

Inhibitory Effect of Kefiran on Ovalbumin-induced Lung Inflammation in a Murine Model of Asthma

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Kefiran is a major component of kefir which is a microbial symbiont mixture that produces jelly-like grains. This study aimed to evaluate the therapeutic availability of kefir on the ovalbumin-induced asthma mouse model in which airway inflammation and airway hyper-responsiveness were found in the lung. BALB/c mice sensitized and challenged to ovalbumin were treated intra-gastrically with kefir 1 hour before the ovalbumin challenge. Kefiran significantly suppressed ovalbumin-induced airway hyper-responsiveness (AHR) to inhaled methacholine. Administration of kefir significantly inhibited the release of both eosinophils and other inflammatory cells into bronchoalveolar lavage (BAL) fluid and lung tissue which was measured by Diff-Quik. Interleukin-4 (IL-4) and interleukin-5 (IL-5) were also reduced to normal levels after administration of kefir in BAL fluid. Histological studies demonstrate that kefir substantially inhibited ovalbumin-induced eosinophilia in lung tissue by H&E staining and goblet cell hyperplasia in the airway by PAS staining. Taken above data, kefir may be useful for the treatment of inflammation of lung tissue and airway hyper-responsiveness in a murine model and may have therapeutic potential for the treatment of allergic bronchial asthma.

Key words: Kefiran, Asthma, Inflammation, Airway hyper-responsiveness, Mucus, Ovalbumin-induced murine model

Abbreviations: OVA, ovalbumin; BALF, broncho-alveolar lavage fluid; AHR, airway hyper-responsiveness; H&E, hematoxylin and eosin; PAS, Periodic acid-Schiff; Dex, Dexamethasone; PBS, phosphate buffered saline; ipNeb, intra-peritoneal administration and nebulization; Th2, type 2 helper T cell

INTRODUCTION

Allergic asthma is characterized clinically by the hypersecretion of mucus, obstruction, and inflammation of the airways; enhanced bronchial reactivity (airways hyper-responsiveness, AHR) (Djukanovic et al., 1990; Bochner et al., 1994) to spasmogenic stimuli. Studies on the mediators of allergic lung inflammation have identified a number of effector cells, such as T cells, mast cells and eosinophils, and inflammatory mediators, such as cytokines, chemokines and leukotrienes that contribute to the

development and maintenance of airway inflammation. Although this inflammatory response is complex, clinical and experimental investigations have highlighted the obligatory role of CD4⁺ Th2 lymphocytes and the importance of eosinophils in the etiology of asthma (Robinson et al., 1992; Walker et al., 1991; Kon et al., 1998; Nakajima et al., 1992). The Th2-type, cytokines IL-4 and IL-5, have been directly linked with the immunopathogenesis of asthma (Robinson et al., 1993), and murine models have also provided corroborative evidence of the importance of these cytokines as central regulators of allergic disease (Cohn et al., 1997; Hogan et al., 1997; Brusselle et al., 1994).

Kefiran is a major component of kefir which is a microbial symbiont mixture that produces jelly-like grains. Kefir is a fermented milk drink produced by the action of lactic acid bacteria, yeasts and acetic acid bacteria,

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trapped in a complex matrix (kefir grains) of microorganisms, exopolysaccharides, and proteins (Farnworth and Mainville, 2003). Early observations of the structure of kefir grains noted that some of the bacteria were encapsulated by an exopolysaccharide called kefiran (La Riviere and Kooiman, 1967). Kefiran is the clear or pale yellow slimy polysaccharide gel and is a water-soluble branched glucogalactan. It has been reported to possess antibacterial, antimycotic and antitumor activity (Micheli et al., 1999). Kefir can be considered a probiotic resource, because it enjoys a variety of health claims besides its nutritional status. There are several studies investigating its immunomodulatory (Vinderola et al., 2005), anti-inflammatory properties (Rogers et al., 2002), anti-neoplastic and pro-digestive effects (Saloff-Coate, 1996). It was also demonstrated that while kefir induced a typical Th2 response by increasing the number of IL-4+, IL-6+, and IL-10+ cells, pasteurized kefir induced simultaneously the production of INF- γ and TNF- α (Vinderola et al., 2004). Although kefir was demonstrated to anti-inflammatory and anti-asthmatic effects in a previous study (Lee et al., 2007), it is not known what component may play a role in anti-asthmatic activity in a murine model.

We have employed a bronchial asthma model to examine the anti-allergic and anti-inflammatory effects of kefiran on asthmatic mice and investigated whether kefiran could influence the development of experimental asthma in already sensitized mice.

MATERIALS AND METHODS

Culturing of kefir grain

The freeze-dried kefir grain of 2% (w/w) was first grown at 25°C for 24 h in 200 mL of milk. Then, activated kefir grain for kefir culture was inoculated the fresh milk of 1 L and cultivated at 25°C for 24 h. The final pH of kefir fermentation was pH 4.8.

Isolation of kefiran

Before the isolation, the cultured kefir grains were washed with sterile distilled water. The kefir grains were treated in boiling water (1:100) for 1 h with stirring. The mixture was then cooled and centrifuged at 16,000 *g* for 15 min. The procedure was repeated once again. The supernatant was decanted and the polysaccharide was precipitated out by addition of an equal volume of cold ethanol and left at 4°C for overnight. The precipitate was resuspended in hot water (1:100) for 1 h with stirring and the precipitation procedure was repeated twice. The precipitate was finally lyophilized and used in this study.

Animals and experimental procedures

Specific pathogen-free female BALB/c mice, aged 8-10

weeks, were purchased from Daehan biolink Co. (Korea). All experimental procedures were carried out in accordance with the National Institutes of Health Guidelines for the care and use of laboratory animals, and were executed according to the National Animal Welfare Law.

Groups of mice (*n*=4 or 5), receiving the following treatment, were studied: (1) sham-sensitization plus challenge with PBS (ipNeb); (2) sensitization plus challenge with ovalbumin (OVA: Sigma A5503, St. Louis, MO) (ipNeb); (3) sensitization plus challenge with OVA and treated with kefiran.

Sensitization and airway challenge procedure was accomplished as our previous report (Lee et al., 2007). Briefly, mice were sensitized by intraperitoneal injection of 20 μ g OVA, which was emulsified in 2 mg aluminum hydroxide (Sigma), in a total volume of 100 μ L on days 1 and 14. The mice were challenged through the airways with OVA (1% in PBS) or PBS for 20 min on days 28, 29, and 30, after the initial sensitization, by ultrasonic nebulization (NE-U12; Omron Corp., Tokyo, Japan). Forty-eight hours after the last challenge (day 32), the mice were sacrificed, and allergic airway inflammation was characterized. Kefiran (50 mg/kg dissolved in PBS) or dexamethasone (4 mg/kg dissolved in PBS), administered through intragastric feeding, three times at 24-h intervals, beginning at 1 h before the OVA aerosol challenge.

Bronchoalveolar lavage (BAL) fluid collection and cell counting

The mice were sacrificed with an overdose of pentobarbital (Sigma P3761) 48 h after the last challenges, and a tracheotomy was performed. After cold PBS (0.5 mL) was instilled into the lungs, bronchoalveolar lavage fluid (BALF) was obtained by aspiration two times (total 1 mL) via tracheal cannulation (Yamazaki, 1968). BALF was centrifuged and supernatants were collected and stored at -70°C before use.

The total inflammatory cell number was assessed by the counting of cells in at least five squares of a hemocytometer after excluding dead cells by staining with trypan blue (Daigle and Simon, 2001). One milliliter of BALF was centrifuged (250 *g*, 4°C, 10 min). The cell pellets was suspended in 1 mL of PBS, and 100 μ L of the solution was spun onto each slide to fix the cells to slide with a cytospin machine (Hanil Science Co., Korea). After slides were dried, the cells were fixed and stained by Diff-Quik® Stain reagents (B4132-1A; Dade Behring Inc. Deerfield, IL), according to the manufacturer's instructions.

Measurement of Th2 cytokines in BALF

The concentrations of interleukin (IL)-4 and IL-5 in BALF were measured by enzyme linked immunosorbent

assay (ELISA) kit from R&D Systems (Minneapolis, MN). All of the procedures were performed according to the manufacturers' directions. The absorbance values of the solutions in each well were recorded by using an ELISA plate reader at 450 nm.

Measurement of airway hyperresponsiveness (AHR)

AHR was assessed in conscious and unrestrained mice at 2 days after the last aerosol challenge by means of whole-body plethysmography (OCP3000, All medicus Co., Korea). Mice were placed in a plastic chamber and exposed to aerosolized PBS. Baseline value was followed by increasing concentrations of aerosolized methacholine solutions (6.25, 12.5, 25 mg/mL; Sigma) for 3 min each using ultrasonic nebulizers.

Broncho-constriction was recorded for an additional 5 min for each increased dose of methacholine aerosol. The average Penh values were expressed for each methacholine concentration as the increase over baseline Penh values (Hamelmann et al., 1997).

Histopathological studies

After BALF was collected, lungs were removed from the mice after they had been sacrificed. Lung tissues were fixed with 10% neutral buffered formalin for 1 day. The lung tissue was embedded in paraffin, then cut into 4 μ m thickness sections, stained with H&E solution (hematoxylin; Sigma MHS-16 and eosin, Sigma HT110-1-32), and with periodic acid-Schiff (PAS) stain (IMEB Inc. San Marcos, CA) for measuring mucus production. The tissue was subsequently mounted and coverslipped with Dako-mounting medium (Dakocytomation; Denmark Carpinteria CA). The degree of airway inflammatory cell infiltration was scored in a double-blind screen by two independent investigators. For the assessment of goblet cell hyperplasia, the number of PAS-positive cells as percentages of total epithelial cells in all tangential bronchi in randomly selected sections were calculated (Myou et al., 2003). For each murine, airway sections that were randomly distributed throughout the left lung were analyzed, and their average cell numbers were calculated. Quantitative analysis of mucus production was performed through use of an image analyzer (Lecia Microsystem Imaging solution Ltd., Cambridge, UK).

Statistical analysis

Data were presented as the mean $\pm$ standard error of the mean (SEM). Statistical significance was estimated using two-tailed Student's *t*-test for independent means. *P* values less than 0.05 were considered to be statistically significant.

RESULTS

Inhibitory effect of kefir on the release of inflammatory cells

To assess the inflammation of lung tissue, BALF was collected 48 h after the last OVA aerosol challenge, and eosinophils and other inflammatory cells were determined. Mice sensitized and challenged had a significant increase in eosinophil cells and other inflammatory cell number. Treatment with kefir showed a potent inhibitory effect on the number of eosinophils and other inflammatory cells in the BAL fluid (Fig. 1).

Inhibitory effect of kefir on the release of IL-4 and IL-5 into the BALF

IL-4 and IL-5 have been known to associate substantially with the etiology of asthma (Robinson et al., 1992). Both cytokines severely impairs the eosinophil migration into the lung tissue, and the expression of adhesion molecules and chemokines, particularly eotaxin (Gonzalo et al., 1996). To determine the effect of kefir on cytokine release in the OVA-challenged mice, the levels of BALF cytokines were determined by ELISA in each experimental group 48 h after the last challenge. As shown in Fig. 2, OVA sensitization and challenge induced significant elevation of cytokines to 11.4 ± 7.8 pg/mL (IL-4) and 20.6 ± 9.2 pg/mL (IL-5) in the BALF. This elevated IL-4 concentration was markedly reduced by kefir treatment (1.8 ± 1.4 pg/mL, $P < 0.05$) (Fig. 2A). In addi-

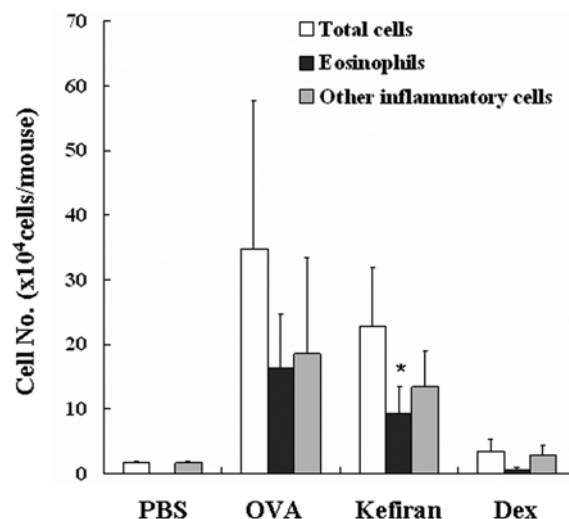


Fig. 1. Effect of treated with kefir on AHR to methacholine in murine models of asthma. AHR was measured as the Material and methods. PBS, PBS-challenged mice; OVA, OVA-sensitized mice; Kefiran, asthmatic mice treated with kefir (50 mg/kg); Dex, asthmatic mice treated with Dexamethasone (4 mg/kg). Data represents the mean $\pm$ SEM. *, $p < 0.05$ compared with OVA.

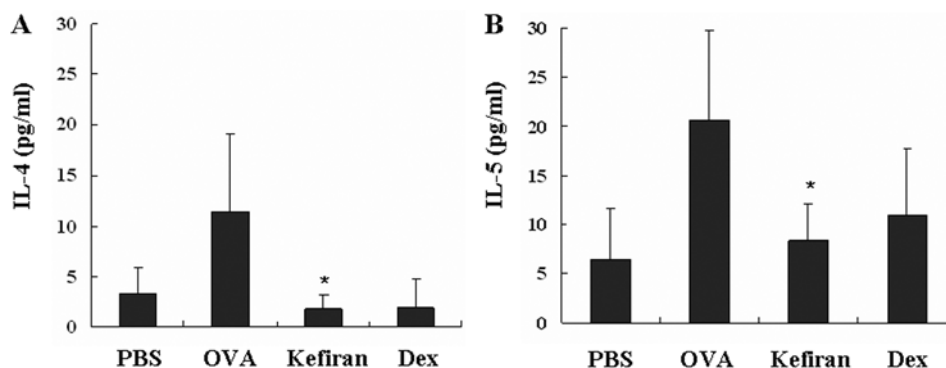


Fig. 2. Effect of treated with kefirin on inflammatory cells in the BAL fluid after OVA challenge. Cell counts were measured as the Methods section. Data represents the mean $\pm$ SEM. *, $p < 0.05$ compared with OVA.

tion, the IL-5 level was significantly reduced after the treatment with kefirin (8.4 ± 3.8 pg/mL, $P < 0.05$) (Fig. 3B). Dexamethasone also showed a significant reduction in both of IL-4 (1.8 ± 3.0 pg/mL) and IL-5 (11.0 ± 6.7 pg/mL). These results indicate that kefirin has an inhibitory effect on BALF IL-4 and IL-5 production by Th2 cells in a murine model of asthma as dexamethasone did.

Inhibitory effect of kefirin on AHR

Airway hyperresponsiveness (AHR) was measured to indicate the bronchial constriction that is commonly found in asthmatics. Twenty-four hours after the final aerosol challenge, the AHR was calculated in enhanced pause (Penh). As shown in Fig. 3, the Penh value of the OVA-treated group was significantly higher than that of the PBS control group at any concentration of 6.25–25.0 mg/mL of methacholine. In the kefirin-treated group ($P < 0.05$), the Penh value was significantly reduced com-

pared with that of the OVA-treated group. This activity of kefirin was similar to that of dexamethasone, a glucocorticoid which is an anti-inflammatory drug as a positive control in this work.

Inhibitory effect of kefirin on eosinophilia in lung tissue

To evaluate the effects of kefirin on airway inflammation and remodeling, histopathological studies were performed. Lung tissue was collected 48 h after the last OVA challenge. The majority of the infiltrated inflammatory cells were eosinophils. Kefiran significantly attenuated the eosinophil-rich leukocyte infiltration compared with OVA-challenged mice. We found marked peribronchial and perivascular inflammatory infiltrates in lung sections

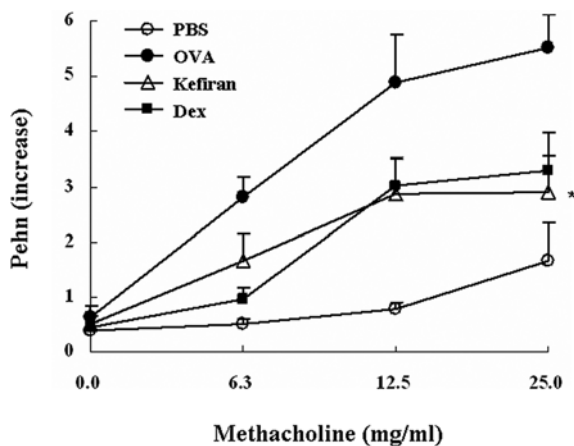


Fig. 3. Effect of treated with kefirin on Th2 cytokine level in BAL fluid. BAL fluids were collected 48 hr after the last OVA challenge. Levels of Th2 cytokines were analyzed using ELISA. Data represents the mean $\pm$ SEM. *, $p < 0.05$ compared with OVA.

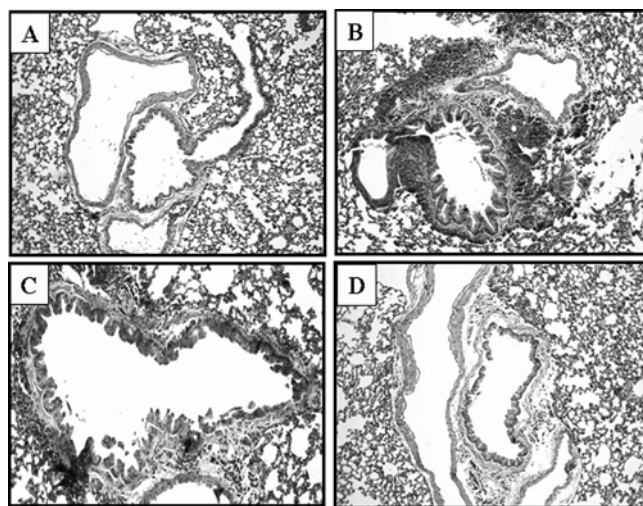


Fig. 4. Effect of treated with kefirin on lung tissue eosinophilia and airway remodeling. The lung tissues were fixed, embedded, cut into slices, and stained with H&E solution which enables to distinguish the inflammatory cells infiltrated into peribronchial and perivascular connective tissue. (A) PBS-challenged mice; (B) OVA-challenged mice; (C) asthmatic mice treated with kefirin (50 mg/kg); (D) asthmatic mice treated with dexamethasone (4 mg/kg).

of OVA-sensitized and challenged mice (Fig. 4B) compared with control mice (Fig. 4A). The induced peribronchial and total lung inflammation were significantly reduced by treatment with kefir and Dex (Fig. 4C and 4D). In addition, kefir blocked similarly the changes structural cells of the airways, including increases in airway smooth muscle mass, epithelial cell disruption, and subepithelial fibrosis. These results demonstrate that kefir inhibits OVA-induced inflammation in the lungs.

Inhibitory effect of kefir on airway mucous cell hyperplasia and mucus production

Airway remodeling is characterized by excessive production and hypersecretion of viscous mucus. In order to

evaluate goblet cell hyperplasia, we stained lung sections with Periodic acid-Schiff (PAS) stain (Fig. 5). Overproduction of mucous and goblet cell hyperplasia were observed in the bronchial airways of OVA-treated mice (Fig. 5B), but not in the PBS-treated mice (Fig. 5A). PAS-positive cells were calculated for each experimental group, as described in the Material and methods. OVA-sensitized/challenged mice had $46.06 \pm 4.18\%$ goblet cells, while Dex-treated mice had $13.70 \pm 8.71\%$ goblet cells, and kefir-treated mice had $31.80 \pm 3.09\%$ goblet cells (Fig. 5E). In particular, there was a greater reduction in the number of PAS-stained goblet cells in the kefir-treated asthmatic murine than OVA-sensitized/challenged murine. These results demonstrate that kefir prevents mucus production in airways.

DISCUSSION

Allergic disease is a major health issue, with the prevalence of allergic rhinoconjunctivitis and asthma increasing steadily worldwide. In the allergic airways, airway inflammation is characterized by increased mucus secretion and infiltration and subsequent activation of inflammatory cells, typically eosinophils (Wood et al., 2003; Bousquet et al., 1990). Previous studies have suggested that activated eosinophils, macrophages and mast cells produce the leukotrienes (Sampson 1996), which play an important role in the pathogenesis of allergic airway inflammation and asthma through inducing Th2 cytokine production by activated T cells, mucus secretion by goblet cells, bronchoconstriction, and airway remodeling (Holgate et al., 2003). These investigations have not only shed light on the immunologic mechanisms underlying allergy, but have also allowed evaluation of potential therapeutic agents (Kumar and Foster, 2002).

Sugars have long been described as T-cell independent antigens that fail to induce immunologic memory and immunoglobulin class-switching (Cobb and Kasper, 2005). In the previous study, immunostimulation of kefir produced by *L. kefiranofaciens* on the gut mucosa was studied by the examination of its effects on the IgA+ and IgG+ B cell populations and cytokines from the gut mucosa and on the cytokines in the circulating blood and small intestine fluid (Vinderola et al., 2006). However, there has been no report for the kefir on the anti-asthmatic effect in OVA sensitization and challenge in BALB/c mice.

The present study is the first demonstration that kefir effectively suppressed OVA-induced airway hyperreactiveness to methacholine, airway eosinophil accumulation, and the elevated IL-4 and IL-5 levels in BALF in a murine model of asthma.

Airway hyperresponsiveness (AHR) to nonspecific

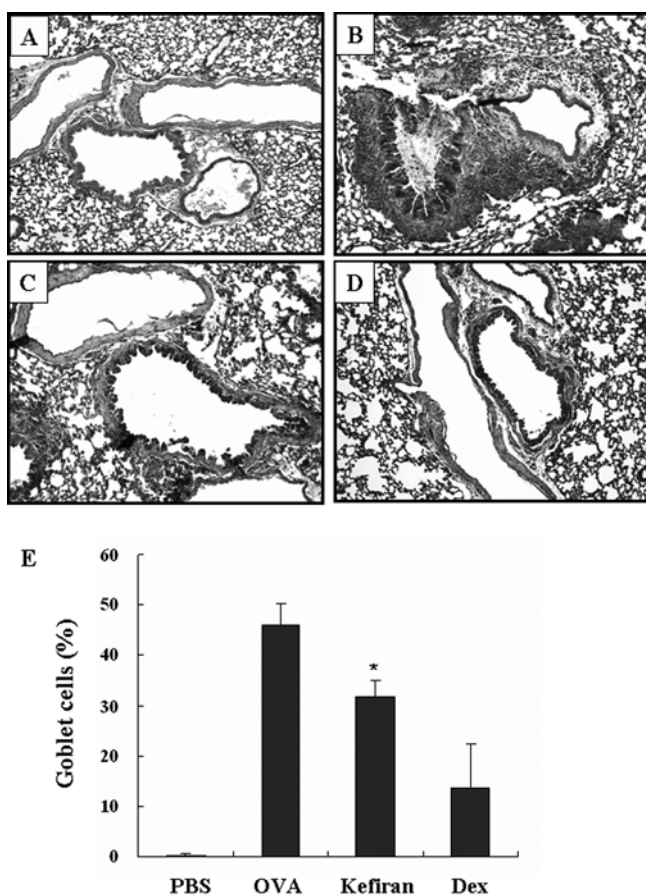


Fig. 5. Effect of treated with kefir on mucus production. The lung tissues were fixed, embedded, cut into slices, and stained with periodic acid-Schiff (PAS) solution which enables to see the hyperplasia of goblet cells, the mucus-producing cells, and mucus within airway or bronchi (A) PBS-challenged mice; (B) OVA-challenged mice; (C) asthmatic mice treated with kefir (50 mg/kg); (D) asthmatic mice treated with dexamethasone (4 mg/kg); (E) quantitative analyses of mucus production in lung sections were performed based on the methods of Nakata et al. and Tanaka et al. Data represents the mean $\pm$ SEM. *, $p < 0.05$ compared with OVA.

stimuli like methacholine is one of the defining symptoms of allergic asthma. Eosinophils is identified as the major effector cells underlying allergic airway inflammation (Wardlaw, 1999). Moreover, the overexpression of IL-4 or IL-5 in the respiratory epithelium led to the development of morphologic changes to the airways; however, airways hyperreactivity was restricted to the IL-5 transgenic mice (Lee et al., 1997; Rankin et al., 1996).

We found that kefir blocked the OVA-induced eosinophil infiltration into the lung and mucus-secreting goblet cells and attenuated AHR which is claimed to correlate with subepithelial fibrosis, which is observed in the airway wall thickening during the remodeling process (Hoshino et al., 1998). We demonstrated that mucus production was dramatically reduced in kefir-treated mice compared with the PBS-treated control. Goblet cells, which are known to produce mucus and are absent in small airways of normal individuals (Jeffery and Zhu, 2002), are observed in chronic airway inflammatory conditions and contribute to bronchiolar obstruction (Vinderola et al., 2005b). Thus, the reduction in mucus production in kefir-treated lung tissue may be due to the substantial drop in Th2 cytokine levels and to the reduction in eosinophilia in asthmatic mice.

In summary, intra-gastric administration of kefir in a murine asthma model effectively reduced OVA-induced cytokine production, pulmonary eosinophilia, mucus hypersecretion, and AHR compared to yeast or bacteria purified from Kefir (data not shown). Histological studies demonstrate that kefir substantially inhibited ovalbumin-induced eosinophilia in lung tissue and mucus hypersecretion by goblet cells in the airway. These findings suggest that kefir may have the potential to block the airway inflammation and remodeling in allergic asthma patients.

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