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Association of Bovine Cellular Immunity with Endotoxin Level in Dust from Korean Beef Cattle Housing Environments

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ABSTRACT

Hazardous biochemical agents in indoor animal husbandry environments promote the occurrence of various illnesses among husbandry workers and industrial animals. The relationship between endotoxin levels in dust collected from Korean beef cattle farms and markers of bovine cellular immunity was investigated. Peripheral blood was obtained from 25 cattle from five cattle farms in Korea. Endotoxin levels present in total or respirable dust were determined by the Limulus Amebocyte Lysate Kinetic method. Cytokine production was evaluated following the stimulation of peripheral mononuclear cells with concanavalin A for 72 h in a 5% CO_2 incubator. Production of both interleukin (IL)-4 and interferon (IFN)γ was significantly higher in the high endotoxin exposure group (100.9 ± 70.6 EU/m^3) compared with the low endotoxin exposure group (17.7 ± 18.6 EU/m^3), with a lower IFNγ/IL-4 ratio in animals from the high endotoxin farms, indicating immunity skewed toward a type-2 response. The proportion of γδ T lymphocyte, important bovine immune cells involved in protection against microbial infection, was lower in cattle from the high endotoxin farms than in those from the low endotoxin farms. The numbers of white blood cells, red blood cells, lymphocytes, eosinophils, and basophils were significantly downregulated in cattle from the high endotoxin farms. Overall, these results suggest a probable negative association between dust endotoxin levels and cell-mediated immunity in Korean beef cattle.

Key words : Endotoxin, Beef cattle, Cellular immunity, Total dust, Husbandry environment

Introduction

Industrial animals, including cattle, pigs, and chicken, can be exposed to various hazardous agents such as dust, microorganisms, toxic gases, and endotoxins in the animal husbandry environment [1-3]. Organic dust in animal husbandry buildings is generated from feces, feed, and animal dander, and its relationship with respiratory illness in farmers has been investigated worldwide; however, few studies have been performed in livestock [3-7].

Among the hazardous agents in organic dusts, there has been much focus on endotoxin owing to its pathophysiological effects on human and animal health, especially in terms of the induction of respiratory allergies [3,5,8]. Endotoxin is found in the outer membrane of Gram-negative bacteria such as *Escherichia, Salmonella*, *Shigella*, and *Pseudomonas*. Endotoxin has been reported to mediate various respiratory diseases, including asthma, organic dust toxic syndrome, and chronic obstructive pulmonary disease in animal husbandry workers [9-11]. Endotoxin exposure levels over 100 EU/m^3 can initiate pul-

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monary inflammation, and at exposure levels over 2,000 EU/m^3, severe toxic pneumonia might occur [12,13]. Endotoxin binds to CD14, a component of the endotoxin receptor, on macrophages, dendritic cells, and monocytes, leading to immune stimulation and the production of inflammatory cytokines, including tumor necrosis factor-(TNF)α, interleukin (IL)-6, IL-8 [14,15].

Compared to studies performed in humans investigating changes in immune function following endotoxin exposure, such studies on livestock are very limited [2,3,7]. We recently reported a negative association between endotoxin levels in dust and cellular immunity in chickens [3]. Based on those findings, we further investigated the association between endotoxin levels in dust collected from Korean beef cattle farms and hematologic and cellular immunologic markers. We determined the concentrations of total and respirable dust from indoor cattle husbandry barns; the levels of endotoxin in dust were also measured. Regarding cellular immunologic markers in cattle, cytokine production from peripheral mononuclear cells (PBMCs) was evaluated and lymphocyte subpopulations were quantified. Furthermore, total immunoglobulin G (IgG) in plasma was determined.

Materials and Methods

1. Cattle farms and blood collection

Five Korean beef cattle (Hanwoo) farms in the Gyeongju area of Gyeongbuk province in Korea were selected. Since Korean beef cattle farms are concentrated in this area, the Gyeongju Bovine Husbandry Association was asked to select cattle farms with similar stock densities and a regional veterinarian collected peripheral blood from cattle. Five heads of cattle were randomly selected from each farm and blood was collected from tail veins and placed in EDTA vacutainer tubes. Blood sampling was approved by the Institutional Animal Care and Use Committee of Catholic University of Daegu (CUD IACUC-2012-10), and performed in August 2015. The cattle were 15-20-months old at the time of blood collection. Hematologic analyses were performed using an automatic blood analyzer (Advia 2120, Siemens, Munich, Germany).

2. Dust collection and endotoxin measurement

The concentration of total dust inside the cattle farms was evaluated using a PVC membrane filter (SKC, Eighty Four, PA, USA) with a two-stage cassette at a flow rate of 2.0 L/min for 8 h. The concentration of respirable dust (PM10) was determined using a PVC membrane filter with a 10-mm Dorr-Oliver nylon cyclone at a flow rate of 1.7 Ls/min for 8 h. Dust was sampled from two different locations (at 1/3 and 2/3 distance from the exit) at each farm.

The method used to determine the endotoxin concentration in dust is described elsewhere [3,5,13]. Endotoxin was extracted from the filters by adding 3 mL endotoxin-free Limulus Amebocyte Lysate (LAL) water (LAL Kinetic-QCL set, Lonza, Walkersville, MD, USA) with 5% Tween 20 followed by shaking for 1 h at 350 rpm. Supernatants were collected and stored in a −80°C freezer until analysis. The endotoxin concentrations in supernatants were evaluated following the company's instruction, using a microplate spectrophotometer (Model Epoch, Bio-Tek, Winooski, VT, USA).

3. PBMC collection and lymphocyte phenotyping

PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation (Ficoll-Paque Plus, GE Healthcare Life Sciences, Uppsala, Sweden). Three-color flow cytometry (FACScan, BD, Franklin Lakes, NJ, USA) was used to analyze peripheral lymphocyte subpopulations. Anti-CD3-FITC and anti-CD21-RPE antibodies (AbD Serotec, Raleigh, NC, USA) were used to identify T- and B-cell populations, respectively. Anti-CD4-FITC, anti-CD8-RPE, and anti-WC1-FITC antibodies were used to sort T-helper cells, cytotoxic T cells, and γδ T cells, respectively. RPE- or FITC-conjugated isotype controls were used to subtract the non-specific background binding of fluorescent antibodies.

4. ELISA for plasma IgG

Plasma IgG levels were determined using a sandwich ELISA kit (Komabiotech, Seoul, Korea) with sheep anti-bovine IgG as the capture antibody and HRP-sheep anti-bovine IgG as the detection antibody. Plasma was diluted 1/50,000 with assay diluent. The lower limit of detection for bovine IgG was 7 ng/mL.

5. T-cell activation and cytokine measurement

PBMCs (10^6/mL) in complete RPMI medium (1 mM nonessential amino acid, 1 mM sodium pyruvate, 1% sodium bicarbonate, 2 mM glutamine, 50 M 2-mercaptoethanol, and 10% heat-inactivated fetal bovine serum) were activated with 5 μg

Table 1. Endotoxin levels in total and respirable dust collected from Korean beef cattle farms*

	Low endotoxin exposure farms	High endotoxin exposure farms	Significance (p value)
Stocking density (m^2/head)†	13.9 ± 1.80	22.15 ± 9.55	0.211
Total dust (mg/m^3)	0.43 ± 0.27	0.64 ± 0.57	0.504
Respirable dust (mg/m^3)	0.22 ± 0.20	0.14 ± 0.07	0.533
Endotoxin in total dust (EU/m^3)	17.67 ± 18.60	100.89 ± 70.55	0.039
Endotoxin in respirable dust (EU/m^3)	0.29 ± 0.18	1.43 ± 1.02	0.042

*Three farms were included in the low endotoxin exposure group and two farms were included in the high endotoxin exposure group.
†Data aree expressed as mean ± SD.

Table 2. Comparison of hematologic parameters in cattle from the low and high endotoxin exposure farms*

	Low endotoxin exposure farms	High endotoxin exposure farms	Significance (p value)
WBC ($10^3/\mu L$)†	11.37 ± 1.80	7.52 ± 1.03	0.000
RBC ($10^6/\mu L$)	9.03 ± 0.02	8.10 ± 1.21	0.039
Platelet ($10^3/\mu L$)	250.47 ± 88.60	173.50 ± 133.36	0.095
Lymphocyte ($10^3/\mu L$)	5.89 ± 0.98	2.69 ± 1.01	0.000
Monocyte ($10^3/\mu L$)	0.39 ± 0.14	0.39 ± 0.17	0.925
Neutrophil ($10^3/\mu L$)	3.50 ± 1.10	3.50 ± 1.09	0.569
Eosinophil ($10^3/\mu L$)	1.39 ± 0.55	0.83 ± 0.38	0.012
Basophil ($10^3/\mu L$)	0.11 ± 0.02	0.07 ± 0.03	0.000

*Blood was collected from five heads from each farm.
†Data are expressed as mean ± SD.

concanavalin A for 72 h in a 37°C 5% CO_2 incubator. IL-4 or interferon (IFN)γ levels in each culture supernatant were determined by a sandwich ELISA kit (ThermoScientific, Rockford, IL, USA). The lower limit of detection was 5 and 25 pg/mL for IL-4 and IFNr, respectively.

6. Statistical analyses

SigmaStat 3.5 (Systat Software, San Jose, CA, USA) was used for statistical analyses. Statistically significant differences between the low endotoxin and high endotoxin farms were evaluated by Student's t-test or Mann-Whitney rank sum test, depending on the normality of the data. Pearson Product Moment correlation or Spearman Rank Order correlation was performed to identify correlations between the endotoxin concentration in total dust and that in respirable dust. The criterion for statistical significance was set at $p < 0.05$.

Results

1. Endotoxin levels in the dust from cattle husbandry buildings

Five cattle farms were tentatively divided into low and high endotoxin exposure groups depending on the endotoxin level in total dust (low endotoxin group: 0.7-47.2 EU/m^3; high endotoxin group: 39.6-178.0 EU/m^3). No significant difference was observed in the total or respirable dust concentration between the low and high endotoxin exposure farms (Table 1). However, the endotoxin level in the total or respirable dust was significantly higher in the high endotoxin exposure farms (100.89 ± 70.55 EU/m^3 for total dust and 1.43 ± 1.02 EU/m^3 for respirable dust) than the low endotoxin exposure farms (17.67 ± 18.60 EU/m^3 for total dust and 0.29 ± 0.18 EU/m^3 for respirable dust). In addition, the endotoxin level in total dust was well correlated with that in respirable dust ($r = 0.94$, $p = 0.0016$).

2. Comparison of bovine hematologic parameters in the low and high endotoxin exposure farms

The average age of the Korean beef cattle was similar between the low endotoxin exposure (17.2 ± 0.8 months) and the high endotoxin exposure (18.0 ± 2.8 months) groups. Although the major hematologic parameters were within the normal range [16], lower levels of white blood cells, red blood cells, lymphocytes, eosinophils, and basophils were observed in the high endotoxin exposure group in comparison with those in the low endotoxin exposure group (Table 2).

The plasma level of total IgG was not significantly different

Table 3. Distribution (%) of lymphocyte subpopulations in peripheral blood*

	Low endotoxin exposure farms	High endotoxin exposure farms	Significance (p value)
$CD4^+$ helper T lymphocyte†	22.54 ± 7.31	22.54 ± 7.55	0.999
$CD8^+$ cytotoxic T lymphocyte	15.56 ± 5.34	15.56 ± 5.83	0.998
$WC1^+$ γδ T lymphocyte	14.99 ± 8.76	8.36 ± 3.46	0.054
$CD21^+$ B lymphocyte	11.28 ± 4.60	10.37 ± 5.18	0.663

*Blood was collected from five heads from each farm.
†Data are expressed as mean ± SD.

Table 4. Cytokine production from peripheral T cells of Korean beef cattle*

	Low endotoxin exposure farms	High endotoxin exposure farms	Significance (p value)
IL-4 (pg/mL)†	412.20 ± 320.93	804.80 ± 431.36	0.016
IFNγ (pg/mL)	2497.84 ± 3002.12	6620.80 ± 4338.98	0.010
Ratio (IFNγ/IL-4)	23.13 ± 66.21	9.28 ± 7.20	0.542

*Peripheral T lymphocytes were stimulated with concanavalin A for 72 h in a 5% CO_2 incubator.
†Data ware expressed as mean ± SD.

between the two groups (low endotoxin exposure group: 3.55 ± 0.88 mg/mL; high endotoxin exposure group: 5.41 ± 3.77 mg/mL).

3. Distribution of lymphocyte subpopulations in peripheral blood

The distribution of helper T lymphocytes, cytotoxic T lymphocytes, γδ T lymphocytes, and B lymphocytes was examined using flow cytometry. No significant differences were found in the percentages of helper T lymphocytes, cytotoxic T lymphocytes, and B lymphocytes between the low and high endotoxin exposure groups (Table 3). Although not statistically significant, the percentage of peripheral γδ T lymphocytes, which act as memory/effector cells against pathogenic challenges [17,18], was apparently lower in the high endotoxin exposure group than in the low endotoxin exposure group.

4. Cytokine production by activated peripheral T cells *in vitro*

We examined the production of IL-4 and IFNγ by peripheral T cells since IL-4 mediates allergic responses and IFNγ plays a critical role in protection against bacterial and viral infection in livestock [19-21]. The production of both IL-4 and IFNγ was significantly higher in the high endotoxin exposure group than in the low endotoxin exposure group (Table 4).

The level of IFNγ relative to that of IL-4 (IFNγ/IL-4) are frequently adopted to evaluate the predominance of type-2 T-helper (Th2)- or type-1 T-helper cell (Th1)-mediated immune responses [3,5]. Th2 cells are a key source of IL-4 production, and Th1 cells are a key source of IFNγ production. Although no statistically significant difference was found, the IFNγ/IL-4 ratio was more than 2-fold higher in the low endotoxin exposure group than in the high endotoxin exposure group, indicating a predominant Th1-mediated immune response in cattle from the low endotoxin exposure group.

Discussion

There is no internationally accepted guideline for the dust exposure threshold in industrial animal husbandry buildings. Considering the exposure limit for chemical and physical hazards set by the Korea Ministry of Employment and Labor (MOEL) [22], or the threshold limit of the American Conference of Governmental Industrial Hygienists (ACGIH) [23], none of the Korean beef cattle farms studied exceeded the exposure threshold value (10 mg/m^3 of MOEL and 10 mg/m^3 for total dust and 3 mg/m^3 for respirable dust of ACGIH). In addition, no occupational guideline is available for endotoxin exposure in agricultural settings. Considering that exposure to endotoxin at levels over 100 EU/m^3 is implicated in the initiation of pulmonary inflammation, and at levels over 1,000 EU/m^3 is associated with potential systemic effects on health [12,13], certain respiratory illnesses could have occurred in the cattle reared in the high endotoxin exposure farms since the average level of endotoxin in total dust was 101 EU/m^3.

The presence of γδ T lymphocyte has been reported in vari-

ous species of livestock including cattle, pigs, and chicken [17]. Even though the average percentage (8.4%) of γδ T lymphocyte in cattle reared in the high endotoxin exposure farms was within the normal range (8-18%), it was apparently lower than that in the cattle (15.0%) reared in the low endotoxin exposure farms. The γδ T lymphocytes expressing the WC1 antigen on their cell surface are known to play an important role in antigen presentation, immune modulation through cytokine production, and memory function in response to micro-organisms to which they have been previously exposed [17,18]. Therefore, the lower proportion of γδ T lymphocyte in cattle from the high endotoxin exposure farms suggests that cattle may exhibit immune-downregulation, resulting in enhanced susceptibility to infection by pathogenic micro-organisms or a decreased immune response following vaccination.

The immune system of swine farm workers exposed to high levels of endotoxin was reported to be skewed towards type-2 T-helper cell mediated responses [5]. Production of IFNγ was also downregulated in chickens from the farms with higher endotoxin levels [3]. In the present study, the IFNγ/IL-4 ratio was substantially lower in cattle from the high endotoxin exposure farms. These results suggest that endotoxin exposure may be associated with an allergic response and dysregulated cell-mediated immunity against virulent bacteria or virus. We recently reported that prevalent micro-organisms present in Korean beef cattle farms were Gram-positive bacteria; *Staphylococcus lentus*, *S. chromogenes*, *Bacillus cereus*, *B. licheniformis*, and *Enterococcus faecalis*, and *Candida albicans* and *Acinetobacter iwoffi* for fungi and Gram-negative bacteria, respectively [24]. Considering that various pathogenic micro-organisms exist in cattle farms, strategies aiming to reduce endotoxin exposure in cattle may be necessary.

In summary, our investigation demonstrated, for the first time in Korea, that cattle reared in husbandry buildings containing dust with high endotoxin levels could be less resistant to pathogenic insults and infection. Since only five cattle farms were investigated in our study, the generalizability of the results may be limited. Therefore, more systemic evaluations should be conducted to demonstrate the association between bovine husbandry environments and immunity in cattle.

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