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*Am J Physiol Lung Cell Mol Physiol* 291:512-522, 2006. First published Mar 23, 2006;  
doi:10.1152/ajplung.00278.2005

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## Early exposure to a nonhygienic environment alters pulmonary immunity and allergic responses

Caroline L. S. George,<sup>1</sup> Misty L. White,<sup>1</sup> Katarina Kulhankova,<sup>2</sup> Aditya Mahajan,<sup>1</sup>  
Peter S. Thorne,<sup>2</sup> Jeanne M. Snyder,<sup>3</sup> and Joel N. Kline<sup>2,4</sup>

Departments of <sup>1</sup>Pediatrics, <sup>2</sup>Occupational and Environmental Health, <sup>3</sup>Anatomy  
and Cell Biology, and <sup>4</sup>Internal Medicine, University of Iowa, Iowa City, Iowa

Submitted 28 June 2005; accepted in final form 15 March 2006

**George, Caroline L. S., Misty L. White, Katarina Kulhankova, Aditya Mahajan, Peter S. Thorne, Jeanne M. Snyder, and Joel N. Kline.** Early exposure to a nonhygienic environment alters pulmonary immunity and allergic responses. *Am J Physiol Lung Cell Mol Physiol* 291: L512–L522, 2006. First published March 23, 2006; doi:10.1152/ajplung.00278.2005.—The hygiene hypothesis suggests that early life exposure to a nonhygienic environment that contains endotoxin reduces the risk of developing allergic diseases. The mechanisms underlying the hygiene hypothesis are unclear and may involve subtle immune system interactions that occur during maturation. Experimental objectives of this study were to use a novel animal model to test the hygiene hypothesis and to characterize early life immune system responses to a nonhygienic environment. Mice were reared in corn dust, a grain-processing byproduct with a high-endotoxin content and microbial products or in a low-endotoxin environment. The influence of early or later life exposure to corn dust on a subsequent allergen stimulus (ovalbumin) was assessed by bronchoalveolar lavage (BAL) cell analysis, lung histology, serum IgE, and BAL cytokine measurements. The influence of the corn dust environment on the developing pulmonary immune system was assessed by BAL cell analysis and immunostaining of lung tissue. The corn dust environment contained significantly more endotoxin ( $P < 0.001$ ), and the dust exposures attenuated the cellular inflammatory response to ovalbumin in the adult mouse ( $P < 0.01$ ) but did not reduce serum IgE levels or alter baseline BAL fluid proinflammatory cytokine levels. The corn dust environment did not induce significant neutrophilia in lavage fluid but significantly increased the number of antigen-presenting cells in alveolar walls early in life by  $\sim 37\%$ . In conclusion, exposure to a nonhygienic environment did not induce significant airway neutrophilia, yet altered the population of immunologically active cells in the lung and reduced subsequent allergic inflammation.

hygiene hypothesis; immune system; development; rodent

IN RECENT DECADES, THE PREVALENCE of asthma has risen dramatically in industrialized nations, whereas inhabitants of underdeveloped countries appear to be protected from this phenomenon. The “hygiene hypothesis” (36) describes an association between the environment and reduced incidence of atopic and asthma disease. It is strengthened by epidemiological data that demonstrate an influence of early life environment on the development of immune tolerance (28). Environmental conditions associated with a reduced risk of developing allergic disease include large family size, rural homes, low antibiotic use, and poor sanitation (4, 5, 36, 47). Each of these conditions can be characterized by an increased exposure to microbes and microbial products early in life.

The innate immune system comprises the first line of defense in the lung and initiates an immune response after exposure to microorganisms or foreign antigens. Portions of the innate immune system are present in the developing fetus or newborn, whereas other components are not yet fully functional at birth. The appearance of dendritic cells in the lung occurs during the first year of life (42). Dendritic cells exist in the lung in an immature state. They respond to antigens in the environment by changing their phenotype (35) and can play a pivotal role in influencing the developing immune system (12, 25), specifically lymphocytes. Lymphocytes, in turn, influence the response to microbial or allergic stresses in the environment (17).

Animal models have been devised to explore the influence of endotoxin, a key component of a nonhygienic environment, on the innate immune system and the development of an atopic response. As a model for endotoxin exposure, purified endotoxin is often delivered directly to the airway via intratracheal instillation or as an aerosolized solution. We and others have used murine models in which endotoxin solutions are inhaled to mimic agricultural or domestic exposures (8, 9, 30, 44). Analyses of environmental endotoxin exposure in most epidemiological studies are performed by quantifying endotoxin from airborne dust and dust collected from mattresses and floors (40, 41, 47). When endotoxin levels observed in these domestic and occupational environments are compared with the levels of exposure in animal models, experimental animals are often exposed to considerably higher levels of endotoxin. Importantly, both domestic and agricultural dusts contain endotoxin in addition to other immunologically active components (32). Therefore, studies concerning the influence of complex, naturally occurring organic dusts on the developing lung and immune system are needed to address questions about environmental exposures that cannot be answered by using purified endotoxin.

One aim of the present study was to explore the influence of an endotoxin-containing nonhygienic environment on the susceptibility to atopic airway inflammation. A second goal was to define the pulmonary inflammatory response to this nonhygienic environment during development. We used grain dust, an environmental model of agricultural exposure that is rich in endotoxin and other microbial components, in this study (32). We hypothesized that early life exposure to organic dust with a high concentration of endotoxin would modulate allergic immune responses later in life and also that this exposure

Address for reprint requests and other correspondence: C. L. S. George, Dept. of Pediatrics, 2JCP, Univ. of Iowa, 200 Hawkins Drive, Iowa City, IA 52242 (e-mail: caroline-george@uiowa.edu).

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would be associated with a neutrophilic response in the bronchoalveolar lavage (BAL) fluid. Our experiments demonstrate that subchronic exposures to corn dust either very early in life or later in life attenuate the allergic inflammatory response of the lung in mature animals without modulating systemic levels of ovalbumin-specific IgE or levels of proinflammatory cytokines in BAL fluid. In addition, early life exposure to a corn dust environment did not result in pulmonary neutrophilia but rather increased the numbers of antigen-presenting cells in the lung.

## MATERIALS AND METHODS

**Materials.** All materials were purchased from Sigma (St. Louis, MO) unless otherwise specified.

**Environmental exposure.** Two types of environmental exposures were created. The hygienic environment (control) consisted of a standard mouse cage lined with Cellu-Dri, a cellulose fiber animal bedding (Shepherd Specialty Papers, Kalamazoo, MI) used by our animal care facility. Dust collected from the grain-drying system located outside at a local grain elevator that processed corn was used as bedding material to create the nonhygienic environment (experimental). Breeding pairs were housed in control bedding or corn dust bedding, and their litters either remained in these environments or were placed into the corn dust bedding as indicated. In preparation for the allergen studies, some mice were born into the control bedding and then were placed into corn dust bedding at 4 wk of age until the end of the study to create a "subchronic" exposure to a nonhygienic environment. Other mice were born and reared into the corn dust bedding and then moved into control bedding at 3 wk of age to create an "early life" exposure to corn dust. Three weeks of age was selected as the end point of the early life exposure to coincide with weaning from the mother.

The control and experimental bedding materials were assessed for endotoxin content by use of the kinetic chromogenic *Limulus* amoebocyte lysate (LAL) assay (Whittaker Bioproducts, Walkersville, MD) (38). Our LAL kit conversion from endotoxin units (EU) to nanograms was 9 EU/ng. Ambient dust particles from both environments were collected with a Button aerosol sampler (Eighty Four, SKC), an aerosol-sampling device specifically designed to obtain a representative sample of airborne dust in an environment. The endotoxin content of the airborne particulate matter was assessed by the LAL assay. This allowed us to calculate the concentration of airborne endotoxin (EU/m<sup>3</sup>) in cages of individual animals. The size of ambient particles from the control and experimental environments was determined by a Grimm portable dust monitor (series 1.100; Grimm Technologies, Douglasville, GA).

**ELISA for (1-6)-branched, (1-3) $\beta$ -D-glucan.** Fungal cell wall components were detected by ELISA, as recently described (22). Briefly, the dust samples were suspended in pyrogen-free PBS with 0.05% Tween 20. Samples were agitated for 1 h at room temperature, autoclaved at 120°C for 1 h, and then centrifuged at 600 g for 20 min. The ELISA plates were prepared by coating with a mouse monoclonal anti-(1-6)-branched, (1-3) $\beta$ -D-glucan antibody (Murex Biotech Limited). A 100- $\mu$ l sample was applied to the coated plates in serial twofold dilutions. The plates were washed, and then a custom rabbit anti-Scleroglucan polyclonal antibody (1:2,500) was added and incubated for 1 h. The washed plates were incubated with goat anti-rabbit IgG horseradish peroxidase (Bioscience, Camarillo, CA). The washed plates were developed with tetramethylbenzidine substrate, and then the reaction was stopped with 0.18 M H<sub>2</sub>SO<sub>4</sub> and read at 405 nm optical density (OD). A standard curve was created with purified Scleroglucan extract (kindly provided by Dr. David William, East Tennessee State University).

**Microorganism determination.** Microorganisms contained within the corn dust bedding were detected by culture assay by previously

described methods (39). Specifically, three types of culture media were employed in duplicate to determine the total bacteria, gram-negative enteric bacteria, and total fungi load within the corn dust bedding.

**Animals.** Mice (6- to 8-wk-old C3HeB/FeJ) were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were housed together in an isolation cubicle with microisolation lids on each cage. Breeder mice were acclimated to either the control or corn dust environment for 2 wk before they were paired. Litters were maintained in either the corn dust or control bedding environments. Some animals were euthanized on day of life (DOL) 10, DOL 16, or DOL 21 to characterize the effects of the control and corn dust bedding environments on the developing immune system. At 21 days of age, all mice were weaned and placed into a control bedding environment. Weaned mice received water-softened mouse chow (Formulab Chow 5008; Purina Mills, Richmond, IN) until it was determined that they could obtain adequate nutritional support from the dry mouse chow. Mice were provided water and food ad libitum. Animal care and housing requirements set forth by the National Institutes of Health Committee on Care and Use of Laboratory Animal Resources were followed. The University of Iowa Institutional Animal Care and Use Committee approved the protocols used in this study.

**Allergen exposure.** At 8 wk of age, mice raised in a control bedding environment and mice exposed to corn dust either early in life or as a subchronic exposure starting at 4 wk of age were sensitized and challenged to the allergen ovalbumin as previously described (14, 16). Briefly, the mice were sensitized to ovalbumin via an intraperitoneal injection (100  $\mu$ l) of 20  $\mu$ g ovalbumin emulsified in 2 mg alum on allergen exposure days 0 and 7. Mice were then challenged by inhalation of ovalbumin (1% solution in normal saline, generated by an Aero-Tech nebulizer, CIS-US) for 30 min on days 14 and 16. Animals were then killed on day 18 of the allergen exposure, at 74 days of age.

**BAL cell collection.** Mice were euthanized with an overdose of Nembutal (150 mg/kg) and their tracheas were cannulated with PE tubing sized according to their age. We adapted our previously published protocol to accommodate smaller mice (9). The lungs of mice 10–21 days of age were lavaged with 2 ml of pyrogen-free normal saline (Baxter), and adult animals were lavaged with 4 ml of normal saline. Normal saline was allowed to passively flow into and out of the lungs in aliquots ranging from 100  $\mu$ l to 1 ml according to the size of the animal. The collected lavage fluid was centrifuged at 830 g for 5 min at 4°C. The pelleted cells were then resuspended in 200  $\mu$ l of HBSS. With the use of a hemacytometer and trypan blue staining, a count of living cells was obtained. In our laboratory, 98% of the cells collected from lavage fluid using this technique were live cells. A cytospin preparation of 75–100  $\mu$ l of the resuspended cells was stained with Hema 3 stain set (Fisher Scientific, Middletown, VA) to obtain a cell type differential count. Cell types counted were macrophages, lymphocytes, neutrophils, and eosinophils. All total and differential cell counts were performed on masked slides by two independent observers and then averaged for each animal.

**Cytokine determination.** Cytokine identification and quantification from BAL fluid collected from 10-wk-old mice were performed using a multiplex protein kit that utilizes fluorescence antibody capture techniques (Novagen, Madison, WI). The cytokines detected in this assay were IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-10, IL-12 p70, granulocyte macrophage-colony stimulating factor (GM-CSF), IFN- $\gamma$ , and TNF- $\alpha$ . BAL fluid was collected, and the cellular components were removed by centrifugation as described above. The supernatant was stored at -80°C until analyzed. Once thawed, the samples were diluted 1:1 with assay diluent and assayed per the manufacturer's instructions. The range of cytokine concentration detection was 5–2,500 pg/ml.

**Immunoglobulin assay.** Our protocol was adapted from a previously described method (29). Briefly, serum levels of ovalbumin-specific IgE were measured by capture ELISA. Ninety-six-well plates

were incubated with 10 mg/ml ovalbumin in RPMI 1640 medium. After an overnight incubation, wash, and blocking step with 10% FCS in PBS, duplicate samples of serum (1:50 dilution) were incubated overnight in the plate at 4°C. After a washing step, ovalbumin-captured IgE was detected with biotinylated rat anti-mouse-IgE (clone R35-118, BD PharMingen, San Diego, CA). After another washing step, avidin peroxidase (BD PharMingen) was added to each well. The reaction was developed by adding tetramethylbenzidine substrate (BD PharMingen) after a final washing step. Units were reported as OD readings at 450 nm. The control, unexposed animal values were made equal to 1, and the values for the exposed animal were adjusted accordingly.

**Immunohistochemistry.** Mice (21 days old) were euthanized, their trachea cannulated with PE-50 tubing, and their lungs inflated with 0.5 ml 70% OCT compound (Sakura Finetek USA, Torrance, CA) in PBS and then frozen in an acetone-dry ice bath. Frozen sections of lung tissue (5  $\mu$ m) were first fixed in cold acetone-ethanol (3:1), pretreated with 0.3% hydrogen peroxide in PBS containing 0.3% horse serum, and then blocked with an avidin and biotin blocking kit (Vector Laboratories, Burlingame, CA). The tissue sections were then incubated with an MHC class II anti-mouse I-E<sup>k</sup> antibody (clone 17-3-3, BD PharMingen). The "Mouse on Mouse" light microscopy or fluorescein kits from Vector were used to process the tissue sections per instructions from the manufacturer. Slides for light microscopy were developed with 4% diaminobenzidine and counterstained with Meyer's hematoxylin. Light microscopy images of lung tissue were captured with a Spot Jr. digital camera. Dendritic cells were similarly identified with an antibody specific for the surface marker CD11c (clone N418, Serotec, Raleigh, NC) and processed with streptavidin Alexa Fluor 568 for immunofluorescent staining. The antibody diluent, PBS, was used without the primary antibody as a control. Confocal microscopy images of immunofluorescence were captured with a Zeiss 510 microscope. For quantitative analysis of the antigen-presenting cells within lung tissue, two individuals blinded to the condition counted the number of I-E<sup>k</sup>-positive cells per  $\times 20$  alveolar field or the number of positive cells within the intraepithelial area of a conducting airway using light microscopy. Consecutive alveolar images from the inferior portion of the left lung were obtained avoiding large blood vessels and conducting airways. As previously described, images of conducting airways were collected, and obliquely cut conducting airways were not analyzed (8). To obtain the ratio of CD11c+ I-E<sup>k</sup>+ cells to CD11c- I-E<sup>k</sup>+ cells, two observers counted 100 consecutive fluorescent cells. The data were collected from four or five images/animal and averaged between the two individuals.

**Assessment of perivascular inflammation.** Adult mice were euthanized, their right heart flushed with PBS, and their trachea cannulated with PE-90 tubing. The lungs were inflated with zinc-formalin solution (LABSCO, Louisville, KY) at a pressure head of 25 cmH<sub>2</sub>O. Whole lungs were processed into paraffin blocks and sectioned posterior to anterior in a caudal-cranial plane. Lung tissue sections 5  $\mu$ m thick, obtained 75  $\mu$ m from the posterior aspect, were mounted onto glass slides. These lung sections were stained with hematoxylin and eosin. A perivascular inflammatory grading scale was adapted from a previously published study that focused on the number and density of mononuclear cells within the perivascular areas of interstitial blood vessels (18). Our scale ranged from 1 to 4, with grade 1 defined as none to few mononuclear cells (as typically seen in the majority of control animals), grade 2 defined as mononuclear cells 1 or 2 cells thick around the vessel, grade 3 defined as perivascular areas two to three cells thick surrounding the vessel, and grade 4 defined as densely packed ( $>5$  cells thick) inflammatory cells of at least  $\sim 50\%$  of the vessel. Two individuals blinded to the condition assessed 100 blood vessels per animal and graded them according to the degree of mononuclear inflammatory cells in the perivascular area. The percent of blood vessels in each grade for each animal ( $n = 7-10$  animals/group) was averaged between the two observers.

**Statistics.** Student's two-sample *t*-test was used to compare means between two groups for normally distributed data. For comparing multiple groups to test for the effect of two factors (i.e., exposure condition and age) and their interaction, 2-way ANOVA was used. Bonferroni adjustment of the *P* value was applied when multiple comparisons were performed. For variables that were not normally distributed, such as cell count, a geometric mean was calculated. A *P* value of  $<0.05$ , or less than the critical *P* value using the Bonferroni adjustment, was considered statistically significant. All data are expressed as means  $\pm$  SD. The degree of interobserver agreement of the inflammatory score was measured by the interobserver coefficient of variation and the intraclass correlation, with an intraclass correlation  $>0.75$  considered to be very good agreement. The interclass correlation between the two observers was 82% with 95% confidence intervals. All the statistical analysis for this study was performed with SigmaStat 3.0 software.

## RESULTS

**Characterization of the environmental exposures.** The corn dust that was used as bedding material contained a 57 times greater concentration of endotoxin compared with the control bedding [ $1,246 \pm 341$  EU/mg in the corn dust (0.14  $\mu$ g/mg) vs.  $22 \pm 13$  EU/mg (0.002  $\mu$ g/mg) in the control bedding;  $P < 0.001$ ]. Endotoxin measurements were also obtained from control bedding animal cages with three mice per cage, at four time points (time 0, 24 h, 72 h, and 7 days). The mean endotoxin concentration in the control bedding over time was  $5.55 \pm 6.42$  EU/mg bedding, with a range of 1.78–15.12 EU/mg. Therefore, the contribution of mice living in a cage over time toward the total endotoxin exposure was negligible compared with the high concentration of endotoxin in the corn dust. The corn dust bedding contained 0.27  $\mu$ g/mg (1–3) $\beta$ -D-glucan, 760 colony forming units (CFU)/mg culturable fungi, 540 CFU/mg culturable mesophilic bacteria, and 30 CFU/mg culturable thermophilic bacteria. In comparison, the control bedding contained  $<10$  CFU/1,000 mg fungi and  $<3$  CFU/1,000 mg mesophilic bacteria. Size distribution analysis of ambient particles obtained from representative animal cages that contained either corn dust or control bedding demonstrated that over 90% of airborne particles were smaller than 1  $\mu$ m aerodynamic diameter in both environments (Fig. 1). The average concentration of airborne particulate matter in the corn dust bedding environments was similar to the control bedding environments ( $0.61 \pm 0.59$  mg/m<sup>3</sup> vs.  $0.49 \pm 0.41$  mg/m<sup>3</sup>, respectively). The inhalable airborne endotoxin concentration averaged  $620 \pm 198$  EU/m<sup>3</sup> (0.07  $\mu$ g/m<sup>3</sup>) in the corn dust bedding environments and was significantly higher ( $P < 0.001$ ) than in the control environments [ $43 \pm 1.78$  EU/m<sup>3</sup> (0.005  $\mu$ g/m<sup>3</sup>)].

**Both early life and later life subchronic exposure to corn dust reduce atopic airway inflammation.** The airway response to sensitization and challenge with ovalbumin in mice raised in control bedding was compared with that of mice who experienced a subchronic corn dust bedding exposure (4 wk of life through the end of the experiment) or an early life exposure to corn dust (from conception through 3 wk of age). Ovalbumin-treated mice raised in control bedding developed significant BAL pleocytosis and eosinophilia compared with untreated mice (Fig. 2A;  $P < 0.001$ ). Mice with a subchronic exposure to the corn dust bedding developed significantly less cellular airway inflammation (Fig. 2A;  $P < 0.01$ ) and BAL eosinophilia (Fig. 2B;  $P < 0.05$ ) than did those mice raised in control

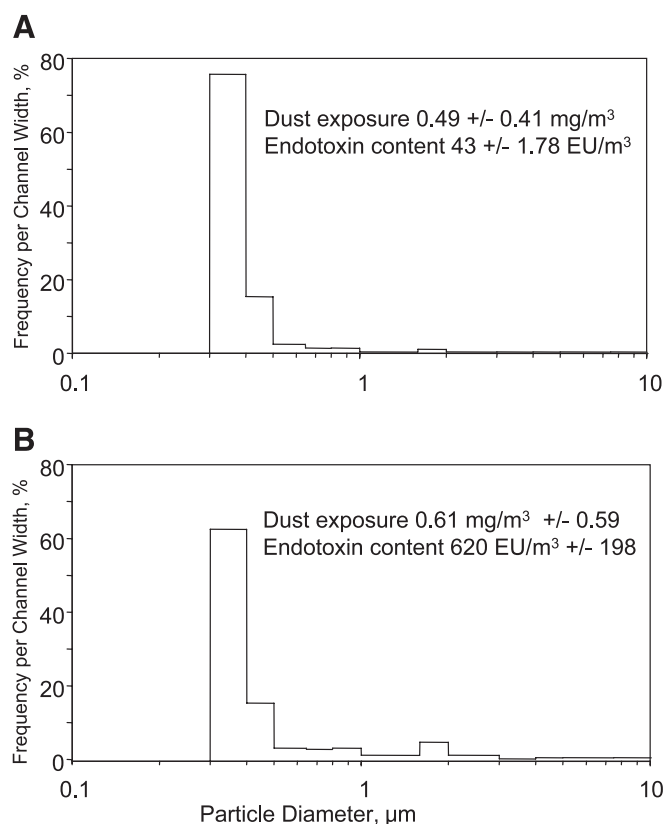


Fig. 1. Graphic representation of particle size data collected from 2 mouse cages containing control (A) or corn dust (B) bedding. Data represent percentage of ambient particles counted in each size channel by a Grimm portable dust monitor. Ambient particle endotoxin concentrations were assayed during five 24-h sample collection periods throughout the experimental period using a Button sampler, gravimetric analysis, and the *Limulus* amoebocyte lysate assay. The dust exposure and endotoxin data are presented as means  $\pm$  SD. EU, endotoxin unit.

bedding. Interestingly, mice with only an early life exposure to corn dust bedding also demonstrated significantly fewer total BAL cells (Fig. 2A;  $P < 0.01$ ) as well as BAL eosinophilia (Fig. 2B;  $P < 0.05$ ) compared with mice reared in control bedding, in response to ovalbumin treatment.

*Early life exposure to corn dust bedding alters the perivascular but not peribronchial inflammatory response.* Histological examination of lung tissue demonstrated peribronchial inflammatory changes after ovalbumin treatment compared with mice not treated with ovalbumin (Fig. 3). Similar inflammatory changes around the conducting airways were observed in ovalbumin-treated mice reared in either control bedding or corn dust bedding. However, a key difference between the groups was seen in ovalbumin-induced development of a perivascular monocytic infiltrates (Fig. 4A). Using an inflammation scoring method for perivascular responses (18) (Fig. 4B), we found that mice with early life exposure to corn dust demonstrated a significant reduction in intensity of perivascular inflammation compared with control mice, after ovalbumin-treatment (Fig. 4C) (18). There was a significant decrease ( $P < 0.05$ ) in ovalbumin-induced grade 3/4 perivascular inflammation in mice exposed to corn dust early in life and a corresponding increase in grade 1/2 perivascular inflammation (Fig. 4C).

*Corn dust exposure does not alter the induction of ovalbumin-induced antibodies.* Despite a modulation of ovalbumin-induced airway inflammation by early life exposure to corn dust, the systemic response to ovalbumin was not affected. This was demonstrated by an ELISA determination of ovalbumin-IgE levels in serum that showed there was no difference between ovalbumin-treated mice with and without an early life exposure to corn dust ( $OD = 15.08 \pm 2.74$  and  $18.40 \pm 2.20$ , respectively;  $P > 0.1$ , determined by Student's *t*-test, from 3 separate experiments).

*Corn dust bedding exposure does not induce pulmonary inflammation.* To evaluate whether exposure to the corn dust alone may induce significant airway inflammation, we evaluated the pulmonary cellular response of adult mice from the four environmental exposure groups. We found no significant difference in total BAL fluid cell number (Fig. 5A) or airway neutrophilia (Fig. 5B) between the control mice or mice exposed to corn dust early in life, later in life, or continuously. The majority of BAL cells in all animals were alveolar macrophages ( $>85\%$ ), whereas neutrophils averaged only 7% of

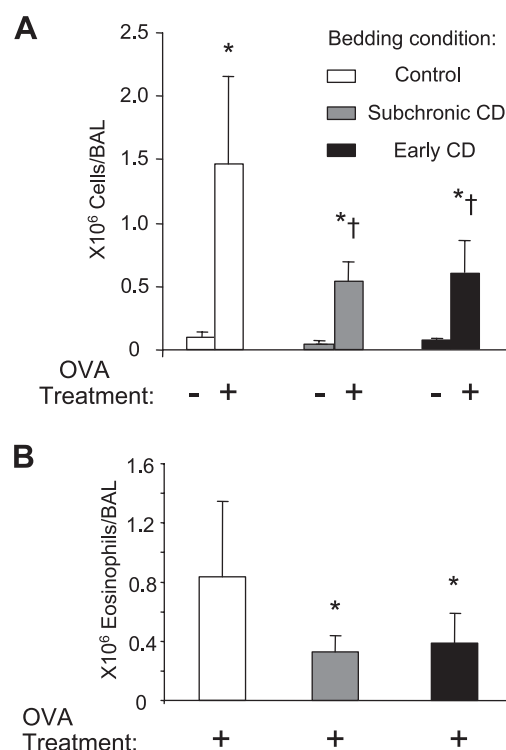


Fig. 2. A: number of total cells in bronchoalveolar lavage (BAL) fluid in 74-day-old mice, with (+) and without (−) ovalbumin (OVA) treatment. Animals treated with OVA had significantly more cells compared with non-OVA-treated animals ( $*P < 0.001$  by 2-way ANOVA). OVA-treated mice with a subchronic exposure to corn dust (CD) bedding (from 4 wk of life until the end of the OVA treatment) had significantly fewer cells in BAL fluid than untreated control mice (control + OVA vs. subchronic CD + OVA;  $\dagger P < 0.01$ , 2-way ANOVA). Ova-treated mice with an early life exposure to corn dust bedding (from conception until 3 wk of life) also had a significant decrease in total cells in BAL fluid compared with OVA-treated control bedding mice (control + OVA vs. early CD + OVA;  $\dagger P < 0.01$ ). B: eosinophils in BAL fluid were significantly reduced in number after OVA treatment in mice with either a subchronic or early life exposure to corn dust ( $*P < 0.05$ , Student's *t*-test) compared with mice reared in control bedding. Mice that did not receive OVA treatment do not have eosinophils in their BAL fluid (data not shown). Data are means  $\pm$  SD;  $n = 10$ –17 animals/condition.

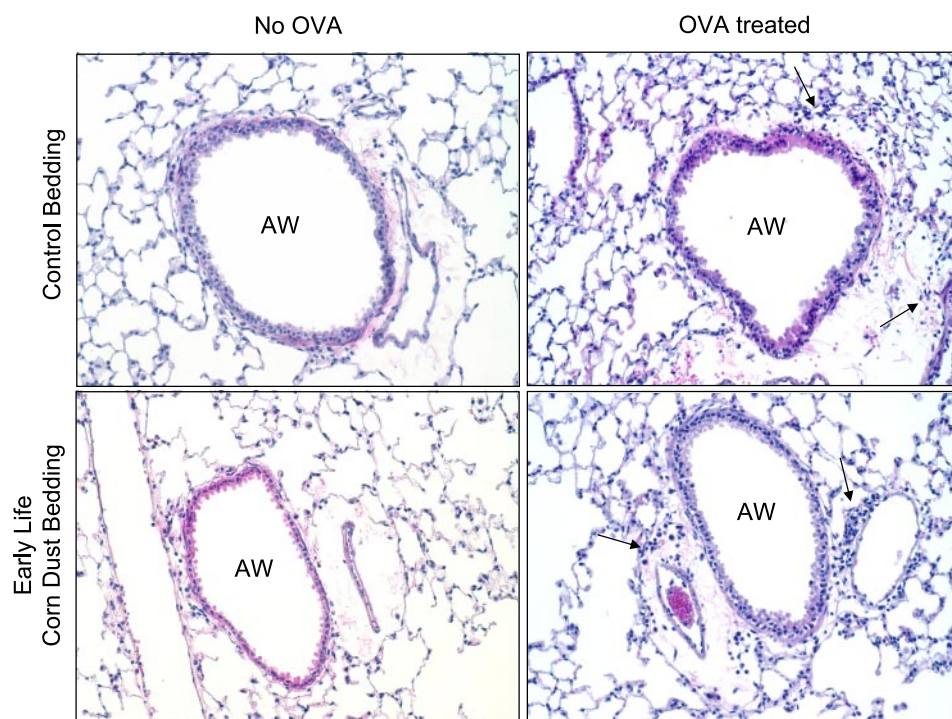


Fig. 3. Representative photomicrographs of hematoxylin and eosin-stained lung tissues from mice with and without OVA treatment, with and without early life exposure to corn dust bedding. OVA-treated mice have mononuclear-infiltrating cells present near the conducting airways (AW) in both early life environmental conditions (arrows), whereas mice not treated with OVA lack these infiltrating cells. No difference in the OVA-induced inflammatory response surrounding the conducting airways was observed when comparing the 2 early life exposure groups.

the BAL cells. Cytokine analysis of BAL fluid similarly demonstrated that exposure to corn dust-bedding did not significantly alter the concentration of IL-6, IL-10, IL-12, GM-CSF, IFN- $\gamma$ , or TNF- $\alpha$ , compared with control bedding exposed animals (Fig. 6). Interestingly, the number of neutrophils and the concentration of TNF- $\alpha$  in BAL fluid were lowest in the subchronic group; however, these changes were not statistically significant. Other cytokine proteins detected in this assay were below the level of detection in the BAL fluid.

**Maturation of pulmonary immune cells.** To better understand the immune effects of early life environment, control and corn dust-exposed mice were compared at several time points. Between DOL 10 and DOL 21, there was a rapid and significant increase in the number of immune cells present in BAL fluid (Fig. 7A). At DOL 10, eosinophils comprised a sizable proportion of the BAL cells isolated from mice raised in control bedding as well as those raised in corn dust bedding (Fig. 7B), consistent with the understanding of the normal early life immune environment being Th2 skewed. During this period of development, mice reared in corn dust bedding demonstrated significantly more eosinophils compared with control mice ( $P < 0.05$ ). As the animals mature, the eosinophil numbers decreased toward adult levels ( $\sim 0.5\%$  of total BAL cells), whereas alveolar macrophages increased proportionately throughout maturation (data not shown). Mice reared in the corn dust bedding tended to have increased numbers of neutrophils in their BAL fluid; however, this difference did not reach statistical significance ( $P = 0.054$ , DOL 10–21) (Fig. 7C). By DOL 21, mice reared in either environmental condition had a BAL fluid cell profile similar to that observed in adult mice (9).

Pulmonary antigen-presenting cells in the lung parenchyma were evaluated by immunostaining. MHC class II (I-E<sup>k</sup>)-positive cells were detected within the epithelium of the conducting airways and surrounding areas as well as within alve-

olar walls (Fig. 8A). At DOL 21, there was a significant ( $P < 0.01$ ) increase in the number of MHC class II-positive cells within the alveolar spaces of mice reared in corn dust compared with the number of cells in mice reared in control bedding (Fig. 8B). There was, however, no significant difference in the number of MHC class II-positive cells within the epithelium of conducting airways in mice reared in control bedding vs. those reared in corn dust (Fig. 8B).

We hypothesized that the increased antigen-presenting cell populations in the lung were likely to be dendritic cells. This was suggested by the localization of the MHC class II-positive cells within the lung tissue walls and colocalization of I-E<sup>k</sup> (MHC class II protein) and CD11c (common dendritic cell surface marker) in all of the cells within the conducting airway epithelium and the majority of cells within the alveolar wall (Fig. 9A). The proportion of CD11c+ I-E<sup>k</sup>+ cells (dendritic cells) to CD11c– I-E<sup>k</sup>+ cells (macrophages) was similar between mice raised in control and corn dust conditions (Fig. 9B).

## DISCUSSION

Many have theorized that environmental exposures can tip the developing immune system toward either the expression of genes associated with allergic disorders or toward nonallergic genes (48). Our murine model of environmental exposure to a “nonhygienic” dry organic dust environment has allowed us to investigate the influence of early life environment on the developing pulmonary immune system. In this study, we first demonstrated that the corn dust environment contains respirable particulate matter. In mice, and other small rodents, particles  $< 1 \mu\text{m}$  aerodynamic diameter are likely to reach the alveolar region (13). The composition of our corn dust bedding was complex. We confirmed that the level of ambient endotoxin in the corn dust environment was significantly higher than in the control bedding environment. Additionally, the corn

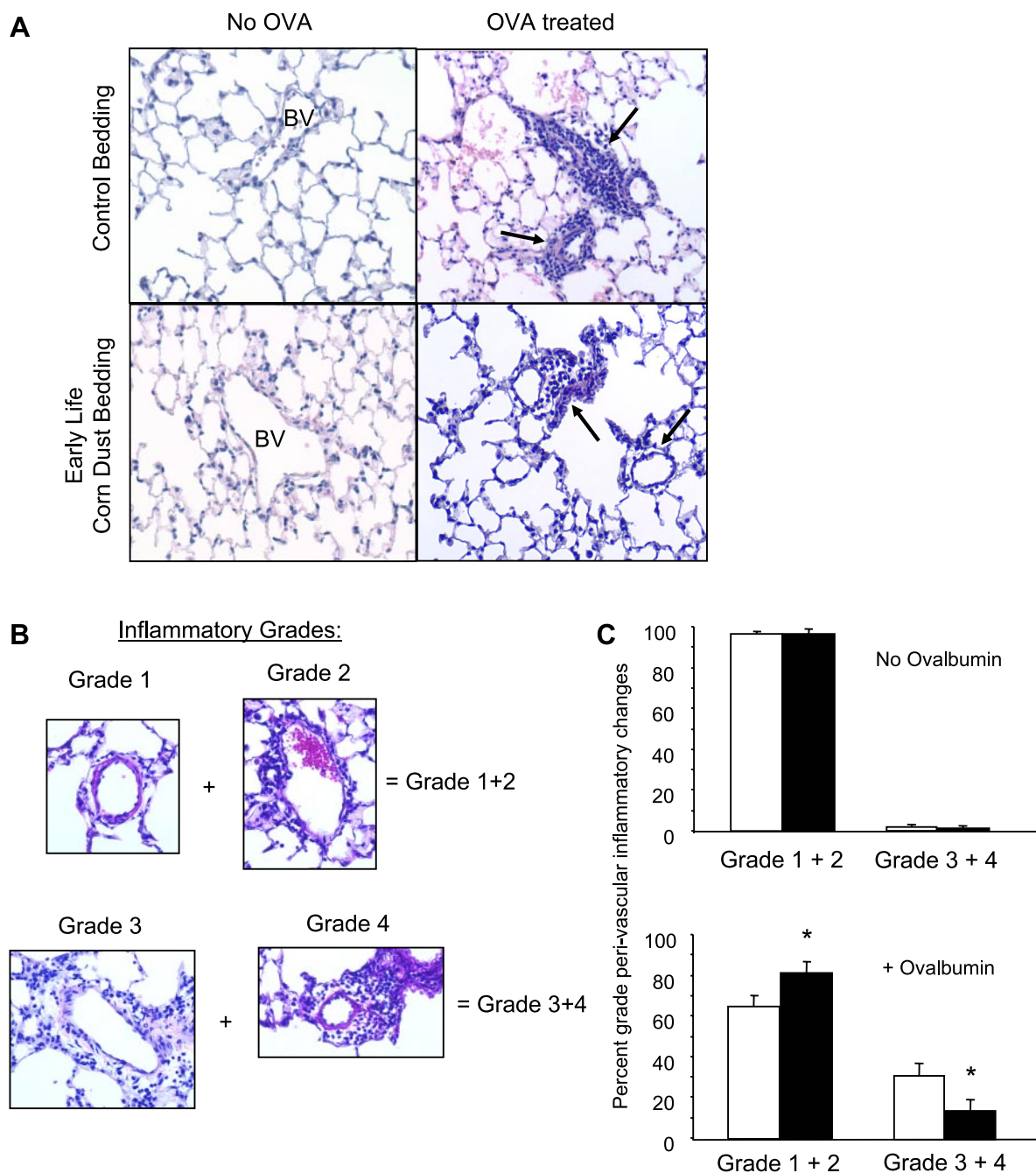


Fig. 4. A: representative photomicrographs of blood vessels (BV) within lung tissue with and without OVA treatment. Mice that were not treated with OVA did not demonstrate perivascular inflammation. Typical inflammatory changes after OVA treatment in the perivascular regions are indicated (arrows). Less perivascular inflammation was observed in the lungs of mice exposed to corn dust bedding early in life compared with those mice that were reared in control bedding. Although both early life exposure groups had OVA-induced mononuclear inflammatory cell infiltrates around blood vessels, mice reared in corn dust bedding had less interstitial inflammation than mice reared in control bedding. B: grading scale used to assess perivascular inflammation. Grades 1–4 represent a progressively increasing density of mononuclear inflammatory cells in a circumferential pattern around blood vessels. C: percentage of blood vessels within each grade of inflammation; 100 blood vessels were graded from each animal. Early life exposure to corn dust (black bars) significantly decreased ( $*P < 0.05$ ) the number of lung blood vessels with grade 3/4 inflammation and increased the number of blood vessels with grade 1/2 inflammation after OVA treatment compared with mice reared in control bedding (white bars). Data are means  $\pm$  SD;  $n = 7$ –10 animals/condition.

dust bedding also contained microorganisms and immunologically active branched  $\beta$ -glucans of fungal origin. Like endotoxin,  $\beta$ -glucans can favor production of a Th1 cytokine profile (37). When administered orally in a swine model of influenza infection,  $\beta$ -glucans both increase IFN- $\gamma$  in the lung and decrease the viral load (15). In summary, our murine model

contains several components in addition to endotoxin that are immunologically active.

We chose to focus on the inflammatory response to an allergen as an endpoint because both animal models and epidemiologic studies of large populations have found a close association between environmental exposure to endotoxin and

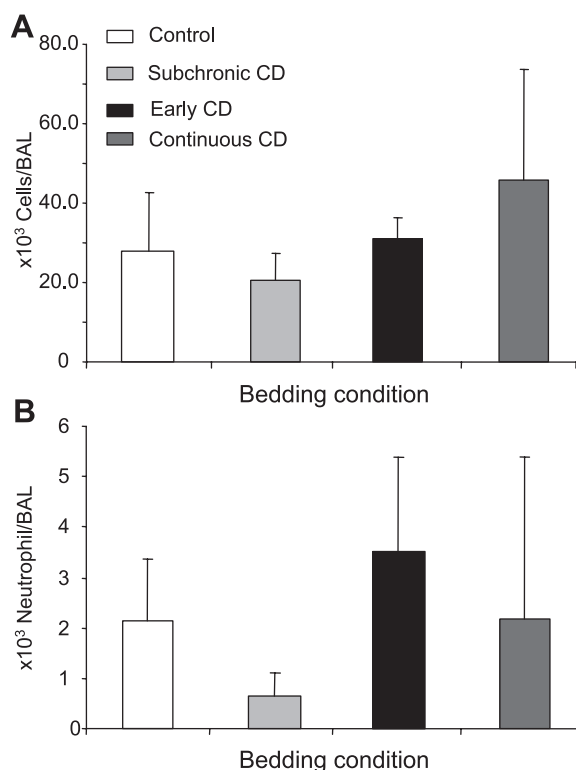


Fig. 5. A: numbers of cells in BAL fluid. Cell counts were determined in 74-day-old mice that experienced 1 of 4 environmental conditions (control bedding, and subchronic, early life, or continuous exposure to corn dust bedding). There were no significant differences in cell numbers between any of the corn dust bedding exposures and the control mice. B: number of neutrophils in BAL fluid. There were no significant differences in the total number of neutrophils in any of the corn dust exposures compared with control mice. Data are means  $\pm$  SD;  $n = 4$  animals/condition.

markers of atopy (5, 10, 11, 21). Mice who experienced a subchronic exposure to corn dust bedding (from 4 to 10.5 wk of life) were characterized by an attenuated lung cellular inflammatory response as reflected by the number of total BAL fluid cells and eosinophils. A similar reduction in eosinophilia

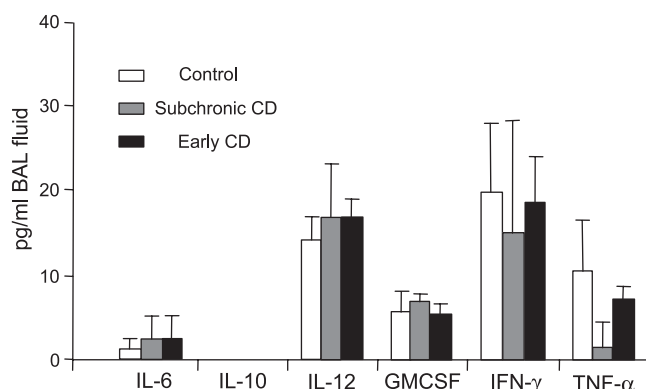


Fig. 6. Cytokine levels in BAL fluid. Cytokines were measured in BAL fluid obtained from 74-day-old mice that had been exposed to either control bedding or subchronic corn dust bedding (from 4 wk to 74 days of life) or to early life corn dust bedding (from conception until 3 wk of life). There were no significant differences between the control mice and the 2 corn dust bedding exposure groups in the levels of IL-6, IL-10, IL-12, granulocyte macrophage-colony stimulating factor (GM-CSF), IFN- $\gamma$ , or TNF- $\alpha$ . Data are means  $\pm$  SD;  $n = 3$  or 4 animals/condition.

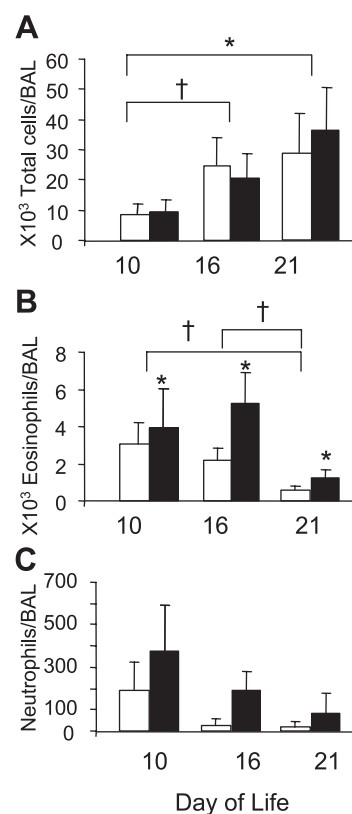


Fig. 7. BAL white blood cell (WBC) populations during development. A: total WBC counts in mice reared in control bedding (white bars) vs. in corn dust bedding (black bars) were determined at day of life (DOL) 10, DOL 16, and DOL 21. The total number of BAL cells increased with increasing age (\* $P < 0.05$ , DOL 10 vs. DOL 21; † $P < 0.01$ , DOL 10 vs. DOL 16; 2-way ANOVA). There was no difference between the number of BAL cells in mice reared in the control and corn dust environments at any stage of development. B: eosinophil numbers in BAL fluid. The total number of eosinophils obtained from BAL fluid was significantly greater in the corn dust-reared mice compared with the control mice (\* $P < 0.05$ , 2-way ANOVA). Over time, the eosinophil number decreased with age in all mice. C: neutrophil numbers in BAL fluid. Mice reared in corn dust bedding tended to have increased numbers of BAL neutrophils compared with the control-bedding mice; however, the difference was not statistically significant ( $P = 0.054$ , 2-way ANOVA). The number of neutrophils in both environments decreased with age. Data are means  $\pm$  SD;  $n = 7$ –13 animals/condition at each age.

has been demonstrated by Tulic et al. (43) in a rat model of ovalbumin-induced lung injury when the animals were treated with intraperitoneal LPS before or just after the intraperitoneal sensitization dose of ovalbumin (43). In our studies, we also bred mice into the corn dust environment and then removed them at 3 wk of age to limit their environmental exposure. With this experimental design, our results show that exposure to the corn dust environment early in life also can attenuate the inflammatory response that the adult mouse develops in response to an allergen. A similar reduction in ovalbumin-induced BAL pleocytosis and eosinophilia has been described recently in a model of in utero and perinatal exposure to LPS (3).

In addition to a reduction in BAL pleocytosis and in eosinophil numbers, the mice exposed to the corn dust bedding environment early in life also had reduced pulmonary interstitial inflammation. Typically, murine models of ovalbumin-induced allergic lung injury are characterized by peribronchial and perivascular inflammation. Whereas all animals treated

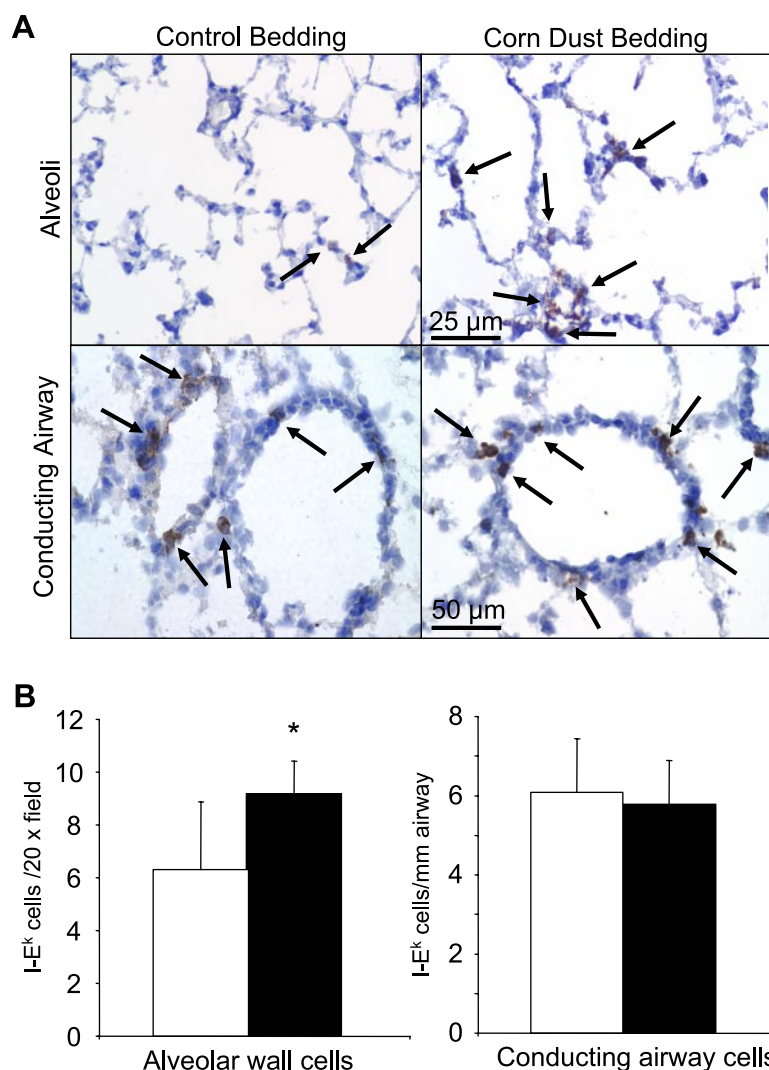


Fig. 8. Major histocompatibility complex (MHC) class II cells in the lung. Representative photomicrographs from 21-day-old mice reared in control and corn dust bedding. *A*: cells that stain positive for the I-E<sup>k</sup> protein (MHC class II positive cells) are brown and are indicated with arrows. *B*: quantitation of MHC class II cells in the lung. Corn dust bedding (black bars) did significantly increase the number of MHC class II + antigen-presenting cells in alveolar wall tissue but did not affect the number of antigen-presenting cells in conducting airways of 21-day-old mice, compared with cell numbers in mice reared in control bedding (white bars). Data are means  $\pm$  SD;  $n = 4$  or 5 animals/condition. \* $P < 0.01$ .

with ovalbumin in the present study were observed to have mononuclear inflammation in the peribronchial region, one obvious difference in the lung histology of mice exposed to corn dust early in life was the reduced degree of mononuclear infiltrating cells surrounding blood vessels. Most studies that used murine models of allergen-induced lung inflammation do not distinguish between various compartments of lung when evaluating interstitial inflammation or limit the focus to the peribronchial compartment. The assumption that the respiratory tract beyond the level of the mainstem bronchi is uniform in immune system function may not be correct. Legge and Braciale (19) demonstrated that the dendritic cell population associated with pulmonary conducting airways have a rate of turnover different from those in the alveolar compartment. Moreover, dendritic cell activity in the conducting airways and peripheral lung tissue may be under the control of local mediators that create unique microenvironments in these sites (46). Finally, in a murine model of inhalation exposure, it was shown that diesel exhaust particles induce perivascular inflammation that develops in the absence of peribronchial inflammation. This is in contrast to ovalbumin-induced inflammatory changes where both lung compartments (perivascular and peribronchial) developed mononuclear infiltrates (34). Therefore, it

is possible that the perivascular and peribronchial immune system compartments in the lung are differentially affected by stimuli and this accounts for the altered perivascular histology that we observed after ovalbumin treatment.

In contrast to the pulmonary response to ovalbumin, serum ovalbumin-IgE levels were unaffected by the corn dust environment. Others have demonstrated that when the endotoxin is delivered systemically, even in the prenatal period, serum ovalbumin-IgE levels are decreased (3). One significant difference between our experimental design and other models is that our endotoxin exposure utilizes spontaneous inhalation of respirable microbial products, whereas others use intraperitoneal injections of LPS (3) or aerosolization of purified endotoxin (44). Our results, in contrast to these other models of endotoxin exposure, do not support the epidemiological studies that found an association with endotoxin-laden dust in agricultural domestic settings and a reduced incidence of atopy (21, 47). One reason that our exposure model did not attenuate the systemic allergic response may be that the corn dust bedding environment is a less potent stimulus compared with the delivery of purified endotoxin, as suggested by the different immune responses associated with high- vs. low-dose endotoxin exposures (6).

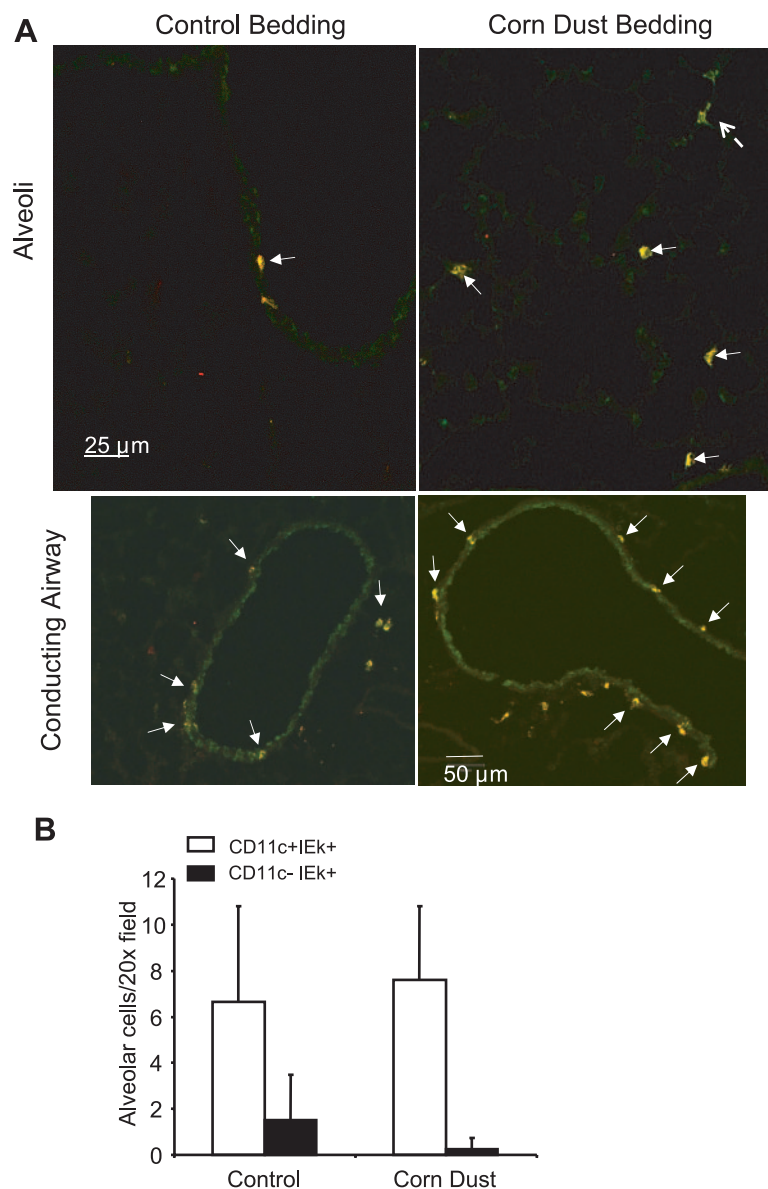


Fig. 9. Antigen-presenting cells in the lung. **A**: representative images of immunofluorescence staining from 21-day-old mice demonstrate that MHC class II-positive cells (I-E<sup>k</sup> antibody, green color) also stain for CD11c (a dendritic cell marker, red color). Colocalization of both antigens is indicated by yellow fluorescence (solid arrows) and was detected in both the conducting airways and in the alveolar walls. A minority of cells in the alveoli stained for the MHC class II protein I-E<sup>k</sup> only (dashed arrows), and these may represent macrophages. **B**: ratio of CD11c-positive MHC class II-positive cells to CD11c-negative MHC class II-positive cells. There was no significant difference in the ratio of the CD11c+ I-E<sup>k</sup>+ to CD11c- I-E<sup>k</sup>+ cells present in the control or corn dust-exposed mice. CD11c+ I-E<sup>k</sup>- cells made up <1% of the total cell population in both groups of mice. Data are means  $\pm$  SD;  $n$  = 3 animals/condition.

The corn dust bedding environment was not associated with an inflammatory response in pulmonary alveoli at any time point examined in this study. We specifically chose the C3HeB/FeJ strain of mouse for our studies because it is particularly sensitive to endotoxin with respect to the recruitment of neutrophils into the lower airways (20). We anticipated that an environment with endotoxin levels comparable to previously described agricultural and domestic settings (10, 47) would be associated with neutrophilia. The lack of a significant increase in the number of neutrophils in BAL fluid both in the very young or mature mouse makes our corn dust endotoxin exposure very different from previously published aerosolized endotoxin models where a significant influx of neutrophils into the lung is observed (8, 44). An increase in the number of neutrophils tends to reflect the endotoxin level delivered to the lung. The endotoxin concentrations in the respirable portion of the airborne corn dust environment was equal to or higher than what has been observed in domestic settings (47). However, it is difficult to compare our dust

exposure to animal models presented in the literature, as many deliver a set amount of purified endotoxin directly to the airway (6), whereas others do not report the airborne concentration when a nebulizer apparatus is utilized (44). Furthermore, it is possible that animal models that use high-dose purified endotoxin exposures to study endotoxin-induced immunomodulation may produce a very different immune system response that might be occurring in the rural household exposures described in the hygiene hypothesis. Finally, because our mice did not demonstrate the expected neutrophil response to endotoxin, yet demonstrated an attenuated response to the ovalbumin, other immunologically active components within a nonhygienic environment may be important in altering allergic immune responses.

We failed to identify alterations in the BAL fluid of either proinflammatory cytokines (TNF- $\alpha$  and IL-6) or in moderating cytokines (IL-10, IL-12, IFN- $\gamma$ , and GM-CSF) compared with levels in control mice. These results were unexpected because we and others have demonstrated that repeated exposure to

aerosolized or intratracheal delivery of endotoxin-containing solutions is associated with the increased production of several proinflammatory cytokines (8, 45). Because the corn dust bedding environment did not alter BAL fluid cytokine levels or neutrophil numbers, we speculated that we might find alterations within the immune cells earlier in life and that with time the animal had developed a tolerance to this environment.

We next focused on the immune-modulating effects of corn dust on immature mice. We report here for the first time that normal inbred mice demonstrate significant airway eosinophilia early in life. This predominance of eosinophils decreases rapidly during postnatal lung development. The high concentration of lung eosinophils early in life may be associated with the Th2 bias (or reduced Th1 presence) previously described in neonatal mice (1, 2, 33) and human infants (49). The presence of relatively large numbers of eosinophils in the alveolar space early in development has not previously been reported in human or murine studies and may be specific to this strain of mouse. This finding may be important because the change in eosinophil numbers occurs during a critical time during immune system development (7) and may influence how this strain of mouse responds to allergen stimulation. Both the control and corn dust environmental exposures were associated with increased eosinophil numbers during the first 3 wk of life compared with numbers in adult mice. In newborns and infants during the first year of life, failing to shift a Th2 cytokine profile to a Th1 profile has been associated with blood eosinophilia and serum markers of atopy (24). Others have demonstrated that there is no relationship between the number of eosinophils and an attenuated late asthmatic response with repeated low-dose allergen exposure (26). Therefore, investigation into the Th1/Th2 balance in very young mice outside of the BAL fluid compartment may help clarify the association between early life eosinophilia and a reduced later life allergic inflammatory response.

On completion of the early life exposure to corn dust at DOL 21, we detected increased numbers of antigen-presenting (dendritic) cells in lung tissue compared with the numbers in mice of the same age reared in control bedding. This increase was observed in alveolar walls but not in the conducting airways. This may reflect a more rapid turnover of dendritic cells in the upper airways compared with lower respiratory tract dendritic cells (19). It is unclear where the corn dust particles impact the lung and come into contact with the immune system in our murine model. However, given the delicate nature of the alveolar spaces required for gas exchange, it was anticipated that components of both the innate and phagocytic immune systems may be increased in animals exposed to the experimental nonhygienic environment.

The exposure to corn dust in our experimental model was complex and unique, involving not only the respiratory system but also the gastrointestinal system because it is likely that dust particles were ingested during grooming. A state of immune system tolerance can be induced after either ingestion or inhalation of immunoreactive substances. Specifically, in a murine model, oral administration of ovalbumin before intraperitoneal sensitization prevents the development of allergen-induced lung inflammation (31). Similarly, exposure of the nasal mucosa to ovalbumin before intraperitoneal sensitization also attenuates the lung's atopic inflammatory response (23). Therefore, it is possible the gastrointestinal tract, in addition to

the upper and lower respiratory tracts, contributed to the immune system changes that we observed in this study.

The original description of the hygiene hypothesis proposed that smaller family size, reduced infections early in life, and exposure to a more hygienic environment were associated with an increased prevalence of atopic disease (36). More recent studies support the idea of a critical period early in development in which environmental or infectious exposures can render the individual prone to either atopy or tolerance (27). Our results support this idea by demonstrating that early life exposure to a nonhygienic environment can influence how the mature individual responds to an allergic stimulus. We have also demonstrated that the corn dust exposure does not cause significant alterations in the cellular and cytokine protein composition of BAL fluid but results in more subtle alterations, specifically in the population of interstitial antigen-presenting cells. This novel animal model will allow investigation into the impact of diverse and complex environmental exposures on the developing immune system in the respiratory tract.

#### ACKNOWLEDGMENTS

The authors acknowledge Marsha O'Neill and Dr. Nervana Metwali for assistance with microbial analysis, glucan, and endotoxin measurements.

#### GRANTS

This work was funded by the American Lung Association; National Institutes of Health Grants ES-05605, HL-59324, HL-079448, and HL-50050; and a Parker B. Francis Pulmonary Fellowship.

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