

Suppression of allergic response by CpG motif oligodeoxynucleotide–house-dust mite conjugate in animal model of allergic rhinitis

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ABSTRACT

Background: Although there have been many therapeutic options for allergic disease, the true allergen desensitization remains a challenging goal. The classic immunotherapy has a limited efficacy, is inconvenient, and has a risk of anaphylaxis. Recent reports revealed that immunostimulatory DNA sequences (ISS-oligodeoxynucleotide [ODN], CpG motif) act as a strong Th1 response–inducing adjuvants and that DNA-based vaccination might be an effective therapeutic option. In this study, we investigate whether ISS-ODN/*Dermatophagoides farinae* (Der f) conjugate has antiallergic effects in the allergic rhinitis mouse model, sensitive to house-dust mites. Der f is the most common allergen-inducing allergic rhinitis in Korea.

Methods: C57BL/6 mice were sensitized with crude extract of Der f. After injection of ISS-ODN or ISS-ODN/Der f conjugate, several parameters of allergic response were evaluated.

Results: Scratching and sneezing symptoms and eosinophilic infiltration into nasal mucosa were suppressed by injection with ISS-ODN only and ISS-ODN/Der f conjugate. Interleukin-5 level was decreased and interferon γ level was increased in nasal lavage fluid by injection of ISS-ODN/Der f conjugate. Der f–specific immunoglobulin E was decreased by injection of ISS-ODN or Der f /ISS-ODN conjugate; however, these were not statistically significant. Transforming growth factor β 1 secreted by cultured splenocyte was increased significantly in ISS-ODN/Der f conjugate group.

Conclusion: These results suggest ISS-ODN/Der f conjugate induces an antiallergic effect and induces an increase in transforming growth factor β 1 level in the allergic rhinitis model using Der f allergen. Allergic response developed by Der f allergen could be more effectively reduced by injection with ISS-ODN/Der f conjugate than by injection with ISS-ODN only. (Am J Rhinol 20:212–218, 2006)

The prevalence of allergic diseases such as asthma and allergic rhinitis is increasing, as societies are becoming more industrialized. The annual cost of these diseases is increasing also. A variety of treatment modalities have been developed and are being developed now.

Currently, three treatment modalities (avoidance, pharmacotherapy, and immunotherapy) are being used to treat allergic rhinitis. Avoidance is the safest way but is difficult to obtain satisfactory results. Pharmacotherapy is the basic treatment modality in allergic rhinitis; however, it can not cure the disease. It just helps to control allergic diseases, and drug adverse reactions often occur. Immunotherapy, the third treatment modality, can be applied using multiple subcutaneous injections of antigen. It is time-consuming, expensive, and painful and has the potential to induce allergic reactions, including anaphylaxis. The mechanism of efficacy of allergen-specific immunotherapy is thought to be the induction of

immunologic tolerance. Unlike pharmacotherapy, we can expect a long-term cure of disease using immunotherapy.

Much effort has been made to reduce the side effects and to enhance the efficacy of immunotherapy including change in route of administration, recombinant allergens, peptide immunotherapy, anti-immunoglobulin E (IgE) therapy, and DNA-based immunotherapy. Change in route of administration such as sublingual, oral, and nasal routes has been attempted to reduce the side effects, and high-dose sublingual immunotherapy has been found to be an effective alternative.¹ Nineteen recombinant allergens with less allergenicity are used for skin test, and the studies of recombinant allergens as a basis for standardization of allergenic products currently are being performed in Europe. Synthetic peptides representing T-cell epitopes can induce anergy to challenges with whole protein, and several clinical trials using Fel d 1, a cat antigen, have been performed.^{2–4} A combination treatment of anti-IgE (omalizumab) and allergen immunotherapy showed additive effects for birch and grass pollen antigen.⁵

Recent studies revealed that allergic diseases occur as a result of increased Th2 response relative to Th1 response and that immunotherapy diverts Th2 response to Th1 response.^{6–8} Immunostimulatory sequences of unmethylated CpG-containing oligodeoxynucleotide (ISS-ODN) have been identified as potent immune inducers that can prevent and/or divert Th2-biased immune responses. ISS-ODN has been shown to attenuate allergic hypersensitivity responses in several animal models.⁹ In this regard, attention has been given to ISS-ODN as a new immunotherapeutic agent in allergic diseases.

ISS-ODN, also known as CpG motifs, is a family of unmeth-

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Supported by Seoul National University Research Fund (800-20030099)

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ylated CpG dinucleotide. Previously, a Japanese group investigating the antitumor growth effect of the Bacille Calmette-Guerin (BCG) vaccination made an observation,¹⁰ which initially were thought to be due to the stimulation of the immune system by BCG. This group later discovered that the major stimulatory effect was caused by mycobacterial DNA and extended their description of bacterial DNA as an immunostimulatory agent.¹¹ Another group revealed that some ODNs induce B-cell proliferation and that these ODNs have an unmethylated central cytosine-guanosine (CG) dinucleotide, two purines in 5' end, and two pyrimidines in 3' end.¹² The motif was proved to be the same one found in BCG and was found to be 20 times more abundant in bacteria than in the vertebrate. Inversion or CG of methylation will completely abrogate the immunostimulatory effect of the motif. The unmethylated form of CG dinucleotide is abundant in bacterial, viral, and invertebrate eukaryotic genome and <30% in vertebrate genome.¹³ This explains how the mammalian immune system can discriminate between bacterial and host DNA. The bacterial DNA is unmethylated and contains many CG dinucleotide. Therefore, when it enters the mammalian immune system, it easily stimulates the host immune system.

It was known in the 1980s that DNA component of BCG or plasmid DNA bias Th1 immune response, and in the middle of 1990s it was revealed that the unmethylated CpG motifs play the major role in that immune response. Studies using artificial CpG motifs in animal models were begun in the middle of the 1990s. Several laboratories revealed that allergen ISS-ODN conjugates are highly immunogenic^{14,15} and more effective in inducing Th1 responses than ISS-ODN alone or by co-injection of ISS-ODN with allergen.¹⁶ We showed that ISS-ODN can bias Th1 response in the allergic rhinitis mouse model in a previous study.¹⁷

The aim of this study was to evaluate the antiallergic activity of ISS-ODN and to determine whether the conjugate of ISS-ODN and whole body extract of *Dermatophagoides farinae* (*Der f*) had an enhanced immunotherapeutic potential in the murine model of allergic rhinitis sensitive to house-dust mites (HDMs), which is the most common allergen-inducing allergic rhinitis in Korea.

MATERIALS AND METHODS

Production of Oligonucleotide

Immunostimulatory sequences of unmethylated CpG-containing oligonucleotide (ISS-ODN), which include CpG DNA

motif and mutant ODN (M-ODN) were supplied by Bioneer Co. (Daejeon, Korea). Three bases of each 5' and 3' end were phosphorothioated. Phosphorothioated ODN is resistant to the DNAase and has a longer half-life than a nonphosphorothioated one. ISS-ODN and M-ODN had the following sequences: ISS-ODN, 5'TGACTGTGAACGTTTCGAGATGA 3'; and M-ODN, 5' TGACTGTGAACCTTAGAGATGA 3'.

Conjugation of ISS-ODN with Crude Extract of *Der f*

Conjugates of ISS-ODN and M-ODN with crude extract of *Der f* were donated from Eyal Raz, San Diego, CA. The detailed procedure was described in an article published previously.¹⁶

Murine Allergic Rhinitis Model and Treatment

Specific pathogen-free C57BL/6 mice were used as experimental animals. Each mouse weighed 20–30 g and were 5 weeks old. Seven groups of five mice were divided into two control groups and five experimental groups (Table 1). Crude extract of *Der f* (donated by C.S. Hong, Research Institute of Allergy and Clinical Immunology, Yonsei University, Seoul, Korea) was stored in a 4°C refrigerator and was dissolved in phosphate-buffered saline (PBS). All groups except for the negative control group were sensitized using *Der f* crude extract. They were immunized intradermally at the base of the tail with 100 µg of *Der f* crude extract and 100 µL of complete Freund's adjuvant. On days 1 and 3, 300 ng of pertussis toxin (Sigma, St. Louis, MO) was injected intraperitoneally and on day 7, 100 µg of *Der f* crude body extracts and 100 µL of incomplete Freund's adjuvant were injected intradermally (Fig. 1). After aforementioned general sensitization, 20 µg of *Der f* crude body extract dissolved in 20 µL of PBS was instilled into the nares of the mice using a micropipette once a week for five times to induce local sensitization. In the negative control group, PBS was injected intraperitoneally and instilled into the nares five times.

Subcutaneous injections were done to two control groups and five treatment groups 24 hours before nasal challenge on days 13, 27, and 41 as follows: (1) group A (negative control group), 100 µL of PBS; (2) group B (positive control group), 100 µL of PBS; (3) group C, 50 µg of M-ODN/*Der f* conjugate; (4) group D, 50 µg of ISS-ODN/*Der f* conjugate (33 µg of ISS-ODN and 37 µg of *Der f*); (5) group E, 50 µg of ISS-ODN; (6) group F, co-injection of ISS-ODN (50 µg) and *Der f* (50 µg); (7) group G, 50 µg of *Der f* only.

Table 1 The Experimental Groups and Characteristics of Each Group

Group (n)		Systemic Sensitization	Local Sensitization	Treatment
A (5)	(–) control	PBS	PBS	50 µL of PBS
B (5)	(+) control	20 µg of HDM	20 µg of HDM	50 µL of PBS
C (5)	M-ODN/ <i>Der f</i> conjugate	20 µg of HDM	20 µg of HDM	50 µg of M-ODN/ <i>Der f</i>
D (5)	ISS-ODN/ <i>Der f</i> conjugate	20 µg of HDM	20 µg of HDM	50 µg of ISS-ODN/ <i>Der f</i>
E (5)	ISS	20 µg of HDM	20 µg of HDM	50 µg of ISS-ODN
F (5)	ISS + <i>Der f</i>	20 µg of HDM	20 µg of HDM	ISS-ODN (50 µg) + <i>Der f</i> (50 µg)
G (5)	<i>Der f</i>	20 µg of HDM	20 µg of HDM	50 µg of <i>Der f</i>

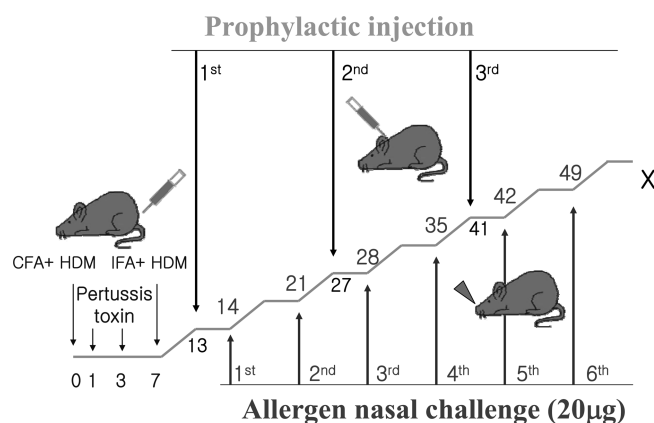


Figure 1. Schematic illustration of experimental protocol. C57BL/6 mice were immunized intradermally at the base of the tail with 100 µg of Der f crude body extracts and 100 µL of complete Freund's adjuvant (on day 0), 300 ng of pertussis toxin intraperitoneally (days 1 and 3), and 100 µg of Der f crude body extracts and 100 µL of incomplete Freund's adjuvant intradermally (on day 7). After general sensitization, 20 µg of Der f of crude body extracts dissolved in 20 µL of PBS was instilled into the nares of the mice using a micropipette once a week for five times to induce local sensitization. Subcutaneous prophylactic injections were done to five treatment groups 24 hours before nasal challenge on days 13, 27, and 41. CFA, complete Freund's adjuvant; IFA, incomplete Freund's adjuvant.

Evaluation of Allergic Response

Symptom Score. On day 49, after last nasal instillation of allergen or PBS, total numbers of nasal scratching and sneezing were counted for 15 minutes.

Histology of Nasal Mucosa and Eosinophil Quantification. On day 50, 24 hours after last nasal challenge, all mice were killed. Heads were separated from the body and the scalps were removed. Whole heads were fixed in 10% formalin, decalcified, embedded in paraffin blocks, and sectioned coronally near the posterior margin of the orbit. Sections were cut at a 4- to 5-µm thickness. Luna staining was performed and under light microscopic vision (400× magnification; Laborlux K, Lekca, Wetzlar, Germany), eosinophils were counted in the submucosal area of the whole nasal septum using an eyepiece reticule. A single observer, not knowing the experimental conditions, counted all of the slides.

Measurement of Cytokines in Nasal Lavage Fluid. Nasal lavages were done after partial tracheal resection using 22-gauge catheters. The catheter was inserted into the tracheal opening in the direction of the upper airway and saline washing was collected from the nares. The nasal lavage fluid was cytospun and the supernatants were assayed using enzyme-linked immunosorbent assay (ELISA). Interleukin (IL)-5 and interferon (IFN) γ concentrations were measured in nasal lavage fluid using the ELISA kit (R&D systems, Minneapolis, MN) according to the manufacturer's instruction. **Specific IgE Levels.** Blood samples were obtained from the portal vein in the abdomen. Serum-specific IgE levels were measured using the ELISA technique. Microtiter plates were coated overnight with 5 µg of Der f extract per milliliter of carbonate-buffered saline (pH 9.6) and then washed with PBS-tw (0.025% Tween 20 in PBS) and nonspecific binding sites were then blocked

with 5% skim milk (Sigma). After washing twice in PBS-tw, serum samples were diluted 1:10 with washing buffer. After 2-hour incubation at 4°C, the plates were washed and incubated with horseradish peroxidase-conjugated rat anti-mouse IgE (Southern Biotechnology Associates, Birmingham, AL) for 1 hour at room temperature. The plates were washed with PBS-tw, and a solution of orthophenyldiamine substrate (Sigma) was added. Absorbance at 490 nm was read 30 minutes after the addition of the substrate.

Transforming Growth Factor β1 in Splenocyte Culture. Single-cell suspensions of the spleen were plated in 96-well tissue culture plates at a final concentration of 10⁵ cells/mL in AIM-V media (Gibco, Grand Island, NY). The cells were incubated in a CO₂ incubator at 37°C for 72 hours, stimulated with crude extract of Der f for 72 hours, and stored at -70°C until the measurement of cytokine. Transforming growth factor (TGF) β1 was assayed in culture supernatant using a sandwich ELISA kit (Mouse/Rat/Porcine TGF-β1 Immunoassay; R & D systems) according to the manufacturer's instructions.

Data Analysis. Using the SPSS 10.0 for Windows (SPSS, Inc., Chicago, IL), we performed mean analysis. The data were expressed as the mean ± SEM. Data analysis was done using the Mann-Whitney test and a value of *p* < 0.05 was considered significant.

RESULTS

Symptom Score

The symptom score, which was the sum of the number of scratching and sneezing for 15 minutes after allergen challenge, of the positive control group was 79.0 ± 9.3 and was increased significantly compared with that of the negative control group (Fig. 2). The symptom scores of the conjugate group and ISS group were 37.0 ± 14.4 and 54.0 ± 8.2, respectively.

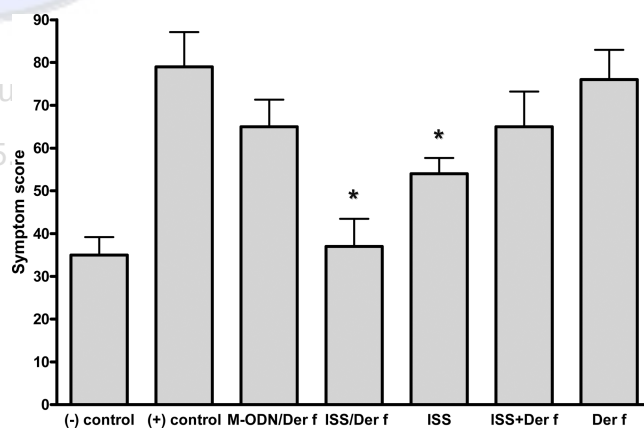


Figure 2. Symptom scores were counted for 15 minutes after allergen challenge. The mean symptom score of the ISS-ODN/Der f conjugate group and that of the ISS group were 37.0 ± 14.4 and 54.0 ± 8.2, respectively, which were statistically significant, compared with that of the positive control group (*p* < 0.05). However, the mean symptom score of neither the ISS + Der f group (65.0 ± 18.4) nor the Der f group was significantly different from that of the positive control group. Five mice were used in each group (**p* < 0.05 versus the positive control group).

tively, which were statistically significant, compared with that of the positive control group ($p = 0.008$ and 0.016 , respectively). But, the symptom scores of ISS + *Der f* group (65.0 ± 18.4) and the of *Der f* group were not significantly different from that of the positive control group.

Histology of Nasal Mucosa and Eosinophil Quantification

The number of eosinophils in the submucosal area in the whole nasal septum was counted under light microscope (400× magnification; Fig. 3). The eosinophil number in the positive control group was 92.4 ± 18.9 , and it was significantly different from that of negative control group ($p = 0.006$). The eosinophil numbers in the conjugate group, ISS group, and ISS + *Der f* group were 21.8 ± 8.41 , 37.8 ± 13.3 , and 22.4 ± 6.54 , respectively, and were significantly reduced compared with that in the positive control group ($p = 0.008$ in all). The numbers in *Der f* group and M-ODN/*Der f* conjugate group were decreased also (47.0 ± 15.1 and 70.8 ± 18.2 , respectively); however, they were not significantly different ($p > 0.05$).

IL-5 in Nasal Lavage Fluid

IL-5 concentration in nasal lavage fluid of the positive control group was 52.1 ± 18.23 pg/mL and it was significantly different from that of the negative control group (Fig. 4). IL-5 concentration in nasal lavage fluid of the ISS-ODN/*Der f* conjugate group was 13.4 ± 3.1 pg/mL and was significantly different from that of the positive control group ($p = 0.008$). Those of other groups were not significantly different from that of positive control group ($p > 0.05$).

IFN-γ in Nasal Lavage Fluid

The IFN-γ concentration in nasal lavage fluid of positive control group was 131.6 ± 69.1 pg/mL (Fig. 5). Those of

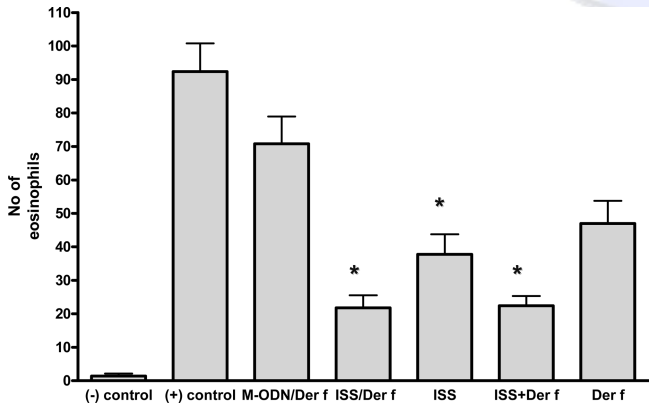


Figure 3. Eosinophil counts in the nasal mucosa. The mean eosinophil count in the positive control group was 92.4 ± 18.9 . The eosinophil counts in the ISS-ODN/*Der f* conjugate group, the ISS group, and the ISS + *Der f* group were 21.8 ± 8.4 , 37.8 ± 13.3 , and 22.4 ± 6.5 , respectively, and were significantly reduced compared with that in the positive control group. The counts in the *Der f* group and the M-ODN/*Der f* group were decreased also. However, they were not significantly different ($p > 0.05$). Five mice were used in each group (* $p < 0.05$ versus the positive control group).

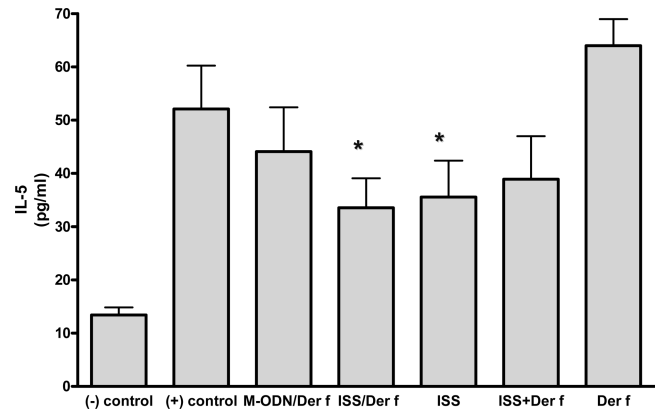


Figure 4. The IL-5 concentrations in nasal lavage fluid. IL-5 concentration of the ISS-ODN/*Der f* conjugate group is 13.4 ± 3.1 pg/mL and is significantly different from that of the positive control group. Those of the other groups were not significantly different from that of the positive control group. Five mice were used in each group (* $p < 0.05$ versus the positive control group).

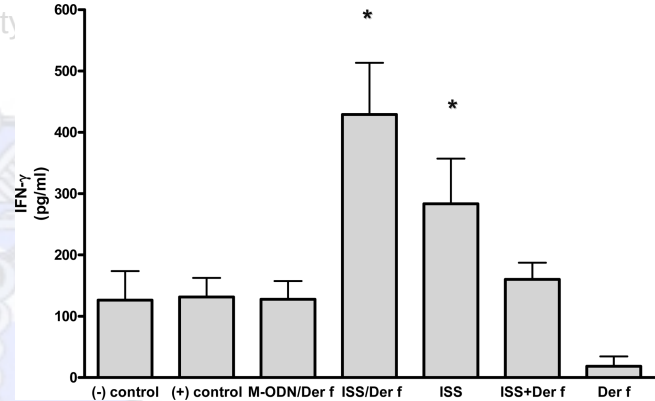


Figure 5. IFN-γ concentrations in nasal lavage fluid. Mean IFN-γ concentration of the ISS-ODN/*Der f* conjugate group and that of the ISS group were 429.0 ± 188.9 and 288.5 ± 164.5 and were significantly different from that of the positive control group. Those of the other groups were not significantly different from that of the positive control group ($p > 0.05$). Five mice were used in each group (* $p < 0.05$ versus the positive control group).

ISS-ODN/*Der f* conjugate group and ISS group were 429.0 ± 188.9 pg/mL and 288.5 ± 164.5 pg/mL, respectively, and were significantly different from that of the positive control group ($p = 0.016$ and 0.045 , respectively). Those of other groups were not significantly different from that of the positive control group ($p > 0.05$). However, there was no significant difference between the positive control group and the negative control group in IFN-γ concentration.

Serum-Specific IgE Levels

The *Der f*-specific IgE level in the ISS-ODN/*Der f* conjugate group was lower than that of the positive control group but was not statistically significant ($p > 0.05$; Fig. 6). The IgE level in the *Der f* group was increased more than that of the positive control group, but this increase was not statistically significant ($p > 0.05$).

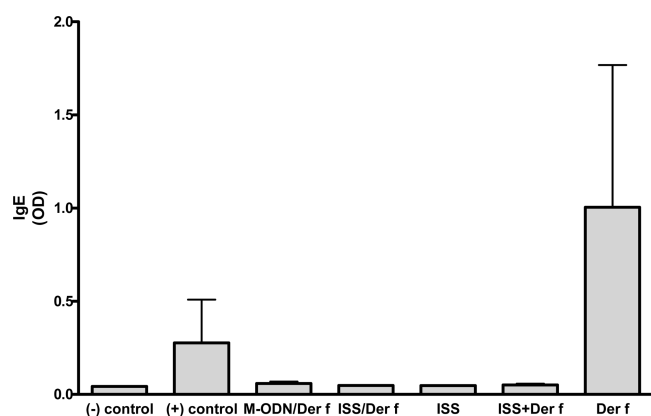


Figure 6. Serum levels of the Der f-specific IgE antibody. No group showed significantly different Der f-specific IgE levels versus the positive control group. Five mice were used in each group.

TGF- β 1 in Splenocyte Culture

The TGF- β 1 levels shown in Fig. 7 are subtraction of TGF- β 1 level in the Der f unstimulated spleen culture from that in Der f stimulated (Fig. 7). The TGF- β 1 level in the ISS-ODN/Der f conjugate group was increased compared to the positive control group and was statistically significant. The TGF- β 1 level of the M-ODN/Der f conjugate group was not increased significantly. However, we could not find any difference between negative control group and positive control group.

DISCUSSION

In this study we evaluated the immunostimulatory effect of ISS-ODN/Der f conjugate. Several studies showed enhanced immunogenicity with the ISS-ODN/allergen conjugate vaccination.^{15,16} They reported 50-fold more increases in the IFN- γ secretion by spleen cells stimulated by ISS-ODN/allergen conjugate than by the mixture of allergen and ISS-ODN. The synergistic effect of ISS-ODN/allergen conjugate is postulated as follows. Allergen is engulfed, processed, and presented to Th0 cells by antigen-presenting cells (APCs), whereas ISS-ODN promotes IL-12 secretion, which directs T-cell development to Th1 phenotype, from APCs. If ISS-ODN/allergen

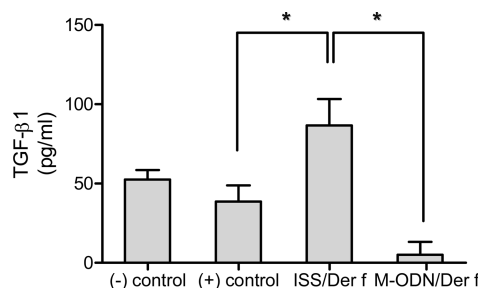


Figure 7. TGF- β 1 levels in the splenocyte culture. These values are subtractions of TGF- β 1 levels in the Der f unstimulated spleen culture from the Der f stimulated spleen culture. The ISS/Der f conjugate group showed significantly different TGF- β 1 levels versus the positive control group. Five mice were used in each group (* $p < 0.05$ versus the positive control group).

conjugate is administered, allergen presentation by APCs to Th0 cells and stimulation of IL-12 secretion to induce Th1 development can occur in the same APCs. Thus, nonspecific ISS-ODN can behave as allergen specific, because the effects of ISS-ODN are concentrated on the T cells specific for the allergen that is conjugated to ISS-ODN.¹⁸ When allergen and ISS-ODN are administered as an unconjugated mixture, the probability of APCs picking up allergen and ISS-ODN simultaneously is lower than when ISS-ODN/allergen conjugate is injected and therefore the immunostimulatory effect would decrease.

In our study, the effect of ISS-ODN/Der f conjugate was higher than that of ISS-ODN alone in allergic symptoms and in eosinophil count, but they were not statistically significant ($p = 0.056$ for both ISS-ODN/Der f conjugate and ISS-ODN alone). The amount of ISS-ODN in ISS-ODN/Der f conjugate group (33 μ g of ISS-ODN) was smaller than that in ISS-ODN group, so if the same amount of ISS-ODN were used, there would be statistical significance between ISS-ODN group and ISS-ODN/Der f conjugate group. When the effect of ISS-ODN/Der f conjugate was compared with that of co-injection of ISS and Der f, ISS-ODN/Der f conjugate reduced allergic symptoms more effectively than the mixture of ISS and Der f, but not eosinophil count. The effect of a simple mixture of allergen and ISS-ODN was variable in the literature. Kline *et al.*¹⁹ reported that mixture of allergen and ISS-ODN had a more pronounced effect than ISS-ODN alone in the murine model of asthma; however, Cho *et al.* found that mixture of ISS-ODN and ovalbumin (OVA) showed slightly better effects in protective immunity than ISS-ODN alone.²⁰ Compared with the effect of ISS-ODN/allergen conjugate, the mixture of allergen and ISS-ODN was less effective and the difference can be as much as 100 times.¹⁸ In this study, the mixture of ISS-ODN and Der f is less effective than ISS-ODN/Der f conjugate and ISS + Der f group showed variable results when compared with ISS-ODN group. Tighe *et al.* showed that the quantity of ISS-ODN in the ISS-ODN/protein mixtures can change the immunogenicity of the mixture.¹⁵ That result also can explain variable results in immunogenicity.

IL-5, which is a Th2 cytokine, was decreased and IFN- γ , which is a Th1 cytokine, was increased in ISS-ODN/Der f conjugate group and in ISS-ODN group compared with that of the positive control group. IL-5 was decreased statistically only in the ISS-ODN/Der f conjugate group, and the greatest increase in IFN- γ also was seen in the ISS-ODN/Der f conjugate group. These results showed that ISS-ODN/Der f conjugate biased the Th1 reaction and is more immunogenic than ISS-ODN alone, which is consistent with previous reports using other antigens.^{15,16}

TGF- β is a pleiotropic cytokine known to affect immune mechanisms such as T-cell proliferation, differentiation, apoptosis, antigen presentation, and B-cell proliferation. It also is known that TGF- β induces peripheral T-cell suppression with cooperation of IL-10 in both during natural exposure to aeroallergens in healthy individuals and during systemic immunotherapy in allergic rhinitis patients.²¹ In our study TGF- β 1 secreted by *in vitro* stimulated splenocyte was increased in the conjugate group, which implied that increased TGF- β 1 level could be one of the mechanisms of peripheral tolerance by the ISS-ODN/Der f conjugate.

The Der f-specific IgE level was increased in the Der f

group, in which *Der f* antigen was treated for three times after sensitization. Those injections might boost the IgE production more than those of the positive control group. In other groups, the IgE level was decreased compared with that of the positive control group, which was not statistically significant. Thigh *et al.* reported that IgE titer in the allergen-ISS conjugate-treated group was significantly less than that of the positive control or allergen-treated group during the immunization period.¹⁶

Most of the animal studies used asthma models using ragweed extract,²² Amb a 1 (one of ragweed Ag),²³ Bet v 1,²⁴ Schistosoma egg antigen,^{19,25} OVA,^{19,26} and conalbumin²⁷ as antigens. Studies of ISS-ODN using HDM allergic rhinitis model are rare. In case of DNA vaccination, it was reported that transfer of DNA gene encoding Der p1²⁸ or *Der f* 11²⁹ induced Th1 responses. There is a report on the positive effect of ISS on Th1 cytokine promotion induced by *Der f* *in vitro*.³⁰ The effect of ISS-ODN in the OVA-induced rhinosinusitis model was reported recently.³¹ They showed that ISS-ODN prevented the development of Th2-mediated eosinophilic inflammation and symptoms in a murine model of allergic rhinosinusitis.

In this study, ISS-ODN or ISS-ODN/*Der f* conjugate was applied three times biweekly and only short-term effects were evaluated and long-term results have to be evaluated. Tighe *et al.* found that ISS-ODN conjugate induces antigen-specific and Th1-biased responses that are maintained at least for 1 year and are resistant to the Th2-biased reaction for at least 5 months in murine models.^{16,21} However, when ISS-ODN was applied without allergen, the immunomodulatory effect lasted <2 months.

A phase 1 clinical trial using ragweed allergen, Amb a 1, found that Th1/Th2 alteration lasted for at least 16 weeks after 6 weeks of consecutive injections of Amb a 1-ISS conjugate.³² In another clinical trial on ragweed hay fever, Tulic *et al.* reported that after 6 consecutive weekly injections of Amb a 1-ISS conjugate, there was a significant decrease in chest pain and a trend toward reduced nasal symptoms in the second ragweed season. Total symptom scores and medication used in the next year trended lower, but the differences were not statistically different.³³ This study provided evidence for long-lasting effects of Amb a 1-ISS conjugate. Additional evidence for efficacy and safety have to be sought for immunotherapy using CpG motif.

In summary, ISS-ODN/*Der f* conjugate reduced allergic symptoms and eosinophil infiltration, diverted Th1 response, and increased TGF- β production by splenocyte in the allergic rhinitis mouse model using *Der f* allergen. Allergen-ISS conjugate can be a promising immunotherapeutic agent of HDM, which is the most common antigen in Korea, and other allergens if its efficacy and safety were proved.

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