Altered immune responses in broiler chicken husbandry workers and their association with endotoxin exposure

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Abstract: Exposure to bioaerosols in indoor animal farms associates with respiratory illnesses, but little is known about the immune modulation to chicken farmers. This study aimed to compare the general immunity of chicken farmers with those of control subjects with non-agricultural jobs. Blood taken from the farmers and controls was subjected to plasma IgE and IgG subclass measurements. Isolated peripheral blood mononuclear cells (PBMC) were stimulated and cytokine production was measured. Indoor total and respirable dust levels and their endotoxin (LPS) and aflatoxin (AF) levels in the farms were measured. In total, 29 chicken farmers on 19 farms and 14 age- and sex-matched office workers participated. Hematological differences were not observed. The farmers tended to have higher serum IgE and IgG subclass levels with significance for IgG1. The cytokines released by PBMC from farmers indicated skewing toward Type-2 helper T-cell responses: interferon (IFN)-γ:interleukin (IL)-4 and IFNγ:IL-13 ratios were significantly lower than for control PBMC. The farms had 707.1 EU/m³ LPS in total dust, and 15.8 EU/m³ LPS in respirable dust. Farmers exhibited immune skewing towards allergic immune responses that correlated with the LPS levels on their farms. Chicken farmers may be at risk of respiratory allergies due to occupational endotoxin exposure.

Key words: Chicken husbandry farmer, Organic dust, Cellular immunity, Endotoxin, Aflatoxin

Introduction

The environment of farms contains a complex mixture of organic, inorganic, and microbial contaminants. Chicken farm environments are particularly strongly contaminated by feces, feathers, bacteria, and fungi as well as by gaseous pollutants such as ammonia, hydrogen sulfide, and carbon dioxide1–3). As a result, the dust generated by poultry farms contains substantial amounts of endotoxin, also known as lipopolysaccharide (LPS). Endotoxin is produced by Gram-negative bacteria and its inhalation reduces respiratory airflow and promotes various respiratory illnesses, including occupational asthma, chronic obstructive pulmonary disease, and hypersensitivity pneumonitis4–9). Other potentially deleterious contaminants in chicken farms are peptidoglycans [cell-wall components of Gram-positive bacteria] and (1-3)-β-D glucans [cell-wall components of molds]; both are now considered pro-inflammatory agents.
on farms that can induce chronic respiratory disease\textsuperscript{(10)}. In addition, the organic dust in animal husbandry environments (including chicken farms) can contain aflatoxin, which is produced by certain molds. Aflatoxin is immunosuppressive and is associated with reduced antibody production, increased susceptibility to infectious diseases, and reduced cell-mediated immunity in many animal species\textsuperscript{(11–13)}.

LPS comprises most of the outer cell membrane of all Gram-negative bacteria. When the bacteria are destroyed in the host body, LPS is released and binds to a soluble protein called LPS binding protein (LBP). This molecule then presents the LPS to surface CD14 on macrophages, neutrophils, and dendritic cells; resulting LPS-mediated signal transduction elicits anti-bacterial immune responses\textsuperscript{(14, 15)}.

Thus, LBP plays a critical role in regulating the acute and chronic airway response to inhaled LPS. However, the function of LBP is antagonized by bactericidal/permeability-increasing protein (BPI) that is primarily expressed by neutrophils and the mucosal epithelia and has a high affinity for LPS\textsuperscript{(16)}). Consequently, the balance between LBP and BPI plays an important role in shaping responses to LPS\textsuperscript{(17)}.

Several studies show that exposure of humans to > 100 EU LPS/m\textsuperscript{3} can initiate pulmonary inflammation. Moreover, exposure to > 2,000 EU LPS/m\textsuperscript{3} can cause severe toxic pneumonia\textsuperscript{(18, 19)}). A key component of LPS-mediated inflammation is an enhanced T-helper-type 2 cell (T\textsubscript{H2}) reactivity\textsuperscript{(20–22)}. Swine farm workers exhibit an immune dysregulation characterized by preferential activation of a T\textsubscript{H2} response\textsuperscript{(23)}. Moreover, peripheral blood mononuclear cells (PBMC) isolated from chickens from farms with relatively high LPS levels exhibited reduced production of interferon (IFN)-\gamma, a classical T\textsubscript{H1} cytokine\textsuperscript{(24)}. However, several studies show that LPS exposure can promote beneficial T\textsubscript{H1} responses\textsuperscript{(22, 25)}. While this appears paradoxical, it seems that the dose, duration, and frequency of LPS exposure may largely determine whether LPS induces a predominant T\textsubscript{H1} response or a predominant T\textsubscript{H2} response\textsuperscript{(22, 26, 27)}.

Little is known about the impact of environmental LPS exposure on immune responses of chicken husbandry farmers. Thus, the present study asked whether occupational exposure to the chicken husbandry environment modulates immune responses and, in particular, whether it drives allergic responses. For this, the current study compared hematological variables, plasma immunoglobulin levels, plasma LBP and BPI levels, and PBMC cytokine production in chicken farmers with those in age-/sex-matched control subjects with non-agricultural jobs. Moreover, LPS and aflatoxin levels in the dust from chicken farms were measured and their correlation with the various immune variables was also assessed.

Subjects and Methods

Collection of blood from participants in this prospective case-control study was approved by the Institutional Review Board of Daegu Catholic University (approval #CUIRB-2014-0013). All subjects consented in writing to participate in the study.

Study subjects

Farmers working on broiler chicken farms in Jinan County (Jeonbuk Province, ROK) were asked whether they would participate in this study. This region in southwest Korea contains the biggest broiler chicken husbandry estates in the country. Age- and sex-matched office-working rural residents from the region were recruited as study controls. For each subject, fasting venous blood (10 ml) was collected once in the period from June to September 2015.

Measurement of immune function

Each blood sample was collected into EDTA vacutainer tubes and the number and proportion of white blood cells (WBC), red blood cells (RBC), platelets, lymphocytes, monocytes, and granulocytes was then determined using an automatic blood analyzer (ADVIA 2120, Siemens, Munich, Germany). Thereafter, each sample was processed to generate plasma. Total IgE titres in plasma were measured using a Total IgE ELISA Kit (IBL International GmbH, Hamburg, Germany). Plasma levels of IgG subclasses (IgG1, IgG2, IgG3, IgG4) were measured in a sandwich ELISA as described in Kim et al.\textsuperscript{(28)} and Heo et al.\textsuperscript{(29)}. Plasma levels of LBP and BPI were analyzed using sandwich ELISA kits (Human LBP Duoset ELISA, R&D Systems, Minneapolis, MN; Human BPI ELISA, BioSource, San Diego, CA).

PBMC were isolated by Ficoll-Hypaque density gradient centrifugation (Ficoll-Paque Plus, GE Healthcare Life Sciences, Uppsala, Sweden). After counting and assessing viability, cells were then placed in 24-well plates at 10\textsuperscript{6} cells/ml/well in complete RPMI medium containing 1 mM non-essential amino acids, 1 mM pyruvate, 1% bicarbonate, 2 mM glutamine, 50 \textmu M 2-mercaptoethanol, and 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), as well as 5 ng phorbol 12-myristate 13-acetate (PMA), 500 ng ionomycin (Sigma, St. Louis, MO), and 10 U
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recombinant IL-2 (Roche, Mannheim, Germany), and cultured for 72 hr at 37°C in a 5% CO₂ incubator. At the end of this period, well supernatants were collected and stored in a -80°C freezer until analyzed for IFN-γ, tumor necrosis factor (TNF)-α, IL-4, and IL-13 using sandwich ELISAs as described previously.

Collection of dust and measurement of endotoxin (LPS) and aflatoxin (AF) levels

The dust in each farm was sampled during working hour at two different locations, namely, one-third and two-thirds from the exit. The amount of dust and endotoxin in the samples was measured as described by Roque et al.

In brief, total dust was measured using a polyvinyl chloride (PVC) membrane filter (SKC, Eighty Four, PA) with a two-stage cassette at a 2.0 L/min flow-rate. Amounts of respirable dust were measured at the same time using a PVC membrane filter in a 10-mm Dorr-Oliver nylon cyclone run at a flow rate of 1.7 L/min.

LPS in total/respirable dust samples was extracted in Limulus Amebocyte Lysate (LAL) water containing 0.5% Tween 20 by shaking at 350 rpm for 1 hr. Supernatants were collected and LPS concentrations analyzed using a LAL Kinetic QCL kit (Lonza, Walkersville, MD) and a microplate spectrophotometer (Epoch, Bio-Tek, Winooski, VT). AF levels in dusts were measured using a competitive enzyme immunoassay kit (Total Aflatoxin ELISA kit, Euro-Proxima, Amhem, the Netherlands), which is known to detect total aflatoxin including AF B1, B2, G1, G2, and M1.

Statistical analyses

All statistical analyses were performed using Sigma Stat 3.5 (Systat Software, San Jose, CA) and graphs plotted using Sigma Plot 10 (Systat). Farmer and control groups were compared in terms of gender ratio using a Fisher’s Exact Test. The two groups were compared in terms of other variables using a Student’s t-test or a Mann-Whitney rank-sum test, depending on the data normality. To determine correlations between dust, LPS, and/or AF concentrations and immune variables, a Pearson Product Moment correlation or Spearman Rank Order correlation test was performed. Differences were considered significant at p<0.05.

Results

In total, 29 farmers (19 men, 10 women) working on 19 broiler chicken farms and 14 age-/sex-matched office workers (9 men, 5 women) from the same region agreed to participate in the study. Since the control subjects were age- and sex-matched with the farmers, the two groups did not in differ in terms of gender composition or mean age: female farmers and controls were 56.1 ± 7.3 and 51.6 ±
15.0-yr-old, respectively, while the male farmers and controls were 50.8 ± 10.2 and 47.7 ± 7.1-yr-old, respectively. The female and male farmers had worked on chicken farms for an average of 7.7 ± 6.4 and 7.2 ± 5.9 yr, respectively; this difference was not significant.

**Hematologic variables**

The farmer and control groups did not significantly differ in terms of any of their cell counts except for RBC levels. Analyses showed the farmers had significantly more RBC than the controls (Table 1). In addition, some parameters such as number of WBC, monocyte, and eosinophil were significantly (p<0.05) lower in the female chicken farmers than the female controls.

**Plasma immunoglobulin levels**

The farmers had 3-fold higher plasma IgE levels than the controls (1,719.5 ± 3,184.5 vs. 697.3 ± 1,315.9 ng/ml), although this difference did not achieve statistical significance (Table 1). This disparity was due to differences between the male farmers and control workers (1,990.9 ± 3,851.8 vs. 389.6 ± 244.5 ng/ml). Female farmers and control workers had similar levels of IgE (1,203.8 ± 1,194.1 vs. 1,251.2 ± 2,236.2 ng/ml).

The farmers also tended to have higher levels of the four IgG subclasses in plasma than the controls. However, this difference was only significant for IgG1 (4.6 ± 1.9 vs. 3.4 ± 1.3 mg/ml, respectively) (Table 1). Up-regulation of plasma IgG1 was observed in male farmer (5.0 ± 1.8 mg/ml) vs. male controls (3.4 ± 1.5 mg/ml) (p = 0.029). Female farmers had IgG1 levels similar to those in female controls (4.0 ± 1.8 vs. 3.5 ± 0.8 mg/ml, respectively). Even though no significant difference in IgG3 level was found between the all chicken farmers and the controls, male farmers (0.3 ± 0.1 mg/ml) had higher IgG3 level (p=0.003) than the male controls (0.1 ± 0.1 mg/ml).
Cytokine production by PBMC

A skewing of PBMC towards TH1 or TH2 is often determined by subjecting the cells to non-specific immune activation, measuring IFNγ along with IL-4 and/or IL-13 production, and then calculating the IFNγ:IL-4 or IFNγ:IL-13 ratios. Farmers had significantly lower mean IFNγ level than control workers irrespective of gender (Fig. 1B), which could contributed to the significantly lower mean IFNγ:IL-4 and IFNγ:IL-13 ratios in farmers than control workers (Fig. 1D and 1E). These observations suggested chicken husbandry work might promote TH2 reactivity. Notably, this alteration was only observed in male farmers. PBMC from the farmers also produced significantly less TNFα than cells from control workers. This difference was significantly observed in both men and women (Fig. 1F).

Regarding spontaneous release of cytokines from PBMC unstimulated, levels of cytokines were very low (IL-4: 1.8 ± 0.6, IFNγ: 428 ± 209, IL-13: 115 ± 28, TNFα: 1,054 ± 140 pg/ml) compared with those from PBMC stimulated (IL-4: 140 ± 26, IFNγ: 30,390 ± 8,884, IL-13: 879 ± 124, TNFα: 6,325 ± 989 pg/ml), respectively. In addition, no significant difference in the unstimulated values was found between the chicken farmers and the control workers (data not shown), except TNFα (chicken farmers: 498 ± 56, control workers: 2,204 ± 172 pg/ml, p=0.000, Fig. 1F).

The farmer and control groups did not differ in terms of plasma LBP (5,800.9 ± 2,005.6 vs. 5,679.7 ± 3,639.4 ng/ml) and BPI (18.3 ± 7.9 vs. 15.9 ± 11.4 ng/ml) levels. However, as expected, plasma LBP levels correlated significantly and negatively with plasma BPI levels.

Dust, endotoxin, and aflatoxin levels

Seven farms were subjected to indoor dust collection analyses. The total indoor dust and respirable indoor dust levels in seven [of the 19 participating] farms were measured along with the LPS and AF levels in the total and respirable dust (Fig. 2). On average, those seven farms had 1.11 ± 0.59 mg total dust/m³ and 0.28 ± 0.10 mg respirable dust/m³ (Fig. 2A). The average LPS levels in total and respirable dust were 707.14 ± 562.56 and 15.79 ± 15.73 EU LPS/m³, respectively (Fig. 2B). Average AF concentrations in total and respirable dust were 2.26 ± 0.39 and 0.77 ± 0.15 ng AF/m³, respectively (Fig. 2C).
Correlation between immune variables and chicken husbandry environmental factors

The immune variables were measured in 14 chicken farmers who worked on the seven chicken farms and who agreed to undergo indoor dust, endotoxin, and aflatoxin measurement. The Pearson Product Moment correlations were calculated.

Fig. 3. Correlations between immune variables and chicken husbandry environmental factors.

The immune variables were measured in 14 chicken farmers who worked on the seven chicken farms and who agreed to undergo indoor dust, endotoxin, and aflatoxin measurement. The Pearson Product Moment correlations were calculated.

Correlation between immune variables and chicken husbandry environmental factors

The immune variables of the 14 chicken farmers who worked on the seven farms that underwent dust, LPS, and AF measurements were determined as described above. They did not differ substantially from the values found in the whole cohort of 29 farmers (data not shown). The correlations between these immune variables and various chicken husbandry environmental factors were assessed. Factors evaluated were: daily working hours indoors in the chicken farms; the head of broiler chicken in the husbandry building where the dust was collected; and, LPS and AF levels in the total and respirable dusts (Fig. 3).

The number of daily working hours demonstrated a significant positive correlation with numbers of eosinophils in the peripheral blood of the farmers (Fig. 3A). Moreover, the head of broiler chickens in the husbandry building in which the dust was collected significantly correlated positively with the TNFα levels produced by the stimulated PBMC (Fig. 3B). In addition, the IFNγ:IL-13 ratio correlated negatively and positively with the LPS and AF concentrations in total dust, respectively (Fig. 3C and 3D).

Plasma IgG2 levels correlated negatively with total dust levels. Neutrophil frequency in peripheral blood demonstrated a significant positive correlation with LPS levels in total dust. Lymphocyte numbers in peripheral blood correlated negatively with total dust LPS levels. Interestingly, while total dust LPS levels demonstrated a significant
negative correlation with respirable dust LPS levels, total dust AF levels showed a significant positive correlation with respirable dust AF levels. No significant correlation was demonstrated between the levels of total or respirable dust collected from the chicken farms with other immune variables besides the immune parameters described above.

Discussion

Agricultural work is one of the three most hazardous jobs worldwide. In particular, animal husbandry is associated with many occupational hazards for farmers. These work hazards include inhalation of bioaerosols or organic dust, which induces a broad spectrum of respiratory illnesses, including occupational asthma or Farmer’s lung. These health risks are due to endotoxins in the dust associated with indoor animal husbandry buildings. It was previously reported that swine farm workers who are exposed to high levels of endotoxin were prone to allergic reactions. Similarly, the current study observed that TH2 responses likely predominated in chicken farmers.

At present, there are no internationally accepted thresholds for dust/airborne endotoxin exposure in animal husbandry buildings. However, the American Conference of Governmental Industrial Hygienists stated that inhalable particles and respirable particles should be kept below 10 and 3 mg/m³, respectively. The present study showed that the average dust levels in the broiler chicken farms (total dust = 1.11 mg/m³, respirable dust = 0.28 mg/m³) were below these levels, but were respectively higher than the total (0.51 ± 0.38 mg/m³) and the respirable (0.19 ± 0.0.17 mg/m³) dust levels evaluated at the same year and season in the beef cattle farms in Korea. Thus, the dust levels alone may not be responsible for the altered immune status of the chicken farmers. Furthermore, the biochemical mixture contaminants including dust particle, microorganisms, and pesticides in chicken husbandry environment could influence on the immune alterations. However, the LPS levels in the farms were on average 707 EU/m³, which greatly exceeds the LPS level that initiates respiratory disorders (50–100 EU/m³). This observation led us to further dissect the pathophysiological effects of LPS on chicken farmers, especially in terms of immune functions.

The chicken farmers and control office workers did not differ significantly in terms of hematological variables (Table 1), and the hematological parameters were within the normal range of Koreans. However, this does not necessarily mean that the immune system of the farmers is completely normal. Indeed, nonspecifically stimulated PBMCs from the chicken farmers showed significantly lower IFNγ:IL-4 and IFNγ:IL-13 ratios and TNFα production than the PBMCs from the controls. IL-4 and IL-13 both play critical roles in inducing the allergic response by antagonizing IFNγ, thereby promoting TH2 paradigms that drive allergic responses. Thus, the lower IFNγ:IL-4 and IFNγ:IL-13 ratios noted here suggest the chicken farmers have TH2-skewed responses. This notion is supported by the fact that the chicken farmers tended to have higher plasma IgE levels than control subjects. Given the two groups did not differ in terms of age and sex distribution, these observations suggested to us that the altered immune responses of the farmers was likely due to factors in their working environment. This viewpoint was supported by the negative correlation between IFNγ:IL-13 ratios and LPS levels in total dust. In addition, this study also noted that farmers tended to have higher plasma levels of IgG1 and IgG4 (difference significant for IgG1) than controls. Both IgG subclasses are associated with a higher risk of allergic diseases. Notably, the current study found that these changes in humoral (IgE, IgG1, IgG3) and cellular (IFNγ:IL-4 and IFNγ:IL-13 ratios) immunity were more prominent in male farmers. This does not appear to correlate with degree of exposure to the chicken husbandry environment, since the female and male farmers had worked in chicken husbandry for similar durations and had similar daily working hours inside the husbandry buildings. It is possible this difference between female and male farm workers reflected behavioral factors such as smoking and husbandry work tasks, but smoking status was not a factor influencing the TH2-skewed responses in the chicken farmers.

Since we did not evaluate clinical symptoms in the chicken husbandry farmers, we are not sure whether the TH2-skewed responses in the farmers could lead to or exacerbate occurrence of occupational respiratory illness including asthma. Concerning the recent reviews on relationship between agricultural dust exposure and occurrence of respiratory illness in farmers, organic dust exposure may be protective against asthma in adult farmers or contribute to development of asthma. This inconclusive observation so far may be originated from a complexity of organic dust components, polymorphism of genes related such as Toll-like receptor 4, or activation of pattern recognition scavenger receptor A/CD204 pathway, or endotoxin tolerance. Endotoxin tolerance may be a confounding factor to explain lower TNFα production in the chicken farmers than the control subjects. According to the endotoxin tolerance hypothesis, production of pro-inflamm-
matory cytokines from monocytes/macrophages such as TNFα could be suppressed following a subsequent endotoxin exposure\textsuperscript{44,45}. Considering the endotoxin concentration in respirable dust (average:15.79 EU/m\textsuperscript{3}, range:1.57–45.64 EU/m\textsuperscript{3}) collected from the chicken farms, which were below the LPS level initiating respiratory disorders (50–100 EU/m\textsuperscript{3}), respiratory allergic symptoms might not be apparently shown. Inhalation of respirable organic dust with diameter of 10 μm or less (PM\textsubscript{10}) has been well documented to be more involved with occurrence of occupational respiratory diseases\textsuperscript{5,33}. Even though the levels of respirable dust (average: 0.28 mg/m\textsuperscript{3}) and LPS in the respirable dust (average:15.79 EU/m\textsuperscript{3}) in the chicken farms were below the threshold initiating respiratory clinical symptoms, LPS in the respirable dust could moved into lung alveoli, which might somewhat contribute to disturbance of lung function. Even though no significant difference in plasma LBP and BPI level was observed between the chicken farmer and control group, relative level of these two proteins could associate with the TH2-skewed responses for LPS-mediated respiratory allergic symptoms since relative level of LBP versus BPI [LBP/(BPI x 10\textsuperscript{2})] was negatively correlated with the IFN\textgamma/IL-4 ratio ($r=-0.335, p=0.075$, data not shown). But, in order to get a clear delineation on magnitude of LBP or BPI’s influence on the immune modulation in chicken farmers, further investigation should be followed on expression of other proteins related with LBP and BPI signaling such as CD14 LPS receptor, TLR4, or myeloid differentiation factor-2\textsuperscript{2,14–17}.

The immune dysregulation seen in the chicken farmers may also be due to inhalation of airborne AF. Though the relationship between AF exposure in occupational settings and immune modulation in workers has not yet been systematically investigated, several reports indicate that administration of AF through oral or inhalational route suppresses TNFα production in rodents or livestocks\textsuperscript{46–49}. Thus, exposure to AF in the chicken husbandry buildings may have contributed to low TNFα production by PBMC from the farmers. However, this possibility should be tested more rigorously in future as the degree of AF exposure in the offices of the control workers was not measured. Moreover, the AF levels in the total dust of the chicken farms was $\approx$ 2.26 ng/m\textsuperscript{3}, a value not markedly higher than AF levels in air that have been reported previously (they are mostly in pg/m\textsuperscript{3} or low ng/m\textsuperscript{3} range)\textsuperscript{50}. Nevertheless, since AF is a carcinogen\textsuperscript{51}, chronic exposure to this toxin in occupational settings should be avoided.

Although the present study was performed with relatively small number of chicken farm workers and farms recruited, the present study showed that airborne endotoxin levels in broiler chicken husbandry buildings were high enough to initiate airway inflammation. Indeed, farmers working in these buildings exhibited an immune imbalance skewed towards TH2 responses, which correlated with exposure to inhaled endotoxin. These immunological changes were more prominent in male farmers. Despite of enhancement in TH2 responses in the male chicken farmers (higher plasma IgE and IgG1 levels, lower IFN\textgamma/IL-4 and IFN\textgamma/IL-13 ratios) compared with the control workers, further systematic studies including clinical investigation, pulmonary function test, and measurement of aeroallergen specific IgE and serum cytokine levels with larger sample sizes are needed to validate these findings and to elucidate the mechanisms by which endotoxin and aflatoxin exposure alter immune responses.

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