

Human Placental Extract Reduces Allergic Inflammation in a Murine Allergic Rhinitis Model

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Objectives/Hypothesis: In this study, we addressed the immunotherapeutic potential of human placental extract (HPE) in a murine allergic rhinitis (AR) model and explored its immunological mechanisms.

Study Design: In vivo study using an animal model.

Methods: HPE was administered to BALB/c mice before sensitization with allergen (*Dermatophagoides farinae* [Derf]) (pre-S group) or after allergen challenge (post-C group). The groups were compared with Derf-treated mice that received no HPE (Derf group) and phosphate buffered saline (PBS)-treated mice (control). Allergic symptom scores, eosinophil counts, and serum Derf-specific IgE levels were measured. mRNA expression levels of interferon (IFN)- γ , T-bet, interleukin (IL)-4, GATA-3, and Foxp3 in nasal mucosa were determined by real-time polymerase chain reaction. IFN- γ , T-bet, IL-4, and GATA-3 were confirmed by Western blotting analysis. Spleen CD4⁺CD25⁺Foxp3⁺ T cells were detected using flow cytometry.

Results: Rubbing motions, serum Derf-specific IgE, GATA-3 mRNA levels, IL-4 mRNA levels, and tissue eosinophil counts were decreased in both pre-S and post-C groups (all $P < 0.05$). Western blots showed decreased expression of GATA-3 and IL-4 in both pre-S and post-C groups as compared to the Derf group. An increased percentage of CD4⁺CD25⁺Foxp3⁺ T cells and an increased level of Foxp3 mRNA were found in pre-S and post-C groups as compared to those in the Derf group (all $P < 0.05$).

Conclusion: Both prophylactic and therapeutic treatments with HPE significantly reduced allergic inflammation in nasal mucosa and had the potential to induce regulatory T cells in a murine model of AR.

Key Words: Allergic rhinitis, human placental extract, allergic rhinitis model, regulatory T cells.

Level of Evidence: N/A.

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INTRODUCTION

Allergic rhinitis (AR) significantly impacts the quality of life in affected patients and is one of the most common reasons for outpatient visits to otolaryngology clinics. Although various medical interventions such as the avoidance of antigen exposure or the administration of topical and/or systemic glucocorticoids are useful treatments for AR, new effective therapeutic options with fewer side effects are needed.

Human placental extract (HPE) (Laennec; Green Cross, Japan Bio Products Ltd, Gyeonggi Province, Korea) has been used as a traditional therapeutic agent in many Asian countries for the treatment of diseases or the replenishment of vital essence.¹ For example, in Korea, HPE has been approved for the improvement of

liver function and menopausal symptoms since 2003. Furthermore, HPE has been used widely for the improvement of fatigue, anti-inflammation, and anti-aging. Despite this popularity, the efficacy and mechanism of HPE has not been sufficiently studied.^{2,3}

Multiple studies have demonstrated the immunosuppressive effects of HPE. It has been reported that HPE inhibited the graft-versus-host disease (GVHD) that is associated with T lymphocytes.⁴ Additionally, HPE might be a useful physiological therapy capable of modulating the xenogeneic immune response. Moreover, an adequate dose of HPE induced the suppression of IgM and IgG responses.⁵

Previous studies have demonstrated the immune inhibitory effect of HPE in allergic dermatitis and arthritis.^{3,6} We hypothesize that HPE might impair the distribution of CD4⁺ T lymphocytes, thereby inhibiting the allergic response. In this study, we addressed the immunotherapeutic potential of HPE in a murine AR model and explored its immunological mechanisms.

MATERIALS AND METHODS

Experimental Animals

Six-week-old healthy female BALB/c mice (20 g–30 g) were used in this study. The experiment was performed with the approval of the Institutional Animal Care and Use Committee at the Catholic University of Korea.

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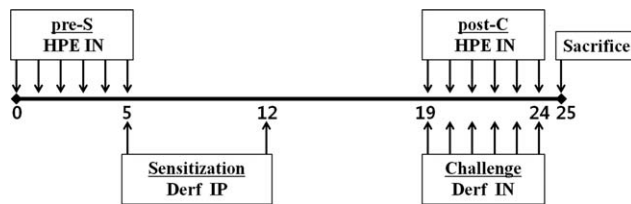


Fig. 1. Schematic representation of the experimental allergic rhinitis model and treatment protocol. Data are expressed as number of days. Derf = *Dermatophagoides farinae*; HPE = human placental extract; IN = intranasal administration; IP = intraperitoneal administration; pre-S = HPE before sensitization; post-C = HPE after challenge.

Reagents

Dermatophagoides farinae (Derf) crude body extract (Arthropods of Medical Importance Resource Bank; College of Medicine, Yonsei University, Seoul, Korea) was used as the allergen. Derf was solubilized and stored at -70°C and dissolved prior to use. The dose of HPE (Laennec; Green Cross Japan Bio Products Ltd, Gyeonggi Province, Korea) was determined in preliminary experiments.⁶

Production of the AR Model and Treatment Protocol

Forty mice were randomized into one of four groups: control ($n = 10$), Derf (AR, $n = 10$), pre-S (Derf administered before sensitization; $n = 10$) and post-C (Derf administered after challenge; $n = 10$). Allergen sensitization and challenge for the development of the AR murine model are summarized in Figure 1. Briefly, on days 5 and 12 all mice except those in the control group were immunized by intraperitoneal injection of $100\text{-}\mu\text{g}$ Derf and 1-mg aluminum hydroxide (Thermo Scientific, Pittsburgh, PA). Mice in the pre-S groups were treated intranasally with 3-mg HPE on days 0, 1, 2, 3, 4, and 5. After 1 week, all sensitized mice were challenged intranasally with $20\text{-}\mu\text{g}$ Derf for 6 consecutive days. Mice in the post-C groups were treated intranasally with 3-mg HPE on days 19, 20, 21, 22, 23, and 24. The control group received phosphate buffered saline (PBS) intranasally instead of Derf.⁷

Evaluation of Allergic Symptoms Induced After Allergen Challenge

The numbers of sneezing and nasal rubbing motions during the 15-minute period after the final allergen challenge were

recorded and compared among the experimental groups by observers who were blinded to the experimental groups.⁸

Nasal Mucosal Tissue Evaluation

Mice were sacrificed and decapitated 24 hours after the final allergen challenge. The heads were fixed in 4% paraformaldehyde for 3 days at 4°C , washed in running water, decalcified for 3 days with Calci-Clear Rapid (National Diagnostics, Atlanta, GA) at room temperature, dehydrated by passage through a graded alcohol series, and embedded in paraffin blocks.⁹ The blocks were cut into $4\text{-}\mu\text{m}$ -thick sections and stained with hematoxylin and eosin to evaluate the general morphology and number of eosinophils in the lamina propria. Eosinophils were counted under a light microscope by an individual blinded to the animal group assignments.

Derf-Specific Immunoglobulin E (IgE) Levels in Serum

Serum Derf-specific IgE levels were measured with an enzyme-linked immunosorbent assay (ELISA) kit (Indoor Biotechnologies, Manchester, UK).

Real-Time Polymerase Chain Reaction

The nasal mucosa was removed and prepared for real-time polymerase chain reaction (PCR) detection of interferon ($\text{IFN-}\gamma$), T-bet, interleukin (IL)-4, GATA-3, and Foxp3. Total RNA was extracted from nasal mucosa using the TRIzol reagent (Invitrogen, Carlsbad, CA), and the first strand was reverse-transcribed using random primers (TaKaRa, Otsu, Japan).^{10,11} The oligonucleotide primer sequences were as follows: $\text{IFN-}\gamma$ forward primer, 5'-AGAGCCAGATATCTCTTTCTACCTCAG-3' and $\text{IFN-}\gamma$ reverse primer, 5'-CCTTTTCGCCTTGCTGTTG-3'; T-bet forward primer, 5'-GCCAGGGAA CCGCTTATA-3' and T-bet reverse primer, 5'-CCTTGTTGTTGGTGAGCTTAA-3'; IL-4 forward primer, 5'-TCAACCCAGCTAGTTGTC-3' and IL-4 reverse primer, 5'-AAATATGCGAAGCACC TTGG-3'; GATA-3 forward primer, 5'-CTGGATGGCGCAAAGC-3' and GATA-3 reverse primer, 5'-GTGGGCGGGAAGGTGAA-3'; Foxp3 forward primer, 5'-GAAAGCGGATACCAATGA-3' and Foxp3 reverse primer, 5'-CTGTGAGGACTACCGAGCC-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward primer, 5'-GCACAGTCAAGGCCGAGAAT-3' and GAPDH reverse primer, 5'-GCCTTCTCCATGGTGGTGAA-3'. The levels of $\text{IFN-}\gamma$, T-bet, IL-4, GATA-3, Foxp3, and GAPDH mRNA expression were determined by

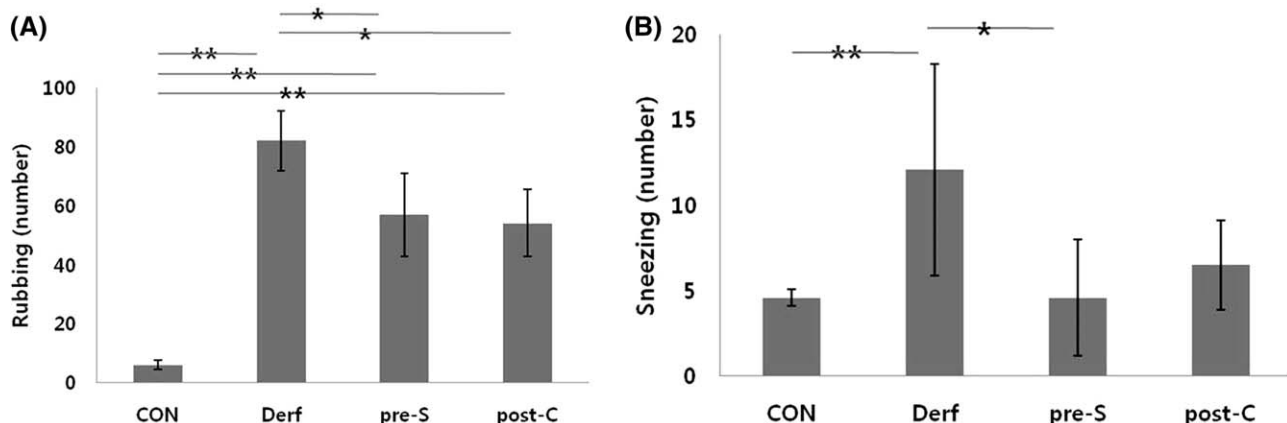


Fig. 2. Nasal symptom score. Rubbing (A) and sneezing (B). Error bars represent standard deviations. * $P < 0.05$ versus the Derf group. † $P < 0.05$ versus the control group. Derf = *Dermatophagoides farinae*.

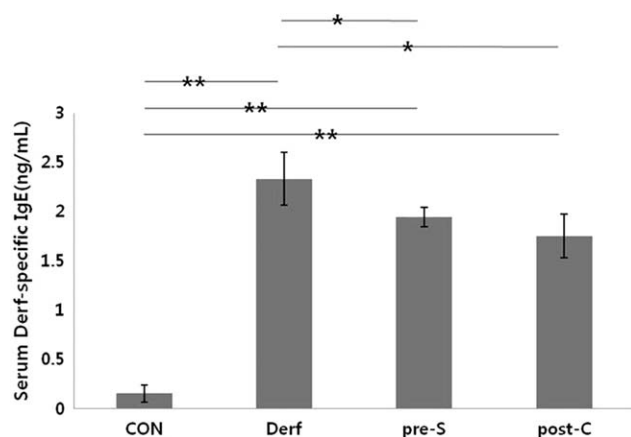


Fig. 3. Serum Derf-specific immunoglobulin E. Error bars represent standard deviations. * $P < 0.05$ versus the Derf group. $^{\dagger}P < 0.05$ versus the control group. Derf = *Dermatophagoides farinae*.

real-time PCR using the CFX96 real-time PCR detection system (BioRad, Hercules, CA) and iQ SYBR Green Supermix (BioRad). The results were normalized relative to GAPDH expression and are shown as the fold increase over the expression of the control group.

Western Blot Analysis

Western blot analysis was used to detect relative protein expression levels of IFN- γ , T-bet, IL-4, and GATA-3 within the nasal mucosa from control, Derf, pre-S, and post-C groups. Results were based on the gray scale ratios between the target gene and GAPDH to indicate the relative protein expressions of target genes (IFN- γ antibody sc-59992, T-bet antibody sc-21003, IL-4 antibody sc-1260, GATA-3 antibody sc-9009; Santa Cruz Biotechnology, Santa Cruz, CA).

Flow Cytometry

The spleen was removed aseptically from each animal 24 hours after the last challenge. For cell-surface staining, aliquots

of 10^6 splenic mononuclear cells were incubated with fluorescein isothiocyanate-conjugated anti-mouse CD4 (GK1.5) antibody (eBioscience, San Diego, CA). For intracellular staining, cells stained with CD4 were incubated with fixation/permeabilization working solution, and Fc receptors were blocked with excess mouse Fc block. Cells were then stained with phycoerythrin-Cy5-conjugated anti-mouse Foxp3 and allophycocyanin-CD25 antibody (eBioscience). CD4 $^{+}$ CD25 $^{+}$ Foxp3 $^{+}$ T cells were analyzed by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA).^{10,11}

Statistical Analysis

All measured parameters are expressed as the mean $\pm$ standard deviation. Differences among groups were analyzed using the Kruskal–Wallis test. In cases of statistical significance, the ranked parameters were compared by one-way analysis of variance and Bonferroni's multiple comparison tests (PASW Statistics 18; SPSS Inc., Chicago, IL). In all analyses, $P < 0.05$ indicated significance.

RESULTS

Allergic Symptoms

Allergic symptoms were determined by the numbers of nasal rubbing motions and sneezes during the 15-minute period after the final challenge. The numbers of nasal rubbing motions were 6.0 ± 1.58 in the control group, 82.1 ± 10.2 in the Derf group, 57 ± 14 in the pre-S group, and 54.2 ± 11.3 in the post-C group. The numbers of sneezes were 4.6 ± 0.5 in the control group, 12.1 ± 6.2 in the Derf group, 4.6 ± 3.4 in the pre-S group, and 6.5 ± 2.6 in the post-C group. The number of nasal rubbing motions was significantly higher in the Derf group than in all of the other groups (control, $P = 0.000$; pre-S, $P = 0.001$; post-C, $P = 0.001$) (Fig. 2A). The number of sneezes was significantly higher in the Derf group than in the control group and pre-S group (control, $P = 0.027$; pre-S, $P = 0.003$) (Fig. 2B).

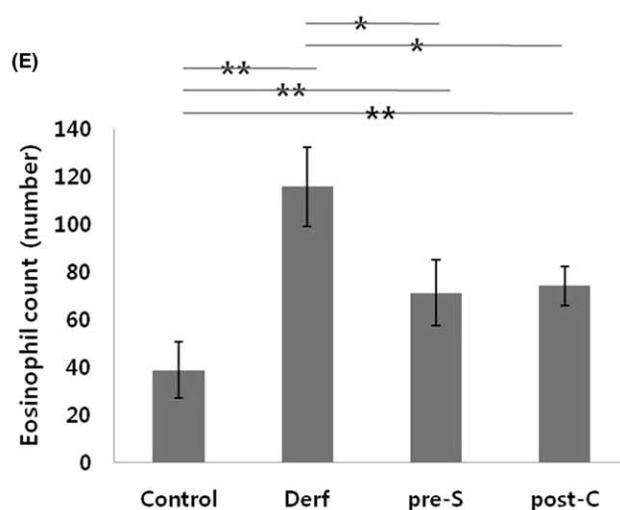
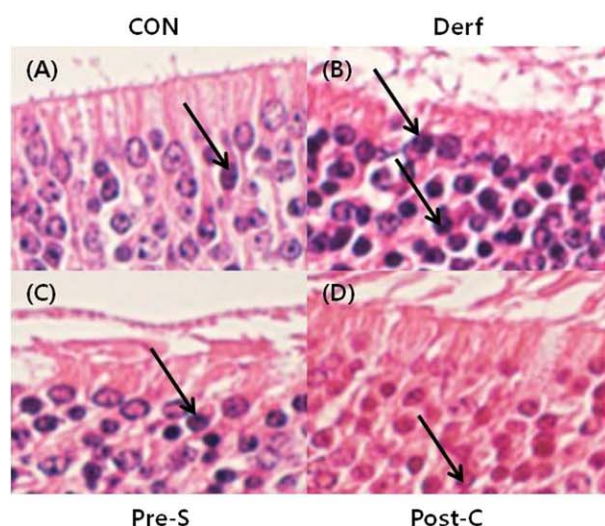


Fig. 4. Infiltration of eosinophils (arrows) in the nasal mucosa of BALB/c mice: (A) control group, (B) *Dermatophagoides farinae* group, (C) pre-S group, and (D) post-C group (hematoxylin and eosin staining; original magnification, $\times 400$). (E) Eosinophil counts in the nasal mucosa of each study group. Error bars represent standard deviations. * $P < 0.05$ versus the Derf group. $^{\dagger}P < 0.05$ versus the control group. Derf = *Dermatophagoides farinae*. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]

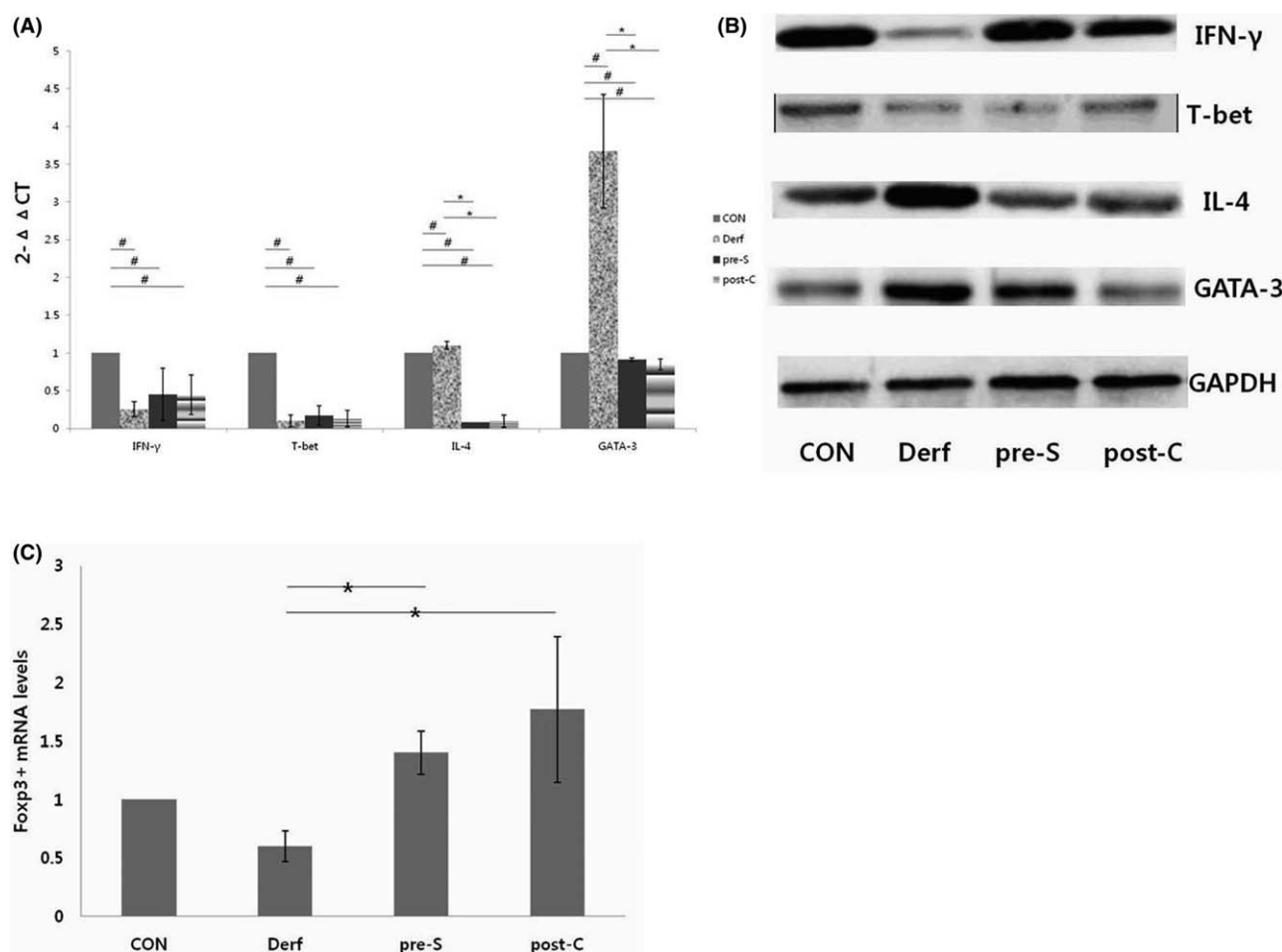


Fig. 5. (A) Quantitative analysis of interferon (IFN)- γ , T-bet, interleukin (IL)-4, and GATA-3. Real-time polymerase chain reaction (PCR) analysis of mRNA expression from nasal mucosa. (B) Western blot analysis was used to detect relative protein expressions of IFN- γ , T-bet, IL-4, and GATA-3. The results were normalized relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. Error bars represent standard deviations. (C) Quantitative analysis of Foxp3. Real-time PCR analysis of mRNA expression from nasal mucosa. * $P < 0.05$ versus the Derf group. $^{\dagger}P < 0.05$ versus the control group. Derf = *Dermatophagoides farinae*.

Serum Derf-Specific IgE

Serum Derf-specific IgE levels were significantly higher in the Derf group (2.33 ± 0.27 ng/mL; $P = 0.000$) than in the control group (0.15 ± 0.09 ng/mL), pre-S group (1.94 ± 0.1 ng/mL; $P = 0.000$), and post-C group (1.75 ± 0.22 ng/mL; $P = 0.000$). Significantly higher levels of Derf-specific IgE were observed in the Derf group as compared to all other groups (all $P = 0.000$) (Fig. 3).

Eosinophil Counts in the Lamina Propria

Figure 4 (A, B, C, D) shows eosinophil infiltration in the lamina propria. Eosinophil numbers were 38.75 ± 11.84 in the control group, 115.8 ± 16.61 in the Derf group, 71.2 ± 13.89 in the pre-S group, and 74.2 ± 8.28 in the post-C group. The eosinophil count was significantly higher in the Derf group than in the control group ($P = 0.000$), pre-S group ($P = 0.001$), and post-C group ($P = 0.005$) (Fig. 4E).

Real-Time PCR and Western Blot Analyses

The IFN- γ , T-bet, IL-4, GATA-3, and Foxp3 mRNA expression levels in nasal mucosa were evaluated by

real-time PCR. The level of IFN- γ mRNA and protein expression was significantly higher in the control group than in all other groups (Derf, $P = 0.001$; pre-S, $P = 0.017$; post-C, $P = 0.016$). The level of T-bet mRNA and protein expression was significantly higher in the control group than in all other groups (Derf, $P = 0.003$; pre-S, $P = 0.025$; post-C, $P = 0.006$). The level of IL-4 mRNA and protein expression was significantly higher in the Derf group than in all other groups (control, $P = 0.031$; pre-S, $P = 0.001$; post-C, $P = 0.000$). GATA-3 mRNA and protein expression was significantly higher in the Derf group than in the control group ($P = 0.002$), pre-S group ($P = 0.000$), and post-C group ($P = 0.000$) (Fig. 5A, 5B). Foxp3 mRNA expression was significantly higher in the pre-S group ($P = 0.000$) and the post-C group than in the Derf group ($P = 0.000$) (Fig. 5C).

Flow Cytometric Analysis of CD4⁺ CD25⁺ Foxp3⁺ T Cells From Splenic Mononuclear Cells

Cells were sorted based on Foxp3 and CD25 expression levels and whether they expressed CD4. The CD4⁺

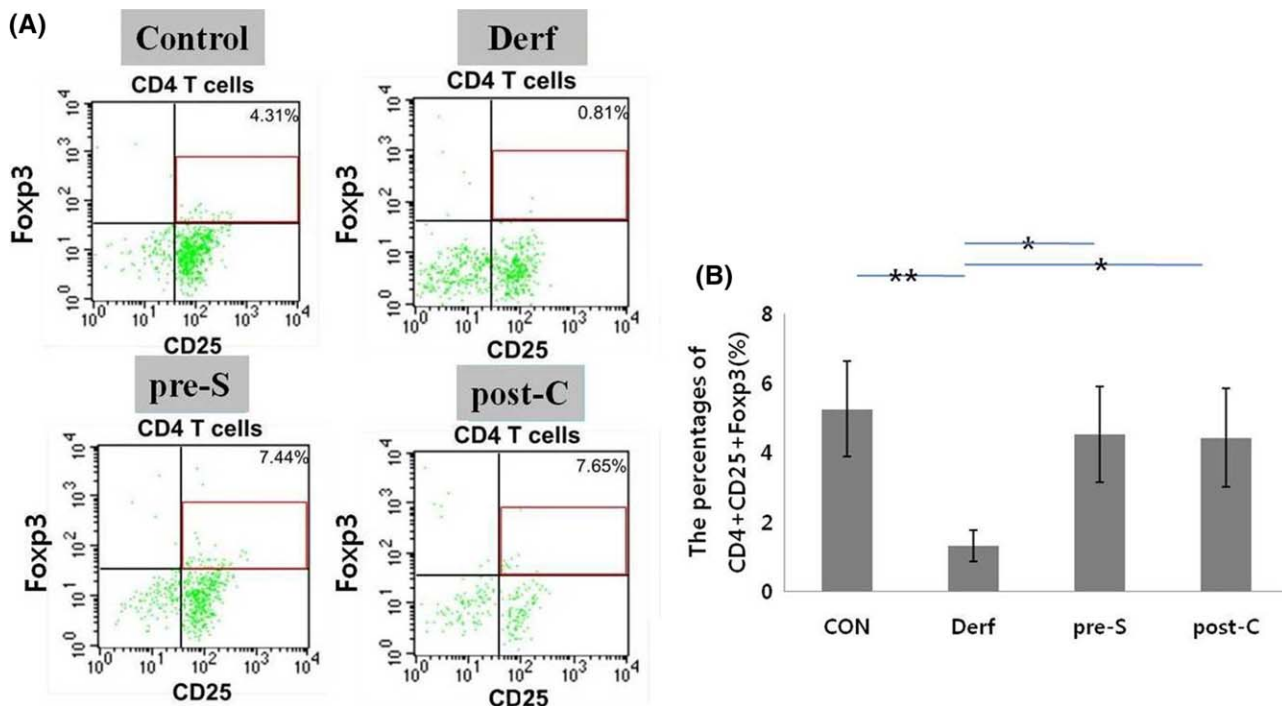


Fig. 6. (A) Flow cytometric analysis of CD4⁺CD25⁺Foxp3⁺ T cell subsets. Representative fluorescence-activated cell sorting analysis in each group. Upper right quadrant, which represents CD4⁺CD25⁺Foxp3⁺ T cells. (B) The percentage of splenic mononuclear cells that were CD4⁺CD25⁺Foxp3⁺ T cells. Error bars represent standard deviations. * $P < 0.05$ versus the Derf group. † $P < 0.05$ versus the control group. Derf = *Dermatophagoides farinae*. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]

CD25⁺Foxp3⁺ T cells accounted for $5.26 \pm 1.37\%$ of all splenic mononuclear cells in the control group, $1.32 \pm 0.45\%$ in the Derf group, $4.53 \pm 1.38\%$ in the pre-S group, and $4.43 \pm 1.42\%$ in the post-C groups. The pre-S ($P = 0.000$) and post-C ($P = 0.000$) groups had significantly higher percentages of these cells than the Derf group (Fig. 6).

DISCUSSION

Numerous studies have demonstrated the biological actions of HPE in various diseases.^{1,6,12} Previous studies reported the role of HPE for maternal–fetal tolerance. They suggested that the inhibitory effect of syncytiotrophoblast extract on the proliferative responses of lymphocytes to stimulants may be due to a cytostatic effect, representing a possible contributing factor in fetal–maternal tolerance.¹³ Another study suggested that HPE exerts an anti-inflammatory function that suppresses chemical mediators, and that these effects might be associated with innate immune functions.¹⁴ A different study suggested that HPE has an IL-8 inductive effect in human monocytes via the activation of jun amino-terminal kinases & stress-activated protein kinases (JNK/SAPK) and enhanced binding of several transcription factors.¹⁵ However, limited information is available regarding the constituents in the HPE immunoregulation mechanism in the AR model.

In this study, we suggest a possible mechanism that explains how HPE exerts immunomodulation in the murine AR model. We hypothesize that HPE might impair the distribution of CD4⁺ T lymphocytes, thereby

inhibiting the allergic response. Previous studies have shown that allergic responses are inhibited by HPE administration prior to antigen rechallenge, suggesting its therapeutic potential in the allergic dermatitis model.⁶ In the present study, repeated exposure with Derf induced a Th2 milieu in mice—with a subsequent increase in tissue eosinophil count and increased levels of serum IgE, GATA-3, and IL-4 in the nasal mucosa. HPE treatment caused a significant reduction in levels of serum IgE, GATA-3 mRNA, and IL-4 mRNA in the pre-S and post-C groups, as compared to the Derf group. Tissue eosinophil counts were reduced significantly in both the pre-S and post-C groups. These results indicated that HPE might reduce the Th2 response.

Treg cells might represent important factors for AR during HPE treatment. Previous studies demonstrated that regulatory T(Treg) cells inhibit allergic dermatitis via downregulation of major histocompatibility complex (MHC) and costimulatory molecules on antigen-presenting cells.^{16–18} In our studies, Foxp3 mRNA levels were increased significantly in both pre-S and post-C groups as compared to the Derf group. Flow cytometry analysis revealed that the percentages of CD4⁺CD25⁺Foxp3⁺ T cells were increased significantly in both pre-S and post-C groups as compared to the Derf group. These results indicated that HPE might induce CD4⁺CD25⁺Foxp3⁺ Treg cells. Our results support the idea that the anti-allergic effect of HPE might be related to the induction of Treg cells.

Cell-surface proteins expressed by trophoblast are involved in immune tolerance at the maternal–fetal

interface. For example, the B7 family members B7-H1, B7-DC, ICOSL, and B3-H4 are likely to play a role in the regulation of immune cell function leading to maternal-fetal tolerance.¹⁹ Similarly, the immune tolerance effect of HPE might play an important role in AR. The interaction of B7-1/B7-2 with CD28 generates a costimulatory signal, and the interaction with CTLA-4 leads to an inhibitory signal in activated T cells.²⁰ Additionally, ligation of CD200R by CD200 leads to the increased production of Treg cells and the inhibition of macrophages.^{21–24}

We assessed whether the timing of HPE administration had any effect on immune modulation. The eosinophil counts, IL-4 levels, and sneezing numbers were decreased in the pre-S group as compared to the post-C group. Moreover, nasal rubbing numbers, GATA-3 levels, and IgE levels were decreased in the post-C group as compared to the pre-S group. However, because no significant differences were observed according to the timing of HPE administration, we suggested that the effect of HPE would have no significant difference between prophylaxis and symptom treatment in the AR model.

CONCLUSION

Both prophylactic and therapeutic treatment with HPE significantly reduced the allergic response. Inhibition of inflammatory cytokines toward Th2-type, Treg cells, and multiple types of cell-surface proteins expressed at the maternal-fetal interface might be associated with the anti-allergic effect of HPE in the AR model. The timing of HPE administration was not significant in exerting an anti-allergic effect.

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