

EFFECTS OF COMBINED BACILLE CALMETTE-GUÉRIN AND DEHYDROEPIANDROSTERONE TREATMENT ON ESTABLISHED ASTHMA IN MICE

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□ *Both bacille Calmette-Guérin (BCG) and dehydroepiandrosterone induce Th1 immune responses and suppress Th2 allergic reactions. To investigate whether a combined administration of BCG and dehydroepiandrosterone treat asthma more effectively, BALB/c mice ($n = 8$ per group) with established airway hyper-responsiveness were treated with BCG and/or dehydroepiandrosterone. Combined treatment with 2×10^5 colony-forming units (CFUs) of BCG and 0.01% dehydroepiandrosterone was the most effective one at suppressing eosinophilia in bronchoalveolar lavage fluids. In addition, this combination also was better at suppressing hypersensitivity as compared to BCG alone (13.7 ± 4.0 - versus 3.6 ± 0.5 -fold increase in the sensitivity index; $P < .05$) in male mice. Similarly, the effect of the combined treatment was superior to that of individual treatments at decreasing the serum ovalbumin-specific immunoglobulin E (IgE) level. However, the addition of 0.1% dehydroepiandrosterone to BCG significantly decreased the efficacy of BCG on hypersensitivity in female mice. In male mice, the suppressive effect of the treatments on hypersensitivity tended to be lower, and the baseline interferon- γ /interleukin-5 (IFN- γ /IL-5) ratio in the splenocyte supernatant was significantly higher as compared to female mice. In conclusion, treatment with an appropriate combination of BCG and dehydroepiandrosterone had additive therapeutic effects on mice with established asthma. Androgens in males and dehydroepiandrosterone overdose might reduce the efficacy of BCG.*

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Although there has been a decrease in the prevalence of infectious diseases, such as tuberculosis, until recently, the prevalence of asthma has increased in western countries. An attractive explanation for the increase in the prevalence of asthma is the so-called 'hygiene hypothesis,' which states that increased hygiene has eliminated environmental cues, such as tuberculosis, skewing adaptive immunity away from Th2 responses [1]. In this context, many studies have shown that bacille Calmette-Guérin (BCG) and other mycobacterial infections suppress allergy and asthma, most likely through Th1 [2, 3] or Treg [4] immune cell responses. In addition, adrenal androgen dehydroepiandrosterone (DHEA) has also been shown to increase the production of Th1 cytokine interferon (IFN)- γ [5] and Treg cytokine interleukin (IL)-10 [6], and suppress asthma in mice [7]. Fortunately, these promising therapeutic modalities that intervene in the early stages of the pathogenic immune process of asthma have been used for many years to prevent tuberculosis and to remedy other diseases, respectively. Therefore, these therapies may be readily applicable to clinical practice, especially if BCG and DHEA combination therapy is found to have an additive or synergistic effect on the treatment of asthma.

Recently, our group reported that a combined BCG and DHEA treatment has an additive effect at suppressing the development of asthma [8]. The investigation by Ribeiro and colleagues [9] describing the DHEA-induced enhancement in the IFN- γ production during immunization of mice to tuberculosis corroborates the results of our study in several regards. Therefore, the present study was conducted to determine if BCG and DHEA combination therapy has an additive effect on the treatment of established asthma.

MATERIAL AND METHODS

Experimental Animals

Specific pathogen-free, 6-week-old BALB/c female and male mice (Daehan BioLink, Eumsung, Korea) were fed standard mouse chow and given water *ad libitum* in an animal care room at Chonnam National University Medical School, Korea. Animal care and treatment followed the guidelines of the Chonnam National University Research Institute of Medical Sciences.

Study Design

The mice were sensitized and provoked with ovalbumin, and those who developed airway hypersensitivity to methacholine were then selected

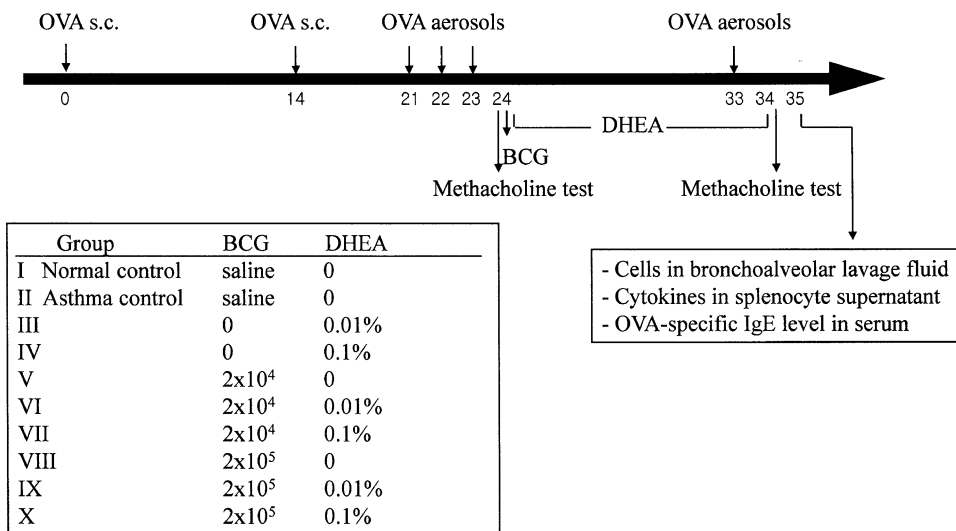


FIGURE 1 Schematic representation of the time course of the experiment. OVA: ovalbumin; DHEA: dehydroepiandrosterone; BCG: bacille Calmette-Guérin.

as experimental animals (Figure 1). Airway hypersensitivity was defined as airway sensitivity of $>95\%$ of the confidence interval of a nonsensitized normal control ($n = 15$), and the asthma thresholds were 19.1 and 16.4 mg/mL for male and female mice, respectively. Thus, 74.8% and 71.0% of the male and female mice that were provoked with ovalbumin developed airway hypersensitivity. The experimental animals were divided into 9 groups ($n = 8$ per group, except for the asthma control group, which contained 10 animals) for each gender: one asthma control group, 2 BCG groups [2×10^4 or 2×10^5 colony-forming units (CFUs)], 2 DHEA groups (0.01% or 0.1%), and 4 BCG combined with DHEA groups. In the treatment groups, BCG was administered on the first day of the study, whereas DHEA was administered throughout the study. After a second provocation with ovalbumin, the mice underwent a methacholine airway hypersensitivity test and were then sacrificed so that the inflammatory cell numbers in the bronchoalveolar lavage (BAL) fluid, the cytokine levels in the culture supernatants of concanavalin A-stimulated splenocytes, and the ovalbumin-specific immunoglobulin E (IgE) levels in the serum could be quantified.

Ovalbumin Sensitization and Provocation, and Administration of BCG and DHEA

The animals were sensitized and provoked with ovalbumin (Grade V; Sigma-Aldrich, St. Louis, MO), as described previously [8]. The experimental animals were then provoked again with 1% ovalbumin aerosols for 1 day after BCG and/or DHEA treatment. Bacille Calmette-Guérin strain Tokyo 172 (Korean National Tuberculosis Association, Seoul) was prepared for

inoculation, as described previously [8]. The mice in the BCG-treatment groups received intraperitoneal injections of 2×10^4 or 2×10^5 CFUs of live BCG after the first methacholine test, whereas the mice in the other groups received PBS injections.

The mice were fed mouse chow containing 0%, 0.01%, or 0.1% (*w/w*) DHEA (Sigma-Aldrich) following the first methacholine test until the end of the study. In a preliminary study, no significant difference was observed in the mean weight of the chow eaten daily by normal mice and DHEA-treated mice, with the total mean weight of the chow eaten being 3.53 g. The body weights of the mice measured at baseline, and on the 14th and 35th days of the study progressively increased (male: 20.4 ± 1.0 , 23.2 ± 1.0 , 24.1 ± 1.7 [mean $\pm$ SD] g; female: 17.3 ± 0.8 , 19.5 ± 1.4 , 19.8 ± 1.6 g, respectively); however, no significant differences were observed among groups in each gender.

Lung Function Tests

Airway responsiveness to 3.125~100 mg/mL of methacholine (Sigma-Aldrich) was measured using an animal body plethysmograph (OCP3000; All Medicus, Ahnnyang, Korea), as described previously [8]. Enhanced pause (P_{enh}) was used to represent airway resistance, as described previously [8]. The methacholine concentration required for a 200% increase in P_{enh} from baseline (PC_{200}) and the maximal response to methacholine for each animal were obtained as indices of airway sensitivity and excessive airway narrowing, respectively. As 3-fold improvement in PC_{20} is considered as significant in occupational asthma [10], the fold increase in PC_{200} —i.e., second PC_{200} /first PC_{200} ratio—was calculated to evaluate the degree of improvement after treatment.

Analysis of Cells in Bronchoalveolar Lavage Fluid, and Assays of Cytokines in Culture Supernatants and Ovalbumin-Specific IgE Antibody in Serum

Twenty-four hours after the last methacholine test, the mice were sacrificed using a 5 mg/kg intraperitoneal injection of thiopental sodium. BAL was then performed, and cell counts in the BAL fluid were determined, as described previously [8]. Measurements of cytokines in the splenocyte culture supernatants and ovalbumin-specific IgE antibody in the serum also were performed, as described previously [8]. The concentrations of IFN- γ and IL-5 were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits (BioSource International, Camarillo, CA). The sensitivities of the assays for IFN- γ and IL-5 were 1 and 3 pg/mL, respectively. Standard serum for ovalbumin-specific IgE antibody was prepared from seven BALB/c mice injected 3 times over 6 weeks with 20 μ g of ovalbumin

plus 2.25 mg of aluminum hydroxide (Imject Alum; Pierce Biotechnology, Rockford, IL).

Statistical Analysis

All statistical results were expressed as the mean $\pm$ SEM. The mean values for PC₂₀₀, cytokines, and ovalbumin-specific IgE antibody were calculated from the log₁₀ transformed values. The Mann-Whitney *U* test and Wilcoxon signed rank test were used to determine the significance of the intergroup and intragroup differences, respectively. A *P* value of less than .05 was considered statistically significant.

RESULTS

Eosinophils in BAL Fluid

The proportions of eosinophils were significantly lower in all of the treatment groups than in the asthma control group in both genders. Combined treatment with 2×10^5 CFUs of BCG and 0.01% DHEA was the most effective one at suppressing eosinophilia (Figure 2). This combination, 2×10^5 CFUs BCG + 0.1% DHEA and 2×10^4 CFUs BCG + 0.1% DHEA treatments were better than treatment with BCG or DHEA alone, respectively. Additionally, treatment with 2×10^4 CFUs BCG + 0.01% DHEA was superior to treatment with BCG alone in female mice. When compared to female mice, male mice showed a significantly lower proportion of eosinophils in the asthma control group ($15.7\% \pm 1.3\%$ versus $9.7\% \pm 1.1\%$; $P < .01$), as well as in most of the groups treated with BCG and/or DHEA.

Airway Responsiveness

Although the baseline P_{enh} values before and after treatment were significantly higher in male mice than in female mice (0.587 ± 0.014 versus 0.497 ± 0.011 and 0.570 ± 0.019 versus 0.486 ± 0.013 , all $P < .001$), the intergroup difference was not statistically significant in each gender. The airway sensitivity was significantly improved in all of the treatment groups in both genders, whereas the maximal response to methacholine was significantly improved only in a part of the treatment groups (Table 1). In addition, the fold increase in PC₂₀₀ after treatment was significantly higher in all of the treatment groups than in the asthma control group for both genders.

In male mice, treatment with 2×10^5 CFUs BCG + 0.01% DHEA provided better results than treatment with BCG alone (13.7 ± 4.0 -versus 3.6 ± 0.5 -fold increase; $P < .05$), and treatment with 2×10^4 CFUs BCG + 0.1% DHEA provided better results than treatment with BCG or DHEA

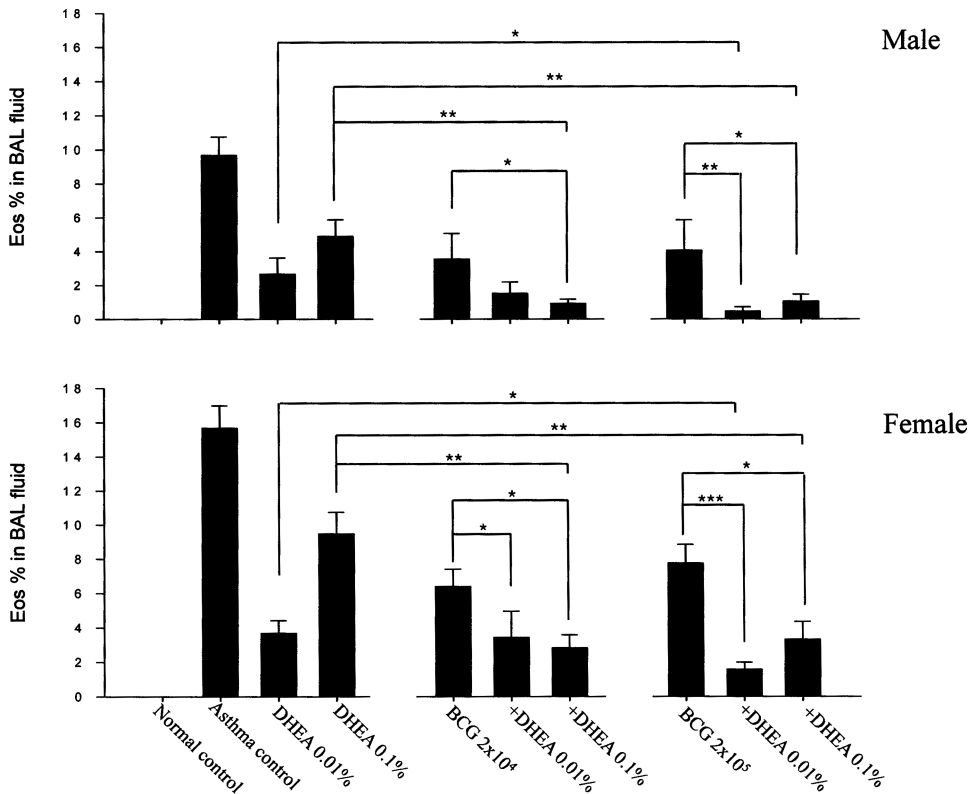


FIGURE 2 Comparisons between individual and combined treatment groups of the eosinophil proportions in bronchoalveolar lavage (BAL) fluid after treatment with bacille Calmette-Guérin (BCG) or dehydroepiandrosterone (DHEA). The proportions of eosinophils were significantly lower in all of the treatment groups than in the asthma control group in both genders. * $P < .05$; ** $P < .01$; *** $P < .001$.

alone (Figure 3). In female mice, treatment with 2×10^4 CFUs of BCG + 0.01% DHEA was superior to treatment BCG alone; however, treatment with 0.01% DHEA alone was already very effective. Conversely, the addition of 0.1% DHEA to 2×10^5 CFUs BCG significantly decreased the efficacy of BCG (5.7 ± 2.1 - versus 16.1 ± 6.2 -fold increase; $P < .05$). The fold increases in PC₂₀₀ after treatment with 2×10^4 CFUs of BCG + 0.01% DHEA ($P < .05$) and after treatment with 2×10^5 CFUs of BCG alone ($P < .01$) were significantly lower in male mice than in female mice.

Cytokine Concentrations in the Supernatants of Cultured Splenocytes

The IFN- γ levels were significantly higher in the mice treated with 2×10^5 CFUs of BCG + 0.01% or 0.1% DHEA, and in the mice treated with 2×10^4 CFUs of BCG + 0.1% DHEA than in the asthma control mice in both genders (Table 2). Treatments with 2×10^5 CFUs BCG + 0.01% DHEA and

TABLE 1 Comparisons of Airway Sensitivity^a and Maximal Responsiveness^b to Methacholine Before and After Treatment With Bacille Calmette-Guérin or Dehydroepiandrosterone

Group	Sensitivity				Maximal response			
	Male		Female		Male		Female	
	Before	After	Before	After	Before	After	Before	After
Normal control	72.8	78.9	74.8	94.5	331 ± 33	342 ± 33	381 ± 42	340 ± 29
Asthma control	8.3	7.8	6.0	4.9	1014 ± 144	1220 ± 219	1212 ± 189	1257 ± 208
DHEA 0.01%	6.4	32.3*	4.3	51.3*	975 ± 250	510 ± 74	813 ± 68	502 ± 96*
DHEA 0.1%	6.7	13.7*	5.1	12.1*	1424 ± 42	779 ± 118	961 ± 153	1418 ± 227
BCG 2×10^4	7.3	23.0*	5.2	20.9*	967 ± 231	645 ± 103	956 ± 195	614 ± 95*
+DHEA 0.01%	10.4	38.6*	5.3	79.6*	669 ± 73	425 ± 32*	1213 ± 263	368 ± 52*
+DHEA 0.1%	7.8	56.1*	5.5	47.9*	951 ± 201	510 ± 71*	763 ± 110	620 ± 95
BCG 2×10^5	9.2	30.9*	3.7	41.1*	832 ± 208	693 ± 83	1486 ± 522	462 ± 43*
+DHEA 0.01%	7.5	73.5*	5.3	79.2*	982 ± 215	439 ± 63*	1044 ± 176	557 ± 128*
+DHEA 0.1%	7.1	43.2*	5.2	19.8*	962 ± 157	582 ± 89	1438 ± 272	721 ± 127*

^aPC₂₀₀, expressed in geometric mean, mg/mL, and is the provocative concentration of methacholine required to induce a 200% increase in enhanced pause (P_{enh}) index from the baseline value.

BCG: Bacille Calmette-Guérin; DHEA: dehydroepiandrosterone.

^bMaximal P_{enh} index, expressed as% of baseline.

* $P < .05$

2×10^4 CFUs BCG + 0.1% DHEA were more effective at increasing the IFN- γ levels than treatment with BCG or DHEA alone, especially in female mice. However, the addition of 0.1% DHEA to 2×10^5 CFUs BCG tended to increase the IFN- γ levels less effectively than the addition of 0.01% DHEA did in female mice.

The IL-5 levels were significantly lower in the treatment groups than in the asthma control group, with the exception of the group treated with 0.1% DHEA alone in male mice. However, the addition of 0.1% of DHEA to 2×10^5 CFUs of BCG increased the IL-5 level over those of mice treated with BCG alone ($P < .01$) in male mice. The baseline IL-5 level was significantly lower (59 versus 144 pg/mL; $P < .01$) and the IFN- γ /IL-5 ratio was significantly higher (30.6 ± 4.3 versus 14.8 ± 4.0 , $P < .05$) in male than in female asthma control mice.

Ovalbumin-Specific Serum IgE Levels

The concentrations of ovalbumin-specific serum IgE were significantly lower in all of the combined treatment groups and in some of the individual treatment groups than in the asthma control group in both genders (Table 2). Treatment with 2×10^5 CFUs of BCG + 0.01% DHEA was superior to treatment with BCG or DHEA alone in male mice. In addition, treatments with 2×10^4 CFUs BCG + 0.1% DHEA in male mice and 2×10^4 CFUs BCG + 0.01% DHEA in female mice were superior to treatment with BCG or

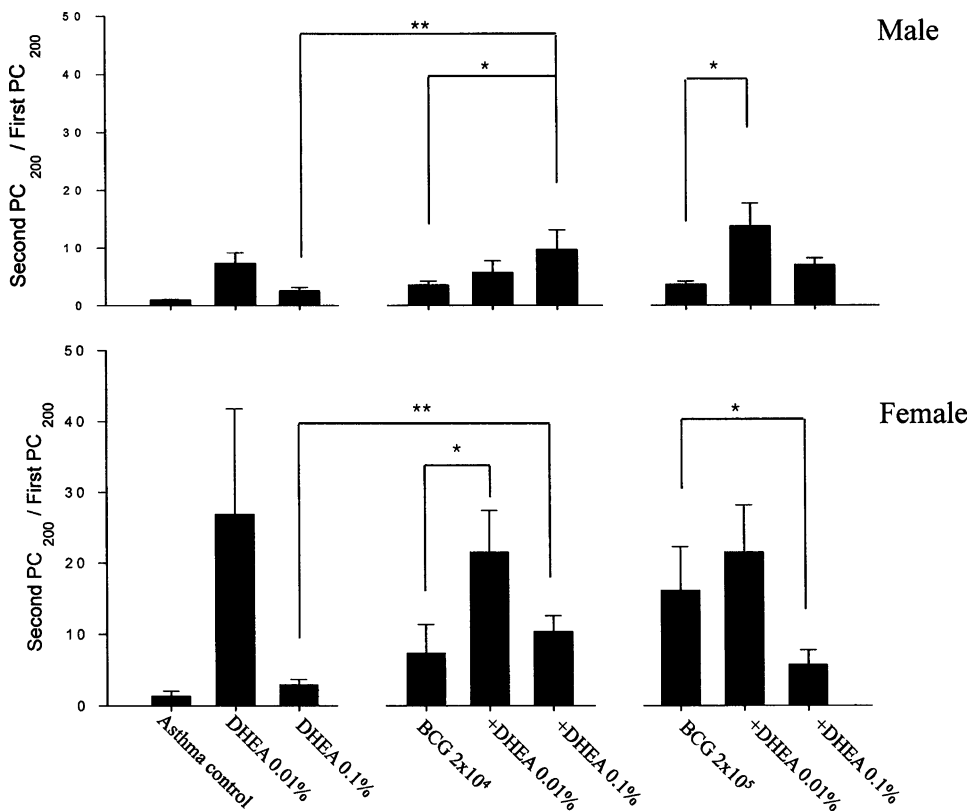


FIGURE 3 Comparisons between individual and combined treatment groups of the fold increases in the airway sensitivity index expressed as the provocative concentration of methacholine required to induce a 200% increase in airway resistance from the baseline value (PC_{200}) after treatment with bacille Calmette-Guérin (BCG) or dehydroepiandrosterone (DHEA). The fold increase in PC_{200} after treatment was significantly higher in all of the treatment groups than in the asthma control in both genders. * $P < .05$; ** $P < .01$.

DHEA alone, respectively. Some of the other combinations also were more effective than treatment with DHEA alone at decreasing the IgE serum level.

DISCUSSION

In the present study, BCG or DHEA treatment significantly suppressed airway eosinophilia and airway hyperresponsiveness, the characteristic features of asthma, in mice with established asthma. These results are consistent with those of previous studies [2–4, 7], even though excessive airway narrowing (maximal P_{enh}) was significantly improved only in a part of the treatment groups in this study.

In addition, we found that BCG and DHEA combination therapy had an additive effect in treating asthma. Combined treatment with 2×10^5 CFUs of BCG and 0.01% DHEA was more effective at suppressing eosinophilia

TABLE 2 Concentrations^a of Interferon (IFN)- γ and Interleukin (IL)-5 in the Splenocyte Supernatants and Ovalbumin-Specific IgE (OVA-sIgE) Levels^b in the Serum

	Male			Female		
	IFN- γ	IL-5	OVA-sIgE	IFN- γ	IL-5	OVA-sIgE
Normal	3044*	24*	1*	4560*	42*	7*
Asthma	1629	59	432	1510	144 [§]	594
DHEA 0.01%	2376	30*	119*	2360	69*	400
DHEA 0.1%	3133	43	100*	2026	73*	269
BCG 2×10^4	2162	27*	121*	2080	61*	365
+DHEA 0.01%	3413	28*	34*	2637	55*	152* ^{#†}
+DHEA 0.1%	3933* [#]	23* [†]	13* ^{#†}	4165* ^{#†}	42*	164*
BCG 2×10^5	3476*	25*	62*	2032	68*	226*
+DHEA 0.01%	3972* [†]	19*	10* ^{#†}	4311* ^{#†}	50*	134* [†]
+DHEA 0.1%	4755*	40* [#]	28* [†]	2902*	53*	129*

^aGeometric mean, pg/mL; ^bgeometric mean, EU/mL.

IFN- γ : interferon- γ ; IL-5: interleukin-5; OVA-sIgE: ovalbumin-specific IgE; BCG: bacille Calmette-Guérin; DHEA: dehydroepiandrosterone.

* $P < .05$ compared with asthma control; [#] $P < .05$ compared with BCG alone;

[†] $P < .05$ compared with DHEA alone; [§] $P < .05$ compared with male.

than treatment with BCG or DHEA alone. Regarding to airway hypersensitivity also, the combination treatment was more effective than treatment with BCG alone (13.7- versus 3.6-fold increase in PC₂₀₀; $P < .05$) in male mice, although it was not more effective than treatment with DHEA alone. Treatment with DHEA alone was already very effective, especially in female mice. Some other combination treatments with BCG and DHEA, especially with 2×10^4 CFUs of BCG and 0.1% DHEA, were also more effective than treatment with BCG or DHEA alone. These results are consistent with those of our previous report [8] showing an additive effect of combined BCG and DHEA treatment in preventing the development of asthma.

Patients with asthma have a reduced serum level of DHEA sulfate [11]; therefore, it may be desirable to at least restore this hormone level in asthmatic patients. However, the addition of a high-dose (0.1%) DHEA to 2×10^5 CFUs of BCG decreased the efficacy of BCG at suppressing airway sensitivity and increased IL-5 production. When compared to the 0.25 to 0.5 mg/kg of DHEA and DHEAS that is secreted daily in humans [12], the actual dose for 0.1% DHEA (approximately 200 mg/kg) that was administered to mice in this study was much too high. Du and colleagues [13] also demonstrated that supraphysiologic levels of DHEA resulted in an increase in IL-4 and a decrease in IFN- γ production in splenocyte cultures. Therefore, the addition of an appropriate, not excessive, dose of DHEA would improve the efficacy of BCG on asthma treatment.

Mycobacterial lipoproteins are recognized by Toll-like receptor (TLR) 2 in association with TLR1/TLR6, which drives the differentiation of naïve

T cells mainly to Th1/Treg cells [14]. Th1 cells secrete IFN- γ [15], and Treg cells secrete IL-10 and transforming growth factor- β [4], thereby conferring protection against Th2-associated allergic airway reactions. DHEA also induces Th1 [5] and Treg [6] immune responses. In addition, DHEA antagonizes glucocorticoid-induced deviation of the immune response towards a Th2 cytokine profile, probably through peroxisome proliferator-activated receptor α [16]. Th1 cells seem to have greater regulatory effects on allergen-specific Th2 responses than Treg cells [17], and in this study also, the therapeutic effects on airway hypersensitivity and eosinophilia were accompanied by changes in the IFN- γ and IL-5 levels toward to Th1 bias. We did not measure the IL-10 level in this study, however, because the results of a preliminary study showed a positive correlation between IL-5 and IL-10 levels, suggesting that a study using this kind of asthma model would be inappropriate to investigate the role of Treg cells.

The effects of BCG on atopic diseases may be influenced by several factors, including the timing of vaccination, the route of delivery, genetic contribution, exposure to environmental mycobacteria [14], and mycobacterial strain [3]. Additionally, we found that a gender-related difference in the effect existed. Verthelyi and Klinman [18] demonstrated that sex hormone levels correlated with the activity of cytokine-secreting cells *in vivo*, and Yu and colleagues [7] showed that DHEA, an androgen, was effective in suppressing asthma, similar to the results of this study. Both BCG and DHEA were less effective in suppressing airway hypersensitivity in male than in female mice in this study. The lower IL-5 level and the higher IFN- γ /IL-5 ratio in male than in female asthma control mice in the present study also suggests that the background DHEA and other androgens in males might influence the Th1/Th2 cytokine levels. Male asthma that developed in spite of the suppression of the background androgens may require more potent Th1-inducing power or other mechanisms to be controlled.

The serum ovalbumin-specific IgE level was also decreased in mice treated with combined regimens in this study. There is some controversy regarding the suppressive effect of mycobacterial antigens on allergen-specific IgE level, and some studies [3] have shown IgE-independent airway eosinophilia and airway hyperresponsiveness. However, Harada and colleagues [19] recently demonstrated that BCG activated murine V α 14 natural killer T cells to secrete IL-21, thereby inducing apoptosis of B ϵ cells and a dramatic decrease in IgE production. In this study, the differences in the cytokine levels were not accompanied very closely by the differences in the effects on airway sensitivity and eosinophilia; therefore, further studies on other mechanisms besides Th1/Th2 cytokines are needed. In addition, the lung function measurements must be validated using more rigorous method even though there are several papers in the literature using P_{enh} values as a measure of airway hypersensitivity and Hamelmann and colleagues [20]

reported that measurement of P_{enh} is a valid indicator of airway hyperresponsiveness. Moreover, asthma is not the same with airway hyperresponsiveness and allergic airway inflammation that were examined using an animal model in this study. Therefore, the information of this study must be very cautiously extrapolated to human asthma.

In summary, BCG and DHEA combination treatment had additive therapeutic effects on established asthma. However, the use of DHEA overdose might reduce the efficacy of BCG. In addition, there was a gender difference in the effects, and these effects were related to Th1/Th2 cytokine levels.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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