2 ml methanol and 2 ml water. Samples were loaded to the column, pulled through dropwise with vacuum, and then dried under full vacuum for 20 min. Ergosterol was eluted from the column with 10% methanol in methyl *tert*-butyl ether. Twenty microliters of 0.1% paraffin oil in acetone was added to the eluent and then dried under a stream of nitrogen. Dried samples were incubated with 50 μl of 1:1 BSTFA/1% TMCS and hexane at 80°C for 30 min to form trimethylsilyl derivatives.

Ergosterol was analyzed with an oven temperature profile of 90 to 280°C at 20°C/min, holding 280°C for 15 min. The inlet temperature was 280°C and the GC–mass spectrometer interface temperature was set at 300°C. The mass spectrometer was operated in single-ion monitoring (SIM) mode targeting *m/z* 363, 337, and 378. For quantification, *m/z* 363 and 365 were monitored for ergosterol and D2-ergosterol, respectively. Results are presented as nanograms ergosterol per milligram dust or per milliliter HBSS extract. Ergosterol was not detected in Tween and HBSS blanks.

Cell Populations

To represent the inflammatory cytokine/chemokine response of lung mononuclear phagocytes (monocytes/macrophages), human peripheral blood monocytes were chosen. Populations of human peripheral blood monocytes were obtained from the UNMC Elutriation Core Facility. Briefly, monocytes were isolated by countercurrent centrifugal elutriation of mononuclear leukocyte-rich fractions of blood cells from healthy donors undergoing leukopheresis (Wahl et al., 1984) and were rested overnight prior to experimental assays. Elutriated monocytes were >99% pure as determined by flow cytometry of cell surface antigen expression of CD14. Peripheral blood was taken with written informed consent, and studies were approved by the Institutional Review Board. Human monocytes were maintained in complete RPMI, which consisted of L-glutamine-RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Biofluids), 2-mercaptoethanol (5×10^{-5} *M*), 50 μg/ml of streptomycin (Invitrogen, Carlsbad, CA), and 80 U/ml of amphotericin B (Invitrogen).

To represent airway epithelial cell inflammatory responses, the human bronchial epithelial cell line BEAS-2B (simian virus 40-immortalized; American Type Culture Collection, Manassas, VA) was chosen as it also allowed enough cells to conduct the described studies. Cells were cultured under serum-free conditions using a 1:1 medium mixture of RPMI 1640 medium (Invitrogen, Carlsbad, CA) and LHC-9 (prepared from LHC basal; Biofluids, Rockville, MD) on 1% type I collagen-coated 100-mm plates as previously described (Romberger et al., 2002). LHC-9 medium contains LHC basal medium (Biofluids), 0.5 μ*M* phosphoethanolamine-ethanolamine (Sigma, St. Louis, MO), 0.11 m*M* calcium (Fisher, Springfield, NJ), 50 U/ml penicillin and streptomycin (Life Technologies, Grand Island, NY), 2 μg/ml fungizone (Life Technologies), trace elements, 5 μg/ml bovine insulin (Sigma), 5 ng/ml epidermal growth factor (Sigma), 10 μg/ml bovine transferrin (Sigma), 10 n*M* 3,3′,5-triiodothyronine (Biofluids), bovine pituitary extract (50 μg protein/ml; Pel Freeze, Rogers, AR), 0.2 μ*M* hydrocortisone (Biofluids), 0.5 μg/ml epinephrine (Sigma), and 0.1 μg/ml retinoic acid (Sigma). Cells were maintained in culture at 37°C in humidified 95% air/5% CO_2 for 48–72 h before each experiment.

Cytokine/Chemokine Assays

Human peripheral blood monocytes and the human bronchial epithelial cell line were stimulated with the various HBSS dust extracts to determine inflammatory cytokine responsiveness. Separate dose and time-course studies determined that a 1% concentration of swine facility dust extract (generally most potent inflammatory agent) at 5 h postexposure elicited optimal monocyte tumor necrosis factor (TNF) α production with no evidence of

cellular toxicity (Poole et al., 2007). Thus, all HBSS dust extracts were diluted in the respective monocyte or epithelial culture medium to a final concentration of 1%. Although it is difficult to directly compare cell culture studies with real-world exposure, using endotoxin as a biomarker of exposure, a 1% concentration of swine facility dust extract equates to approximately 37.28 EU/mg of endotoxin (Table 1), and it was suggested that this corresponds to a "low" swine barn exposure condition (mean endotoxin ± SEM over 5 h, 452.3 ± 65.8 EU/m^3; Dosman et al., 2006). Cells were also incubated with polymyxin B (50 mg/ml; Sigma) plus HBSS dust extracts diluted to a final concentration of 1% to determine endotoxin-independent dust-induced inflammatory cytokine responsiveness (referred to as endotoxin-depleted HBSS dust extracts 1%). Human peripheral blood monocytes (1×10^6 cells/ml) in duplicate were challenged with swine confinement facility, dairy barn, grain elevator, house with dog, or house without pets HBSS dust extracts (1%) and media (control) for 5 h (optimal time) in adjacent experiments. Cell-free culture supernatant was harvested and stored at −20°C for later cytokine/chemokine assays. For airway epithelial cell studies, confluent monolayers of epithelial cells in 6-well cell culture plates were exposed to the HBSS dust extracts (1%) for 24 h, the optimal time to elicit cytokine production in adjacent experiments. Cell-free supernatant was then harvested and stored at −20°C for later cytokine/chemokine assays. Cell viability was assessed by trypan blue exclusion method after stimulation.

TNFα, interleukin (IL)-6, and CXCL8/IL-8 protein levels were assayed by sandwich ELISA as previously published (Poole et al., 2007). Monocyte cytokine/chemokine secretion was reported as concentration (pg/ml) per 1×10^6 viable cells. Epithelial cytokine/chemokine secretion was normalized to total protein (mg) of the cell layer using a Nanodrop spectrophotometer (Applied Biosciences) and reported as picograms per milligram per milliliter.

Statistical Analysis

Endotoxin levels determined by rFC bioassay were normalized to the total amount of dust sampled (EU/mg). Endotoxin's 3-OHFA (pmol/mg or pmol/ml), muramic acid (ng/mg or ng/ml), and ergosterol (ng/mg or ng/ml) concentrations were normalized to the total amount of dust sampled or to concentration in a 1% HBSS extract solution. The sum of all 3-OHFA constituents measured is reported and the percent of total 3-OHFA for carbon chain length is reported. Cytokine/chemokine data are presented as the mean ± standard deviation (SD). Differences in dust-stimulated inflammatory mediator secretion were compared to no stimulation (media control) and to

TABLE 1. Recombinant Factor C (rFC) Bioassay Results of Endotoxin Measurements of the Various Dusts (EU/mg), as Mean (Standard Deviation) for Each Dust Type and Extraction Type ($n = 3$)

	rFC (EU/mg)		
Dust type	Tween extract	HBSS extract	Polymyxin B-treated HBSS extract
Swine facility	1338.7 (176.4)[a]	372.8 (30.5)[a]	3.1 (1.1)
Dairy barn	433.5 (26.1)[b]	22.9 (4.0)	<LOD
Grain elevator	1130.3 (635.2)[a]	295.2 (29.3)[a]	13.8 (2.3)
House, dog	64.0 (19.0)	20.9 (2.1)	0.1 (0)
House, no pets	45.9 (7.3)	12.5 (1.1)	0.1 (0)

Note. LOD, limit of detection.

[a]Statistically difference ($p < .05$): Tween and HBSS swine facility and grain elevator extracts significantly greater endotoxin as compared to other dusts, but not different from each other.

[b]Statistical difference ($p < .05$): Tween dairy barn extract significantly greater than domestic house dust extracts.

each other. Differences in inflammatory responses between complete (untreated) dust and endotoxin-depleted (polymyxin B-treated) dusts were also made. Statistics were performed using one-way parametric analysis of variance (ANOVA) with post hoc Bonferonni test for multiple comparisons. To determine difference between complete (untreated) and endotoxin-depleted (polymyxin B-treated), a two-tailed, paired *t*-test was utilized using SPSS (16.0) software. Due to sample size, correlation testing was not conducted. Significance was set at $p < .05$.

RESULTS

Endotoxin Measurements by Recombinant Factor C (rFC)

In these studies, endotoxin concentration and distribution of endotoxin among the various dusts were measured using the recombinant factor C (rFC) to compare results with GC-MS/MS. Table 1 shows the relative endotoxin activity of the dusts as measured by the bioassay (EU/mg dust). In all dust extracts, significant endotoxin activity was detected. Endotoxin was significantly higher in the swine facility and grain elevator dust extracts as compared to the other dust extracts, but not different from each other. Dairy barn Tween extract was significantly higher than for domestic homes. Higher endotoxin values were measured in the Tween extractions compared to the HBSS extractions. As expected, endotoxin was significantly depleted to the lower limit of detection with polymyxin B treatment (Table 1).

Endotoxin and Proportions of 3-OHFA in Dust Samples and Dust Extracts

In these studies, total and specific hydroxylated fatty acids of varying carbon chain lengths (3-OHFA; endotoxin lipid A component) were measured by GC-MS/MS as described earlier. Figure 1A depicts the total concentration (pmol/mg dust) of 3-OHFA measured in aliquots of the dust sample Tween extractions (average of three dust extractions). The swine facility dust demonstrated the highest concentration, with a predominance of long-carbon-chain-length 3-OHFA (Figure 1B). There was no statistical difference in total 3-OHFA concentration between the dairy barn, grain elevator, and domestic homes. Figure 1B depicts the distribution of the 3-OHFA (percent of total 3-OHFAs) measured in the dust samples from the various agricultural and domestic home dusts. Even-numbered carbon-chain-length 3-OHFA was consistently elevated in the dairy barn, grain elevator, and domestic homes as compared to odd-numbered 3-OHFA. The variability of 3-OHFA concentration within the replicate (triplicate) dust extractions had an average coefficient of variation (CV) of 25% over all dust types. As anticipated, 3-OHFA were undetectable in dust samples + polymyxin B by GC-MS/MS, consistent with the property of polymyxin B to bind/inhibit endotoxin (data not shown).

Next, dust samples were extracted with HBSS as described in the Methods section for utilization in cell culture studies at a 1% concentration. These HBSS extracts were also analyzed by mass spectrometry for 3-OHFA. Figure 2A depicts the total 3-OHFA concentration (pmol/ml) measured in an HBSS dust extract preparation of each dust, and Figure 2B depicts the distribution of 3-OHFA (percent of total 3-OHFA) measured in each dust extract. The concentration of HBSS dust extracts was overall decreased respective to the Tween dust extracts. There were some differences in the fingerprint pattern of the 3-OHFA between the HBSS dust extracts as compared to the Tween dust extracts. There was relatively higher swine facility C_8, C_{14}, C_{16}, dairy barn C_8, and grain elevator C_{14}. Grain elevator C_8, C_{10}, C_{12}, C_{16}, and C_{18}, house with pet C_8, C_{10}, C_{14}, and C_{18}, and house without pet C_8, C_{12}, C_{14}, C_{16}, and C_{18} were below the limit of detection for the HBSS dust extracts.

Together, these studies demonstrate that each dust has a unique 3-OHFA fingerprint, and that overall swine facility dust 3-OHFA were significantly increased with a notably different carbon chain pattern (long chains) as compared to the other agricultural and domestic house dusts, which were more evenly

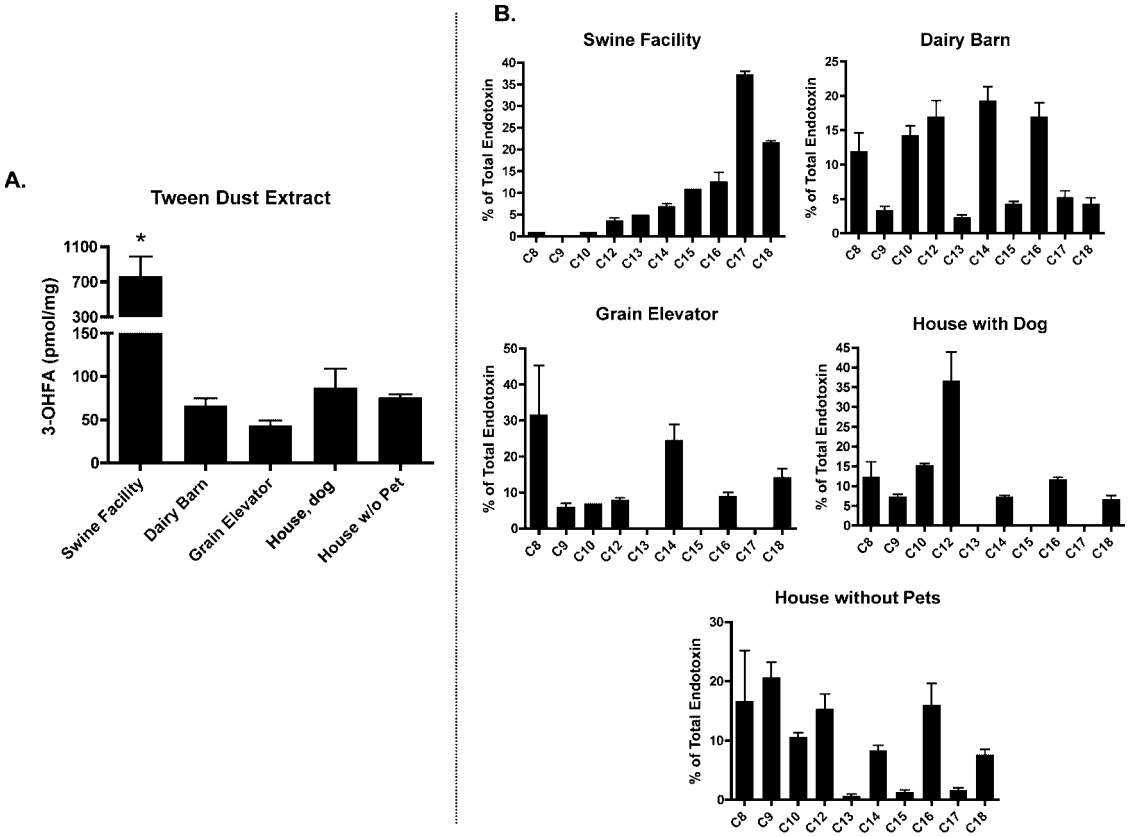

FIGURE 1. Endotoxin's 3-hydroxy fatty acid (3-OHFA) analysis of various agricultural and domestic house dusts after Tween extraction. (A) Total 3-OHFA normalized to the total amount of dust (pmol/mg). (B) The percent of total 3-OHFA for each carbon chain length reported for each individual dust extract. Mean results ± SEM are presented ($n = 3$). Asterisk indicates statistically different from other dust extracts ($p < .05$).

distributed. Differences in extraction methods for the chemical-analytical approach (Tween) versus HBSS for the cell culture approach also resulted in a decrease in recovery of total 3-OHFA and quantitative differences in the 3-OHFA carbon chain patterns.

Muramic Acid in Dust Samples and Dust Extracts

In these studies, muramic acid was measured by GC-MS/MS as described earlier to determine relative distribution of the gram-positive microbial component load in the dusts. As shown in Figure 3A, muramic acid was detected primarily in large-animal farming environments, with greater amounts in swine facility dust than in the dairy barn dust samples (Figure 3A; Tween extraction). In the HBSS dust extract utilized in cell culture studies, muramic acid detection was significantly elevated in the swine facility and dairy barn extracts, with minimal detection in the grain elevator and domestic house dusts (Figure 3B). These data demonstrate that large animal farming environments are a rich source of peptidoglycans (muramic acid), which are primarily found in gram-positive bacteria.

Ergosterol in Dust Samples and Dust Extracts

Ergosterol was next measured by GC-MS in the dust samples and cell culture dust extracts to determine relative distribution of fungal burden. Although ergosterol was detected in all dust samples, grain elevator dusts contained markedly increased levels of ergosterol as compared to the other dusts (Figure 4A). In the 1% dust extracts utilized for the cell culture studies,

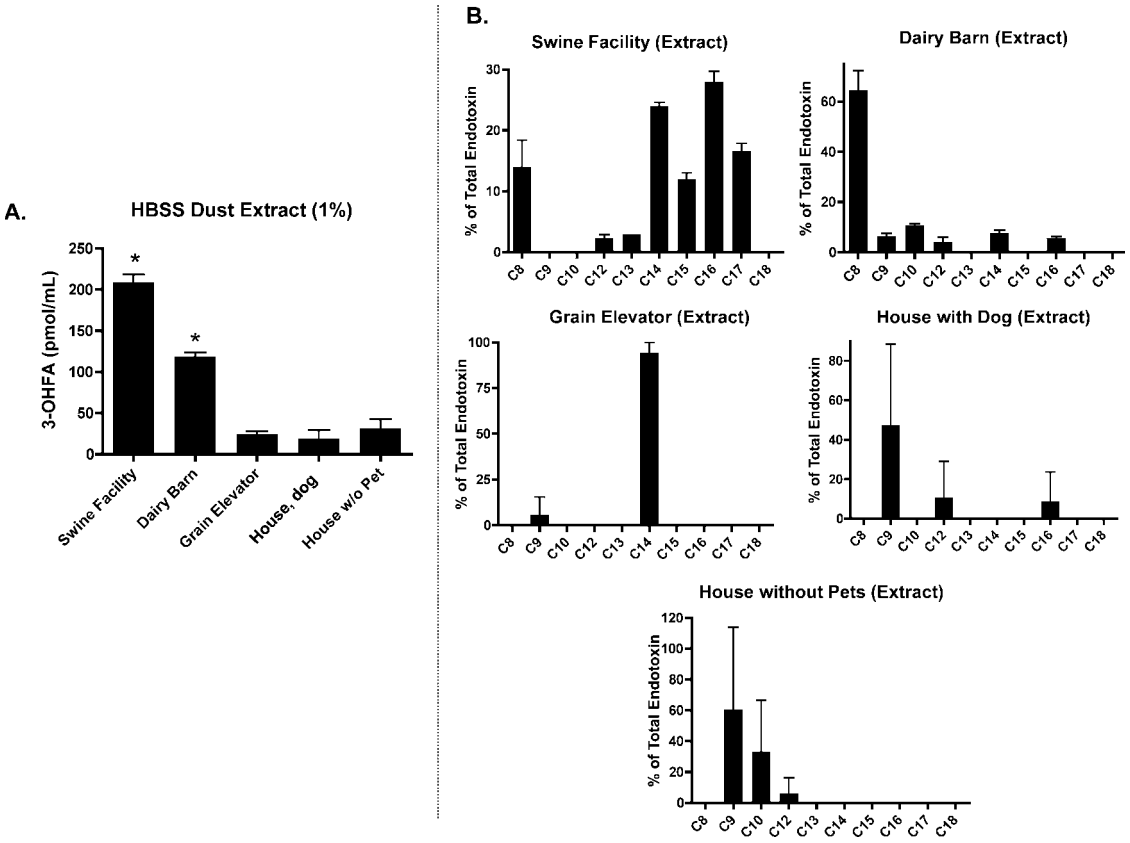

FIGURE 2. Endotoxin's 3-hydroxy fatty acid (3-OHFA) analysis of various agricultural and domestic house dusts with HBSS extraction, which was utilized in cell culture studies at a 1% concentration. (A) Total 3-OHFA normalized to the total amount of dust in a 1% concentration (pmol/ml). (B) The percent of total 3-OHFA for each carbon chain length reported for each individual dust extract. Mean results ± SEM are presented ($n = 3$). Asterisk indicates statistically different from all other dust extracts ($p < .05$).

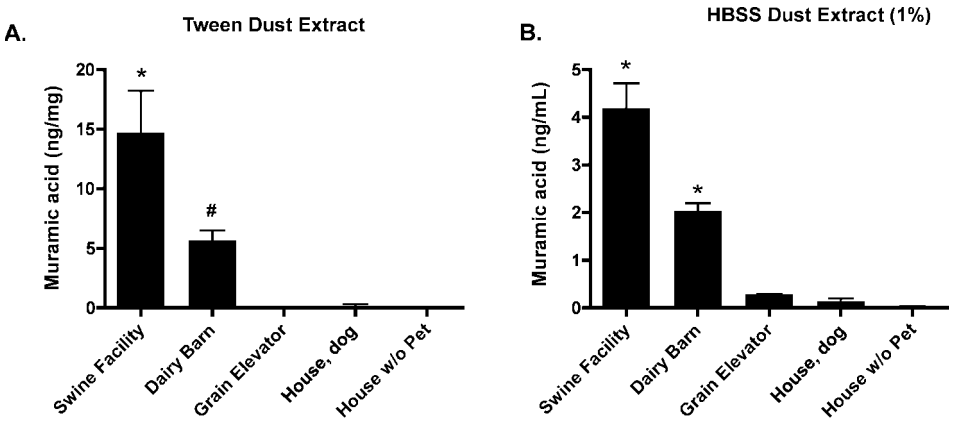

FIGURE 3. Muramic acid analysis of the various agricultural and domestic house dust extracts. (A) Muramic acid concentration normalized to the total amount of dust extracted in Tween (ng/mg). (B) Muramic acid concentration normalized to total volume in the 1% HBSS dust extract (ng/ml). Mean results ± SEM are presented ($n = 3$). Asterisk indicates significantly different from all other dust extracts ($p < .05$).

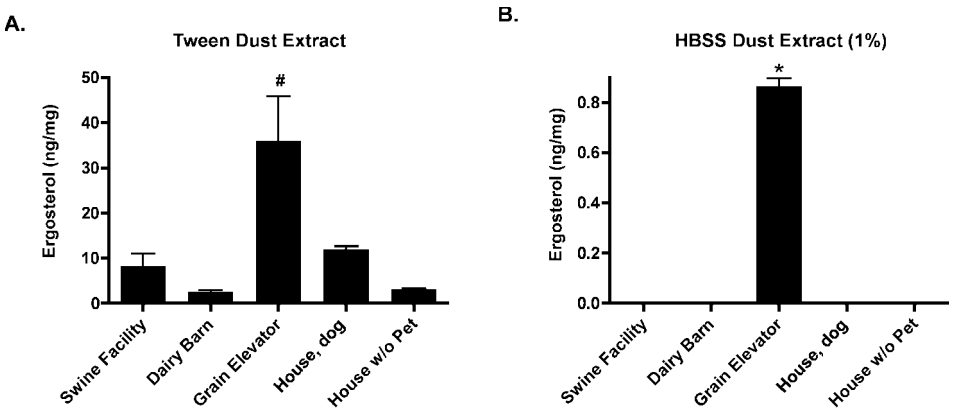

FIGURE 4. Ergosterol analysis of the various agricultural and domestic house dust samples. (A) Ergosterol concentration normalized to the total amount of dust extracted in Tween (ng/mg). (B) Ergosterol concentration normalized to total volume in the 1% HBSS dust extract (ng/ml). Mean results ± SEM are presented ($n = 3$). Asterisk indicates significantly different from all other dust extracts ($p < .05$).

ergosterol was only detected in the grain elevator dust extracts (Figure 4B). These results are consistent with other studies demonstrating a strong presence of fungi in grain farming/grain elevators (Swan et al., 1998).

Dust Extracts Stimulate Inflammatory Cytokine Secretion in Human Monocytes

In these studies, the effect of the various HBSS dust extracts on inflammatory cytokine responsiveness in human monocytes was investigated. As described in the Methods section, human monocytes were stimulated with 1% dilutions of HBSS dust extracts and media control for 5 h in adjacent experiments. All dust extracts resulted in significant monocyte secretion of TNFα, IL-6, and CXCL8/IL-8 as compared to control (Figure 5, A–C; $n = 3$ separate studies). To determine endotoxin-independent responses, 1% HBSS dust extract cultures were treated with polymyxin B (endotoxin-depleted HBSS dust extracts), and differences in the ability to induce cytokine/chemokine secretion were observed. Endotoxin-depleted agricultural HBSS 1% dust extracts resulted in significant secretion of TNFα, IL-6, and IL-8 as compared to media plus polymyxin B control (Figure 5, A–C, $n = 3$ separate studies). With the all dust extracts, there was a significant dampening of TNFα secretion with endotoxin depletion, but there was no significant change in IL-6 and IL-8 secretion. However, there was a quantitative reduction in IL-8 in the endotoxin-depleted grain elevator dust. There was no marked difference in cell count or cell viability between treatment conditions to explain these results.

In comparing dust extract-induced cytokine secretion among the dust groups, the endotoxin-depleted swine facility dust extract demonstrated significantly increased TNFα as compared to all other endotoxin-depleted dust extracts (Figure 5A; $n = 3$). There was also a significant increase in IL-8 with the endotoxin-depleted swine facility dust extract as compared to the endotoxin-depleted domestic house dust extracts (Figure 5C; $n = 3$).

Dust Extracts Stimulate Inflammatory Cytokine Secretion in Bronchial Epithelial Cells

In these studies, the effect of the various dust extracts on inflammatory cytokine responsiveness of human bronchial epithelial cells was investigated. Bronchial epithelial cells were stimulated with and without a 1% dilution of various HBSS dust extracts for 24 h in adjacent experiments. The agricultural dust extracts, particularly swine facility and dairy barn, induced significant secretion of epithelial cell IL-6 and IL-8 as compared to control (Figure 6, A and B; $n = 3$separate studies). There was no significant change in IL-6 and IL-8 secretion in epithelial cells stimulated with

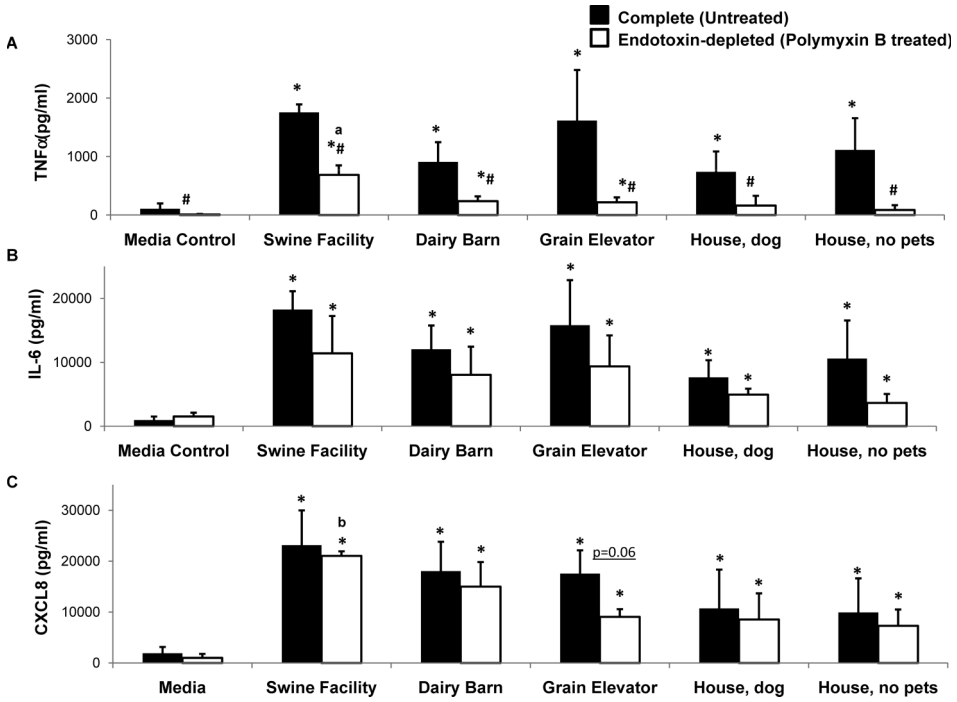

FIGURE 5. Secretion of (A) TNFα, (B) IL-6, and (C) CXCL8/IL-8 in human peripheral blood monocytes stimulated with complete (untreated) and polymyxin B-treated (endotoxin-depleted) various agricultural and domestic house 1% HBSS dust extracts for 5 hr Asterisk indicates significantly different from respective media control ($p < .05$); #, significantly different from matched endotoxin-depleted dust extract (polymyxin B-treated vs. untreated dust extract; $p < .05$), $n = 3$ separate experiments, with mean results presented per 1×10^6 cells ± SD; *a*, statistically different from all other endotoxin-depleted dust extracts; *b*, statistically different from endotoxin-depleted domestic house dust extracts.

endotoxin-depleted swine confinement facility or dairy barn dust extract, although with endotoxin-depleted dairy barn dust extract, a quantitative reduction in IL-6 secretion was observed (Figure 6A; $n = 3$). The grain elevator and domestic house dust extracts- induced inflammatory responses were overall weak. There was no marked difference in cell viability to account for these differences. TNFα was not detected in epithelial cell culture supernatants.

In analyzing complete dust extract-induced cytokine secretion among the groups, the swine facility dust extract demonstrated significantly increased IL-6 as compared to grain elevator and domestic house dust extracts (Figure 6A; $n = 3$). In analyzing endotoxin-depleted (polymyxin-B treated) dust extract-induced cytokine secretion among the groups, the swine confinement dust extracts induced significantly elevated IL-6 as compared to all other endotoxin-depleted dust extracts. There was significantly increased IL-8 secretion with endotoxin-depleted swine facility dust extracts as compared to endotoxin-depleted grain elevator and domestic house dust extracts, with a quantitative observed rise when compared to endotoxin-depleted dairy barn dust extracts.

DISCUSSION

Organic dust exposure is an important environmental factor that has been implicated in increased morbidity among repetitively exposed subjects (Von Essen & Romberger, 2003). Although it is recognized that airway inflammatory immune effects occur with agricultural work exposures, the relative contribution of different constituents of the dust and the effect of these dusts on innate immune cells remain to be fully elucidated. In this

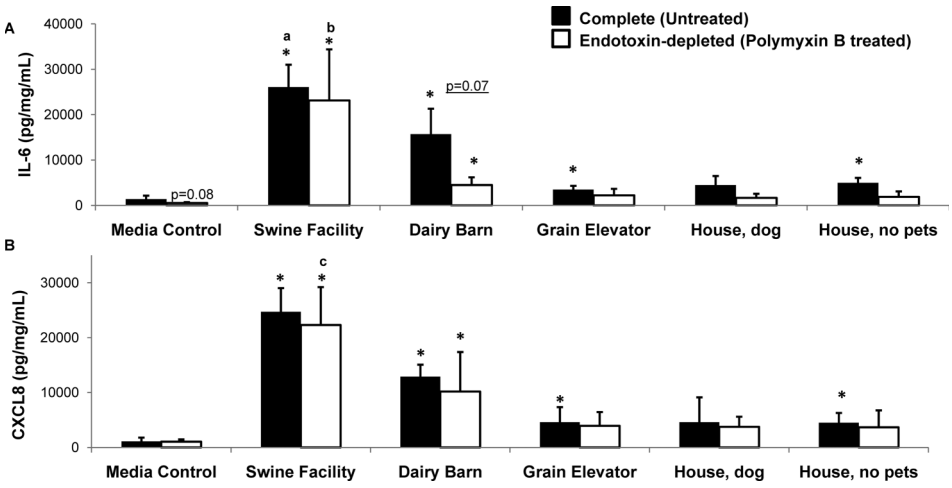

FIGURE 6. Secretion of (A) IL-6 and (B) CXCL8/IL-8 in human bronchial epithelial cells (BEAS-2B) stimulated with complete (untreated) and polymyxin B-treated (endotoxin-depleted) various agricultural and domestic house 1% HBSS dust extracts for 24 h. Asterisk indicates significantly different from respective media control ($p < .05$); $n = 3$ separate experiments. Mean results ± SD are presented. *a*, Statistically different as compared to grain elevator and domestic house dust extracts; *b*, statistically different as compared to all other endotoxin-depleted dust extracts; *c*, statistically different as compared to endotoxin-depleted grain elevator and domestic house dust extracts.

study, agricultural dusts associated with respiratory disease (Bailey et al., 2007) in Colorado and Nebraska were compared to domestic house dusts to determine the relationship between endotoxin's 3-OHFA, muramic acid, and ergosterol. The dusts' abilities to elicit inflammatory cytokine responsiveness in human monocytes and bronchial epithelial cells, cells important in respiratory disease, were also investigated.

Endotoxin, derived from gram-negative bacteria, is a well-described inflammatory agent that has been inconsistently linked to inflammatory respiratory outcomes in exposed workers (Mayeux, 1997). Endotoxin in agricultural settings can be measured by the *Limulus* amebocyte lysate bioassay, but also by the possibly more sensitive rFC bioassay. Unlike the bioassays that fail to detect cell-bound endotoxin, mass spectrometry analysis allows determination of total amounts of both cell-bound and non-cell-bound endotoxin with the chemical marker 3-OHFA (Sonesson et al., 1990; Reynolds et al., 2005). Although correlations between the bioassay and mass spectrometry were previously reported for 3-OHFA in house dust (Hines et al., 2000; Park et al., 2004; Saraf et al., 1997, 1999), patterns of 3-OHFA distribution vary by sampling locations (Hines et al., 2000; Szponar et al., 2001). Bacterial composition varies by environment (Walters et al., 1994), as several studies suggested that the presence of house pets significantly increases the concentration of endotoxins in indoor household air samples (Walters et al., 1994; Zucker et al., 2000). Similarly, livestock dusts contain much higher levels and more variable 3-OHFA distributions than grain dusts (Reynolds et al., 2005; Sebastian et al., 2003). Species differences in livestock may contribute to differences in 3-OHFA distributions, but few studies compared 3-OHFA compositions among various agricultural dusts (Reynolds et al., 2005). This is one of the first studies to use mass spectrometry in the United States to investigate the specific 3-OHFA associated with specific bacterial sources in various agriculture settings and utilize these various dust extracts in cell culture studies.

Although endotoxin and 3-OHFA were detected in all dust samples, 3-OHFA was strikingly increased in swine facility dust as compared to the other dust samples. The chemical structure of the swine facility dust was also markedly different with a predominance of longer chain 3-OHFA (14 to 18 carbons in length). Previous reports indicated that shorter chain (C_{10}–C_{14}) 3-OHFA are positively

correlated with endotoxin activity in the *Limulus* bioassay, while longer chain (C_{16}–C_{18}) 3-OHFA tend to have lower or even no correlation in house dust samples (Saraf et al., 1999; Park et al., 2004). Saito et al. (2009) found the opposite trend in a recent study of agricultural dusts demonstrating that longer chain (C_{15}–C_{18}) 3-OHFA were strongly positively correlated with endotoxin activity in livestock dusts. It was also suggested that longer chain (C_{12}–C_{14}) 3-OHFA may elicit more significant potent immunological effects (Dehus et al., 2006). It is well recognized that workers of swine confinement facilities have a high prevalence of chronic respiratory disease affecting up to one-third of exposed workers (Spurzem et al., 2002).

In contrast to the swine facility dust, the dairy barn, grain elevator, and domestic house dusts had a predominance of even-numbered carbon chain length 3-OHFA with a lesser predominance of odd-numbered 3-OHFA. These results are consistent with our recent investigation that found that even-numbered carbon chain length 3-OHFA were more likely to be associated with nasal airway inflammation in agricultural workers than odd-numbered carbon chain length 3-OHFA (Burch et al., 2009). The domestic house dusts utilized here were utilized solely as a domestic dust sample comparative to samples of agricultural dusts. However, it was observed that the total endotoxin concentration determined by mass spectrometry was no different with the house dusts as compared to the dairy barn and grain elevator endotoxin. This suggests that non-endotoxin components in the agricultural dust may be critical in eliciting disease.

One such non-endotoxin component is peptidoglycan. Peptidoglycan, derived primarily from gram-positive bacteria but also to a lesser degree from gram-negative bacteria, cannot be directly measured, but its chemical marker, muramic acid, can be measured by mass spectrometry. Muramic acid was abundantly detected primarily in the large-animal farming environments, swine facility and dairy barn dusts, and only minimally detected in the grain elevator and domestic house dusts. Using the described methods, our detection of muramic acid in the dusts was overall lower (10-fold) than the amounts of muramic acid detected by others in Sweden (Sebastian et al., 2003). The reasons for this are not clear, but it may limit the ability of this assay to discriminate subtle differences between environments where muramic acid levels are expected to be lower, such as domestic homes. The relative trend of high muramic acid in U.S. swine confinement facilities as compared to domestic homes has been previously reported (Poole et al., 2008). This study confirms those findings and adds dairy barn farming to the list of work environments enriched with gram-positive bacteria. The finding of elevated ergosterol in grain elevators is consistent with the known increased risk of fungal exposure with handling and storing grains (Swan & Crook, 1998). Our study underscores the predominance of fungal elements in grain elevator dust.

To understand the relative contribution of these various dust constituents to inflammatory outcomes, the ability of the dust extracts (complete and endotoxin depleted dust extracts) to elicit cytokine/chemokine secretion from representative airway inflammatory cells was investigated. In human monocytes, agricultural and domestic house dust extracts induced significant TNFα, IL-6, and CXCL8/IL-8 secretion. Removal of endotoxin significantly reduced TNFα responsiveness of all the dusts, but had no significant effect on IL-6 and CXCL8 responsiveness, suggesting a selective role for endotoxin in inducing inflammation. IL-6 and CXCL-8 are two mediators that have been implicated in agriculture-induced respiratory disease (Larsson et al., 1994; Jagielo et al., 1996; Clapp et al., 1994). CXCL8 is a potent neutrophil chemoattractant, and the finding of increased neutrophils in bronchial alveolar lavage fluid and nasal lavage fluid is a consistent finding among workers exposed to agricultural environments (Cormier et al., 2000; Larsson et al., 1994; Clapp et al., 1994). In large-animal farming environments, peptidoglycan likely drives the innate immune inflammatory response, with possible synergy with endotoxin. In contrast, in the grain elevator environment, endotoxin plus ergosterol appears to be important.

In contrast to the human monocytes, the bronchial epithelial cells displayed significant cytokine responsiveness to the agricultural dusts, primarily from the swine confinement facility and dairy barn, whereas the grain elevator and domestic house dusts were weakly or not IL-6 and IL-8 responsive. Depletion of endotoxin did not alter swine facility dust extract-induced cytokine responsiveness, but endotoxin depletion did modulate IL-6, but not CXCL8, responsiveness of the dairy barn extract. Although the swine facility had significantly increased levels of endotoxin as compared to the other dusts, elevated muramic acid levels predominated in the swine confinement facility and dairy barn dusts. Consistent with the notion that endotoxin is not the critical player in the agricultural dust-induced bronchial epithelial cell responses, under normal conditions, epithelial cells lack significant expression of the TLR4–MD-2 complex (LPS receptor; Elson et al., 2007). However, bronchial epithelial cells weakly express TLR2, a pattern recognition receptor that recognizes the structurally broadest range of different bacterial compounds such as PGN, lipoproteins, lipoarabinomannans, lipomannans, glycosylphosphatidylinositol, lipoteichoic acids, and zymosan (Zahringer et al., 2008). Uehara et al. (2007) also found in epithelial cells nucleotide-binding oligomerization domain (NOD) proteins including NOD1 and NOD2, which are intracellular sensors for bacterial molecules and mediate inflammation of specific motifs in PGN. Taken together, these data suggest that in bronchial epithelial cells, the inflammatory cytokine response to large animal farming dusts is being driven by gram-positive microbial components such as peptidoglycan.

A limitation of this study is that air samples were not utilized. Settled dust samples were utilized in order to obtain sufficient mass to determine detection limits in this initial study, and also to obtain sufficient mass for cell culture studies. In this study there were differences in the microbial constituents detected in the dust sample using Tween extracts and the cell culture HBSS dust extracts. In addition to simple dilution effects, the HBSS cell culture extracts were also sterile filtered (0.2 μm pore), which eliminates large particulates, but could result in an underprediction of inflammatory potential. This standard process prevents cell culture contamination and is argued to represent the potential lung deposition of inhaled airborne agents. In addition, HBSS extracts, namely, swine facility dust extracts, have been shown to demonstrate *in vivo* biological activity in a murine model (Poole et al., 2009a, 2009b). It is also possible that other unknown constituents are involved in mediating the organic dust inflammatory consequences, constituents such as particles and/or metals. However, Poole et al. (2008) previously reported that swine facility dust heat-inactivated at 120°C (a process that inactivates the biologics and leaves the metals intact) did not result in inflammatory cytokine secretion. Finally, the inflammatory outcome of this study was investigation of various dust-induced cytokines/chemokines; other inflammatory indices such as free radical or oxidant production were not determined, but could be investigated in future studies.

In conclusion, utilization of this chemical-analytical approach can next be applied to human population environmental studies in the United States to determine the relative contribution of microbial components and to determine whether single or synergy of multiple microbial components is mediating chronic inflammatory respiratory disease in exposed workers. The addition of muramic acid and possibly ergosterol chemical analysis to that of endotoxin is warranted when performing agricultural environmental samplings for developing health hazard control standards or guidelines and for developing effective interventions.

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