
Immunostimulatory sequence oligodeoxynucleotide/cholera toxin B conjugate: a novel allergen-independent intranasal vaccine for allergic rhinitis

Tae-Bin Won, MD*; Song-Hua Quan, PhD†; Dong-Young Kim, MD†‡; Chae-Seo Rhee, MD†‡; and Chul Hee Lee, MD, PhD†‡

Background: Recent studies have shown that by acting as strong T_H1 response-inducing adjuvants, DNA immunostimulatory sequence oligodeoxynucleotides (ISS-ODNs) can be used in the treatment of allergic diseases, and efforts are being made to enhance these T_H1 adjuvant actions.

Objective: To determine whether intranasally delivered ISS-ODN/cholera toxin B (CTB) conjugate has enhanced antiallergic effects in an allergic rhinitis mouse model.

Methods: BALB-c mice were sensitized with ovalbumin. Chemical conjugation of ISS-ODN and CTB was performed. After a single local intranasal administration of 50 µg of ISS-ODN or high- and low-dose (50- and 5-µg) ISS-ODN/CTB conjugate, we measured the allergic response in terms of sneezing events, eosinophil infiltration in the nasal mucosa, serum ovalbumin specific IgE and IgG2a levels, and T_H1 and T_H2 cytokine levels in nasal lavage fluid and spleen cell cultures.

Results: A single local administration of 50 µg of ISS-ODN did not suppress the allergic phenotype. However, 50 and 5 µg of ISS-ODN/CTB conjugate significantly attenuated allergic symptoms, eosinophil infiltration in the nasal mucosa, and interleukin 4 production from nasal lavage fluid and cultured splenocyte supernatant compared with the allergic control. Serum specific IgG2a and interleukin 12 production in nasal lavage fluid and spleen cell cultures was significantly increased.

Conclusions: In a mouse model of allergic rhinitis, a single intranasal delivery of low-dose ISS-ODN/CTB conjugate effectively protects previously sensitized mice from allergic hypersensitivity responses. With further research, ISS-ODN/CTB conjugate may serve as a new allergen-independent intranasal vaccine for the treatment of allergic rhinitis.

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INTRODUCTION

Bacterial DNA has inherent immunostimulatory activities. The molecular basis lies in the content of unmethylated CpG deoxynucleotides (CpG motifs) found in the microbial genome.¹ Synthetic immunostimulatory sequence oligodeoxynucleotides (ISS-ODNs) with CpG motifs have been shown to induce immune responses dominated by cytokines that are known to inhibit the allergic phenotype and to promote T_H1-polarized innate and adaptive immune responses.^{2,3} These findings have prompted the use of ISS-ODN in the treatment of allergic diseases.^{4,5} Although temporary, allergen-independent immunomodulation with ISS-ODN has the clinical advantage of avoiding the limitations of allergen

immunization, such as anaphylactic reactions, extensive allergy testing, and the need for several months for the therapeutic effects to develop. Allergen-independent delivery of ISS-ODN can provide almost immediate protection against allergic responses in T_H2-sensitized mouse models of allergic conjunctivitis and asthma, the efficacy of which has proved to be equal to or higher than that of corticosteroid therapy.^{6–9} Recently, Rhee et al¹⁰ found that intranasal delivery of ISS-ODN attenuates hypersensitivity responses associated with allergic rhinitis in a mouse model of allergy. Efforts have been made to augment the immunostimulatory effects of ISS-ODNs in the form of structural modifications of the DNA itself or adsorption to the surface of cationic microspheres.¹¹ A recent study¹² has shown that using tetanus toxoid as a vaccine antigen for subcutaneous immunization, ISS-ODN conjugated to cholera toxin B (CTB) markedly enhanced the antigen specific IgG antibody response and altered the specific pattern of antibody isotypes toward a T_H1-type response. However, studies on immunomodulation of type 1 allergy by ISS-ODN/CTB conjugate have not been reported, to our knowledge.

This is the first study, to our knowledge, to compare the effects of a single dose of intranasally delivered ISS-ODN conjugated to CTB with those of ISS-ODN alone in attenu-

Affiliations: * Department of Otorhinolaryngology–Head and Neck Surgery, Hallym University College of Medicine, Seoul, Korea; † Department of Otorhinolaryngology–Head and Neck Surgery, Seoul National University College of Medicine, Seoul, Korea; ‡ Institute of Allergy and Clinical Immunology, Seoul National University Medical Research Center, Seoul, Korea.

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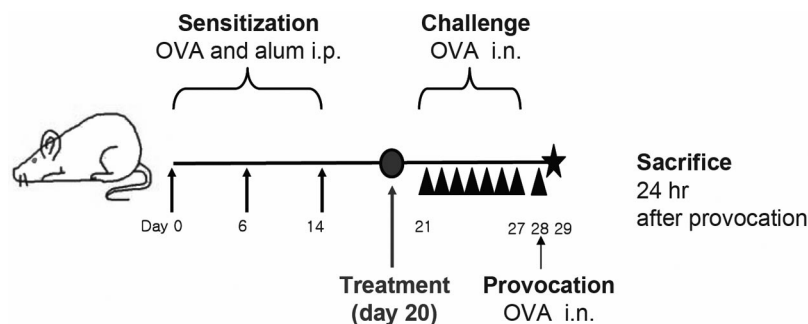


Figure 1. The allergic rhinitis mouse model. BALB-c mice were sensitized by means of intraperitoneal (i.p.) injection of 25 μ g of ovalbumin (OVA) and 2 mg of aluminum hydroxide (alum) in 300 μ L of phosphate-buffered saline (PBS) on 3 occasions spaced 1 week apart. One week after the last sensitization (day 21), mice received a series of 7 daily intranasal (i.n.) OVA challenges consisting of 200 μ g of OVA in 20 μ L of PBS (10 μ L per nostril). Subsets of mice received control oligodeoxynucleotide/cholera toxin B (C-ODN/CTB) conjugate, high- and low-dose immunostimulatory sequence (ISS)-ODN/CTB conjugate, ISS-ODN, ISS-ODN mixed with CTB, or CTB via the i.n. route 1 day before initiating i.n. OVA challenges (day 20). Mice were humanely killed for analyses 24 hours after nasal provocation with OVA (day 29). Five mice were used in each group. Arrows indicate OVA and alum i.p. injection; black circle, i.n. treatment; black triangles, OVA or PBS challenge; and star, humanely killed.

ating hypersensitivity responses in a mouse model of allergic rhinitis. We report that the immunomodulatory activities of intranasally delivered ISS-ODN that attenuate the allergic phenotype can be enhanced when coupled with CTB.

METHODS

ODN and Reagents

Immunostimulatory sequences of unmethylated CpG-containing oligodeoxynucleotide (ISS-ODN; N1018), which included 1 CpG DNA motif and control ODN (C-ODN; N1019) with no CpG motif were supplied by Trilink Biotech (San Diego, California). Three bases of each 5' and 3' end were phosphorothiolated. The ISS-ODN and C-ODN had the following sequences: 5'TGACTGTGAACGTTTCGAGATGA 3' and 5' TGACTGTGAACCTTAGAGATGA 3', respectively. Ovalbumin (grade V) and aluminum hydroxide were purchased from Sigma-Aldrich Corp (St Louis, Missouri). The CTB was supplied by List Biological Laboratories Inc (Campbell, California)

Conjugation of ISS-ODN to CTB

The conjugation of ISS-ODN to CTB (ISS-ODN/CTB) and of C-ODN to CTB (C-ODN/CTB) was performed by mixing thiolated ISS-ODN with maleimide-activated CTB. Maleimido groups were introduced to exposed lysines of the CTB molecule by means of incubation with sulfosuccinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (Pierce Chemicals, Rockford, Illinois) for 2 hours. A thiol group was added to the ODNs by means of incubation with tricarboxyethylphosphine (Pierce Chemicals). Maleimide-activated CTB and thiolated ODNs were incubated together overnight at room temperature, and ODN/CTB conjugates were purified using a Superdex 200 gel filtration column (GE Healthcare, Chalfont St Giles, England). Qualitative measurement of the composition of the ODN/CTB conjugates was analyzed by means of sodium dodecyl sulfate–polyacrylamide gel elec-

trophoresis. The CTB and ODNs in the conjugate were visualized with Coomassie blue (Sigma-Aldrich Corp) and DNA silver (Amersham Biosciences) staining, respectively. The final conjugation ratio of ISS-ODN:CTB was 5.314:1. The GM1-binding activity of ODN/CTB conjugates was confirmed by a solid-phase GM1 enzyme-linked immunosorbent assay (ELISA). The lipopolysaccharide content of ODN/CTB conjugates was 0.1 EU/mL.

In Vitro Effect of ISS-ODN/CTB Conjugate on Lymphoid Cells

To evaluate the immunostimulatory properties of the ISS-ODN/CTB conjugate in vitro, spleen cells of BALB-c mice (4×10^6 cells in 100 μ L/well) were treated with medium, ISS-ODN (1 μ g/mL), ISS-ODN (1 μ g/mL) + CTB (1.46 μ g/mL), ISS-ODN/CTB conjugate (1 μ g/mL), or C-ODN/CTB conjugate (1 μ g/mL). They were incubated at 37°C, and 5% carbon dioxide and culture supernatants were harvested after 24 hours for interleukin 12 (IL-12) ELISA. Results reflect 3 separate experiments.

Experimental Animals

Five-week-old, female, specific pathogen-free, BALB-c mice each weighing 20 to 30 g were used as experimental animals. The studies reported herein followed the principles for laboratory animal research as outlined in the Animal Welfare Act and the Department of Health, Education, and Welfare (National Institutes of Health) guidelines for the experimental use of animals, and the experimental protocols were approved by the Seoul National University Hospital-Animal Welfare Committee.

Sensitization, Intervention, and Allergen Challenge

Eight groups of 5 mice each were divided into 2 control groups and 6 experimental groups. Allergen sensitization and challenge for the development of the allergic rhinitis murine model are summarized in Figure 1. Briefly BALB-c mice

Table 1. Sensitization and Challenge and Treatment of the 8 Experimental Groups

Group (N = 5)	Sensitization and challenge	Treatment
Negative control	PBS	25 μ L of PBS
Positive control	Ovalbumin	25 μ L of PBS
C-ODN/CTB	Ovalbumin	50 μ g of C-ODN/CTB IN
50 ISS/CTB	Ovalbumin	50 μ g of ISS-ODN/CTB IN
5 ISS/CTB	Ovalbumin	5 μ g of ISS-ODN/CTB IN
50 ISS	Ovalbumin	50 μ g of ISS-ODN IN
50 ISS + CTB	Ovalbumin	50 μ g of ISS-ODN + 73.2 μ g of CTB IN
CTB	Ovalbumin	73.2 μ g of CTB IN

Abbreviations: C-ODN, control-oligodeoxynucleotide; CTB, cholera toxin B; ISS-ODN, immunostimulatory sequence ODN; IN, intranasal delivery; PBS, phosphate-buffered saline.

were sensitized by intraperitoneal injection of 25 μ g of ovalbumin and 2 mg of aluminum hydroxide in 300 μ L of phosphate-buffered saline (PBS) on 3 occasions spaced 1 week apart. One week after the last sensitization (day 21), mice received a series of 7 daily intranasal ovalbumin challenges consisting of 200 μ g of ovalbumin in 20 μ L of PBS. Twenty-four hours before the first nasal challenge, immunomodulatory intervention was performed in subsets of mice via the intranasal route. Mice were humanely killed for analyses 24 hours after nasal provocation with ovalbumin (day 29). The different groups and doses are given in Table 1. In the present study, 50 μ g of ISS-ODN/CTB conjugate contained 50 μ g of ISS-ODN and 73.2 μ g of CTB.

Evaluation of Allergic Responses

Sneezing score. On day 28, after the final ovalbumin challenge, the frequency of sneezing was counted in each mouse for 15 minutes by 2 blinded observers, and the average score was calculated.

Measurement of cytokines in nasal lavage fluid. On day 29, 24 hours after the last nasal challenge, nasal lavage was performed after partial tracheal resection using 22-gauge catheters. The nasal passages were gently perfused with 500 μ L of PBS, and fluid was collected from the nares. Levels of IL-4, interferon γ (IFN- γ), IL-12 (IL-12p70), and IL-10 in nasal lavage fluid supernatants were determined using commercial ELISA kits (R&D Systems, Minneapolis, Minnesota) according to the manufacturer's instructions.

Histologic evaluation of nasal mucosa and eosinophil quantification. Twenty-four hours after final nasal challenge, whole heads were fixed in 10% formalin, decalcified, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. Coronal sections, 4 μ m thick, were performed 5 mm from the nasal vestibule. Eosinophils were counted on both

Table 2. In Vitro Immunostimulatory Effects of ISS-ODN and ISS-ODN/CTB Conjugate on Lymphoid Cells^a

Group	IL-12 (pg/mL) (4 $\times$ 10 ⁶ cells/24 h)
PBS	ND
ISS-ODN (1 μ g/mL)	879 (178)
ISS-ODN (1 μ g/mL) + CTB	1,252 (267)
ISS-ODN/CTB (1 μ g/mL)	9,032 (143) ^b
C-ODN/CTB (1 μ g/mL)	125 (110)

Abbreviations: C-ODN, control oligodeoxynucleotide; CTB, cholera toxin B; ISS, immunostimulatory sequence; ND, not detected.

^a Data are given as mean (SD) of triplicate assays and represent 3 separate experiments.

^b Significant difference compared with the ISS-ODN group ($P < .05$).

sides of the nasal septum using light microscopic vision (magnification $\times 400$) (Laborlux K; Lekca, Wetzlar, Germany) using an eyepiece reticule. The mean number on each side was considered to be the eosinophil count. A single observer, unaware of the experimental conditions, counted all the slides.

Serum ovalbumin specific immunoglobulin levels. Serum samples were collected 24 hours after the last nasal ovalbumin challenge for ovalbumin specific IgG1, IgG2a, and IgE determinations, as previously described.¹⁰ For IgE analysis, sera were first mixed with a 50% slurry of protein G-Sepha-

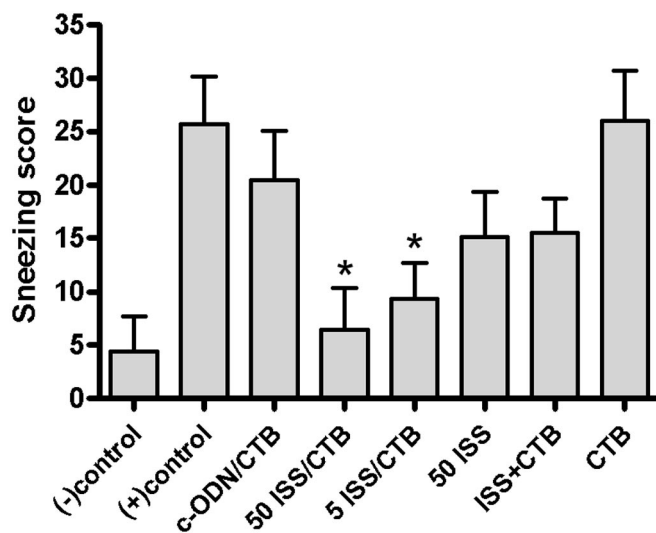


Figure 2. Mean number of sneezes counted for 15 minutes after ovalbumin provocation. The sneezing score in the positive (+) control group shows a significant difference compared with that of the negative (-) control group. The sneezing scores of the 50- and 5- μ g conjugate groups (6.42 [1.9] and 9.3 [1.6], respectively) were significantly lower than that of the positive control group. The sneezing score of the 50- μ g immunostimulatory sequence (ISS) group (15.2 [1.5]) tended to be lower than that of the positive control group but did not show a statistically significant difference. Five mice were used in each group. * $P < .05$ vs the positive control group. c-ODN indicates control oligodeoxynucleotide; and CTB, cholera toxin B. Error bars represent SEM.

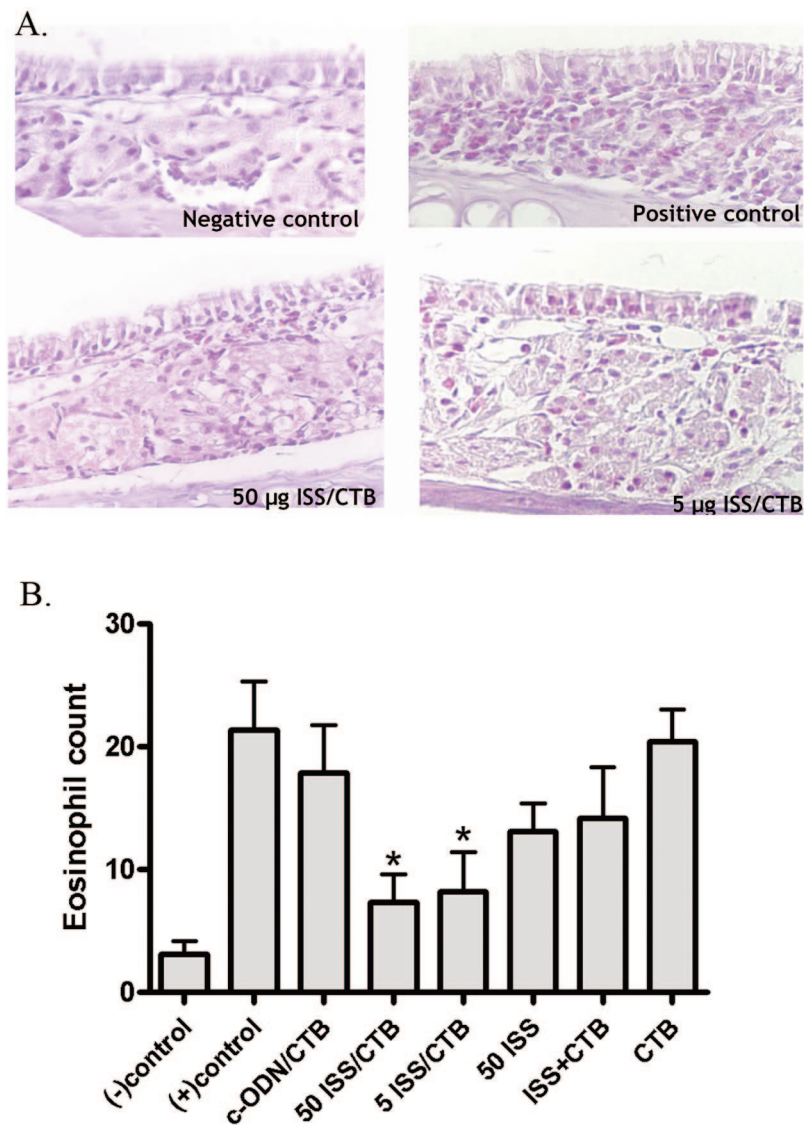


Figure 3. Eosinophil infiltration in the nasal mucosa. A, Representative slides of the negative and positive controls and the 50- and 5- μ g immunostimulatory sequence oligodeoxynucleotide/cholera toxin B (ISS-ODN/CTB) conjugates (magnification $\times 400$). Heavy eosinophil infiltration is seen in the submucosa of the positive control group, whereas it is decreased in the high- and low-dose ISS-ODN/CTB conjugate groups. B, The mean eosinophil counts in the 50- and 5- μ g ISS-ODN/CTB conjugate groups (7.3 [2.3] and 8.2 [3.2], respectively) were significantly reduced compared with that of the positive (+) control group. It was decreased in the 50- μ g ISS-ODN group (13.1 [2.3]) but not to a statistically significant level. Other groups did not show significant differences compared with the positive control group. Five mice were used in each group. $*P < .05$ vs the positive control group. Error bars represent SEM.

rose beads (Pharmacia, Piscataway, New Jersey) to remove IgG and improve ovalbumin specific IgE detection sensitivity. Subsequent ELISA techniques were routine. Ovalbumin specific IgG1 and IgG2a levels were determined using similar methods, but serum samples were not pretreated with protein G-Sepharose beads.

Measurement of cytokines in splenocyte culture. Splenocytes were isolated to evaluate ovalbumin specific cytokine production. At death, spleens were harvested aseptically and

were teased to make single-cell suspensions. Splenocytes were cultured in a 96-well tissue culture plate at a final concentration of 10^5 cells/mL in RPMI-1640 supplemented with 10% fetal calf serum, 100 μ g/mL of streptomycin, and 100 U/mL of penicillin. The cells were incubated in a carbon dioxide incubator with ovalbumin (50 μ g/mL) at 37°C, 5% carbon dioxide for 72 hours, and supernatants were harvested at 72 hours for cytokine ELISA. Results reflect 3 separate experiments.

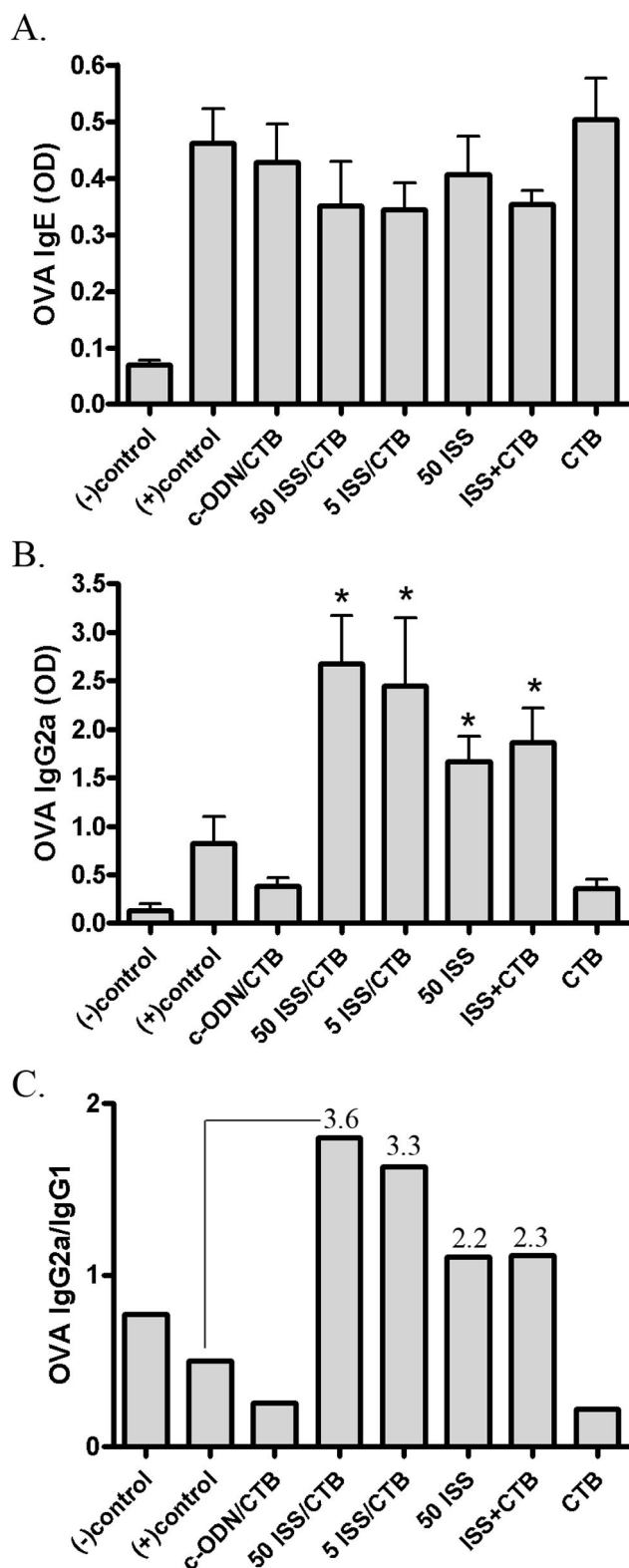


Figure 4. Mean serum ovalbumin (OVA) specific immunoglobulin levels. A, Serum levels of OVA specific IgE antibody. No group showed signifi-

Data Analysis

Data analysis was performed using a software program (SPSS 12 for Windows; SPSS Inc, Chicago, Illinois), and data are expressed as mean (SEM). Results in the different groups of mice were compared using the nonparametric Kruskal-Wallis test followed by post hoc testing using the Dunn multiple comparison of means, and $P < .05$ was considered statistically significant.

RESULTS

IL-12 Assay of In Vitro Stimulation With ISS-ODN/CTB Conjugate

When incubated with BALB-c splenocytes, ISS-ODN/CTB conjugate (1 $\mu\text{g/mL}$) induced an approximately 10-fold-greater increase in IL-12 production than did an equal amount of ISS-ODN (Table 2).

Sneezing Score

The sneezing score of the positive control group was 25.7 (2.1), which was significantly higher compared with that of the negative control group (4.4 [1.7]) (Fig 2). The sneezing scores of the 50- and 5- μg conjugate groups were 6.42 (1.9) and 9.3 (1.6), respectively, which were significantly lower than that of the positive control group ($P = .01$ for both).

Eosinophil Infiltration in the Nasal Mucosa

The eosinophil count in the positive control group was 21.4 (3.9), which was significantly different from that of the negative control group ($P = .001$) (Fig 3). The eosinophil count of the 50- μg ISS-ODN group was decreased (13.1 [2.3]) compared with the positive control group but not to a statistically significant level. The eosinophil counts in the 50- and 5- μg conjugate groups (7.3 [2.3] and 8.2 [3.2], respectively) were significantly reduced compared with that of the positive control group ($P = .02$ and $.03$, respectively). The eosinophil counts in the other groups did not show significant differences.

Serum Ovalbumin Specific Immunoglobulin Levels

The ovalbumin specific IgE level in the positive control group was significantly elevated compared with that of the negative control group. The IgE levels in all of the treatment groups, including the 50- and 5- μg ISS-ODN/CTB conjugate groups, did not differ significantly with those in the positive control group (Fig 4A). The ovalbumin specific IgG2a levels

cantly different levels compared with the positive (+) control group except for the negative (-) control group. Five mice were used in each group. B, Serum levels of OVA specific IgG2a antibody. It was significantly increased in the 50- and 5- μg conjugate groups, and immunostimulatory sequence + cholera toxin B (ISS + CTB) groups compared with the positive control group. Five mice were used in each group. * $P < .05$ vs the positive control group. C, Serum levels of the OVA specific IgG2a:IgG1 ratio. It showed a 3.6-, 3.3-, and 2.2-fold increase compared with the positive control group in the 50- and 5- μg conjugate groups and in the 50- μg ISS group, respectively. c-ODN indicates control oligodeoxynucleotide. Error bars represent SEM.

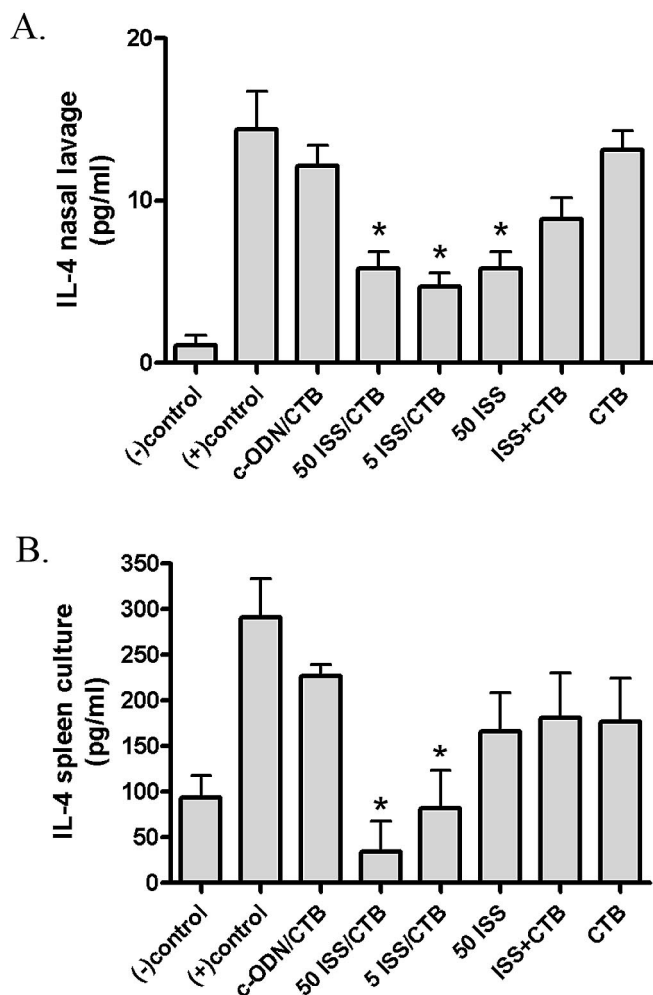


Figure 5. Mean interleukin 4 (IL-4) production in nasal lavage fluid and cultured spleen cells. A, The IL-4 concentrations in nasal lavage fluid show significant differences in the 50- and 5- μ g immunostimulatory sequence oligodeoxynucleotide/cholera toxin B (ISS-ODN/CTB) conjugate groups (5.82 [1.0] and 4.72 [0.83] pg/mL, respectively) and in the 50- μ g ISS group (5.93 [0.82] pg/mL). Other groups were not significantly different from the positive (+) control group. Five mice were used in each group. * $P < .05$ vs the positive control group. B, The IL-4 production in the spleen cells cultured with ovalbumin shows significant decrease compared with the positive control group (290.8 [42.6] pg/mL) in the 50- and 5- μ g ISS-ODN/CTB conjugate groups (33.7 [23.7] and 81.5 [41.6] pg/mL, respectively). Other groups did not show significant differences. Five mice were used in each group. * $P < .05$ vs the positive control group. c-ODN indicates control ODN. Error bars represent SEM.

of the 50- and 5- μ g conjugate groups, 50- μ g ISS group, and 50- μ g ISS + CTB group were significantly increased compared with those of the positive control group ($P = .005$, .02, .03, and .03, respectively) (Fig 4B). The IgG2a:IgG1 ratio showed a 3.6-, 3.3-, and 2.2-fold increase compared with the positive control group in the 50- and 5- μ g conjugate groups and the 50- μ g ISS group, respectively (Fig 4C).

IL-4 Production

The IL-4 measured in nasal lavage fluid 24 hours after ovalbumin provocation showed significantly increased levels in the positive control group (12.49 [0.62] pg/mL) compared with the negative control group (1.1 [0.62] pg/mL). Significant decreases in the levels of IL-4 were seen in the 50- and 5- μ g ISS-ODN/CTB conjugate groups (5.82 [1.0] and 4.72 [0.83] pg/mL, respectively) and in the 50- μ g ISS group (5.93 [0.82] pg/mL) ($P = .02$, .002, and .02, respectively) (Fig 5A). In the supernatant of spleen cells cultured with ovalbumin, the level of ovalbumin specific IL-4 production was significantly decreased compared with that of the positive control group (290.8 [42.6] pg/mL) in the 50- and 5- μ g ISS-ODN/CTB conjugate groups (33.7 [23.7] and 81.5 [41.6] pg/mL; $P = .002$ and .03, respectively). Other groups did not show significant differences (Fig 5B).

IFN- γ Production

The level of ovalbumin specific IFN- γ production in the spleen cell supernatant was increased compared with that of the positive control group (227.8 [135.7] pg/mL) in the 50- and 5- μ g ISS-ODN/CTB (368.2 [138.2] and 338.2 [106.6] pg/mL, respectively) and 50- μ g ISS-ODN (330.6 [68.9] pg/mL) groups but did not reach statistically significant values.

IL-12 Production

The IL-12 measured in nasal lavage fluid did not show a significant change between the positive (12.1 [2.5] pg/mL) and negative (8.5 [2.0] pg/mL) control groups. A significant increase in the level of IL-12 was seen in the 50- and 5- μ g ISS-ODN/CTB conjugate groups (24.3 [5.6] and 32.5 [3.9] pg/mL, respectively) ($P = .02$ and .004, respectively) (Fig 6A). The level of ovalbumin specific IL-12 production in the splenocyte supernatant was significantly increased compared with that of the positive control group (213.4 [58.4] pg/mL) in the 50- and 5- μ g ISS-ODN/CTB conjugate groups (468.4 [102.2] and 395.6 [51.5] pg/mL, respectively) ($P = .002$, and .03, respectively) (Fig 6B). The IL-12 levels in nasal lavage fluid and spleen cell culture supernatant in the 50- μ g ISS group (18.6 [2.8] and 241.0 [49.1] pg/mL, respectively) were increased but not to a statistically significant level.

IL-10 Production

Measurements of IL-10 in nasal lavage fluid did not show a significant difference between the positive control and treatment groups (Fig 7A). However, the level of ovalbumin specific IL-10 production in the splenocyte supernatant was significantly increased compared with that of the positive control group (158.3 [39.5] pg/mL) in the 50- μ g ISS-ODN/CTB conjugate group (284.8 [51.49] pg/mL) ($P = .04$) (Fig 7B). Other treatment groups did not differ significantly from the positive control group.

DISCUSSION

Studies^{1,2,6} have shown that ISS-ODN attenuates allergic responses in different models of allergy. The antiallergic properties have been attributed to its activation of innate and

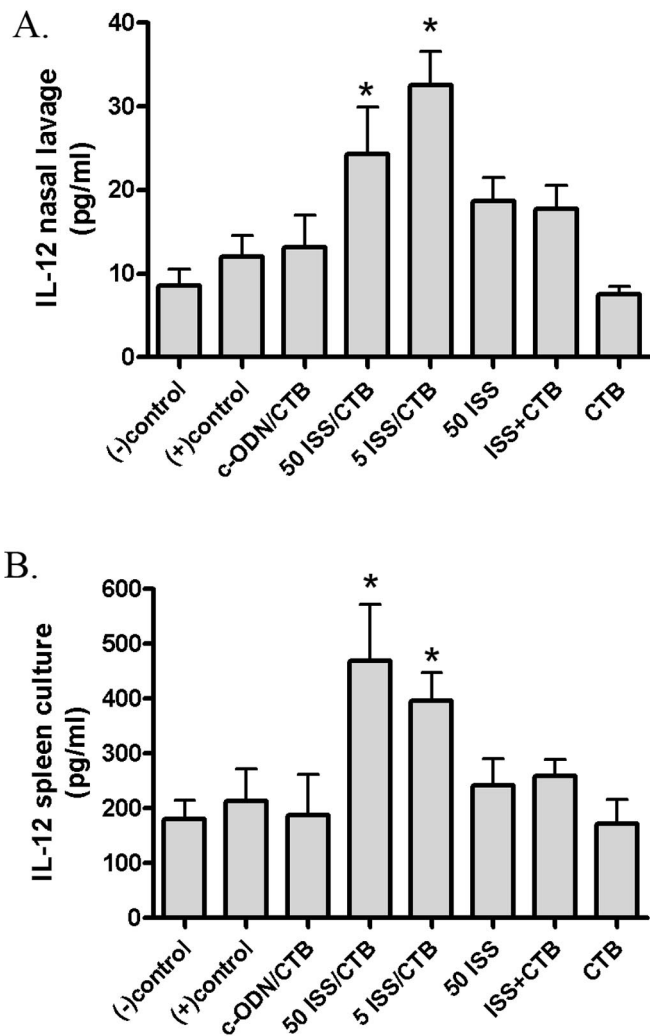


Figure 6. Mean interleukin 12 (IL-12) production in nasal lavage fluid and cultured spleen cells. A, The IL-12 concentrations in nasal lavage fluid show significant differences in the 50- and 5- μ g immunostimulatory sequence oligodeoxynucleotide/cholera toxin B (ISS-ODN/CTB) conjugate groups (24.3 [5.6] and 32.5 [3.9] pg/mL, respectively). Other groups did not show significant differences compared with the positive control group. Five mice were used in each group. * $P < .05$ vs the positive control group. B, The IL-12 production in the spleen cells cultured with ovalbumin shows a significant increase compared with the positive control group (213.4 [58.4] pg/mL) in the 50- and 5- μ g ISS-ODN/CTB conjugate groups (468.4 [102.2] and 395.6 [51.5] pg/mL, respectively). The IL-12 level in the 50- μ g ISS-ODN group (241.0 [49.1] pg/mL) was elevated compared with that of the positive control group but not to a statistically significant level. Five mice were used in each group. * $P < .05$ vs the positive control group. Error bars represent SEM.

adaptive immunity, promoting the T_H1 immune response. The effects of ISS-ODN are mediated through toll-like receptor 9 (TLR-9),¹³ which triggers a cascade activation of adaptor molecules and protein kinases. The TLR-9 resides intracellularly in the endosome, and association of ISS-ODN with

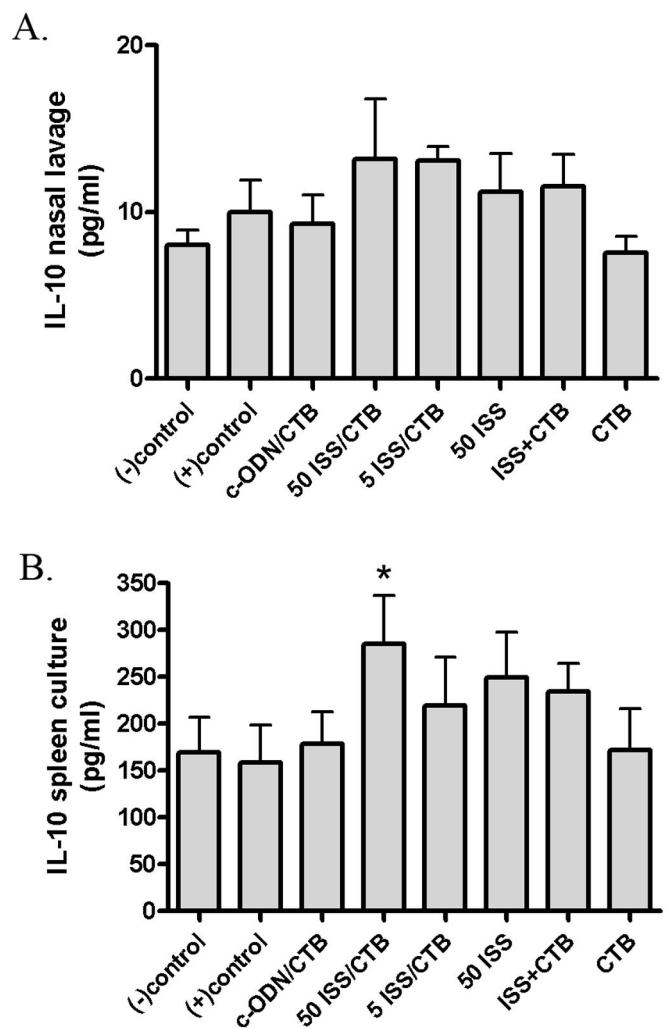


Figure 7. Mean interleukin 10 (IL-10) production in nasal lavage fluid and cultured spleen cells. A, The IL-10 concentrations in nasal lavage fluid did not show significant differences among the different treatment groups. Five mice were used in each group. B, The IL-10 production in the spleen cells cultured with ovalbumin shows a significant increase in the 50- μ g immunostimulatory sequence oligodeoxynucleotide/cholera toxin B (ISS-ODN/CTB) conjugate group (284.8 [51.49] pg/mL) compared with the positive control group (158.3 [39.5] pg/mL). The IL-10 levels in the other groups did not show significant differences compared with the positive control group. Five mice were used in each group. * $P < .05$ vs the positive control group. Error bars represent SEM.

TLR-9 occurs within lysosomes. Therefore, ISS-ODN must be delivered to this intracellular compartment for interaction to occur. Currently, it is believed that internalization occurs spontaneously.¹⁴

Cholera toxin (CT) is the protein responsible for the severe cholera diarrhea caused by *Vibrio cholera*.¹⁵ The CT consists of 5 identical B subunits and a single A subunit. Cell recognition and binding of CT are conducted by the B pentamer via specific binding to the ganglioside GM1 receptor, a glyco-

sphingolipid found ubiquitously on the surface of mammalian cells. High-affinity interaction of CTB with GM1 ganglioside receptors of the cell membrane triggers toxin internalization into smooth endocytic vesicles targeted to the endoplasmic reticulum.

Reports indicate that the immunogenicity of protein antigens can be enhanced when they are co-delivered with CTB.^{16,17} However, co-delivery of ISS-ODN with CTB has shown no synergistic effect for the strength of the immune response.^{18–20} Recently, Adamsson and colleagues¹² suggested that the immunostimulatory effect of ISS-ODN can be improved when chemically conjugated to CTB. Whereas *in vitro* treatment of human peripheral blood mononuclear cells with ISS-ODN did not elicit appreciable levels of CC chemokine responses, ISS-ODN chemically conjugated to CTB elicited strong CC chemokine responses.

In this study, we compared the antiallergic effects of intranasally delivered ISS-ODN conjugated to CTB with those of ISS-ODN alone in a mouse model of allergic rhinitis. Although intranasal treatment with a single dose of 50 μ g of ISS-ODN showed a significant difference in serum levels of IgG2a and nasal lavage fluid IL-4 levels, it did not effectively attenuate allergic symptoms, eosinophilia in nasal mucosa, serum ovalbumin specific IgE levels, and IL-4, IFN- γ , and IL-12 production in spleen cells. On the other hand, intranasal therapy with ISS-ODN/CTB conjugate with an equivalent amount of ISS-ODN (50 μ g) was effective in reducing nasal symptoms, eosinophil infiltration, and immunologic variables of allergy. Production of IL-10, a cytokine with regulatory properties, was also increased in the spleen cell culture. When the amount of ISS-ODN in the conjugate was reduced 10-fold (5 μ g of ISS-ODN/CTB conjugate), equivalent antiallergic effects were observed in the clinical and immunologic variables, except for the increase in IL-10 production. The ISS-ODN mixed with CTB mimicked the effects of ISS-ODN alone, but additional synergistic effects were not found. Other treatment groups did not demonstrate any significant change in the clinical and immunologic variables.

To our knowledge, this is the first study to show that intranasal administration of ISS-ODN/CTB conjugate is more effective than intranasal administration of ISS-ODN alone for attenuating allergic rhinitis. Rhee et al¹⁰ previously reported that intranasal administration of ISS-ODN alone can reduce T_H2 responses in mice with allergic rhinitis. In the present study, however, we observed partial effects in the ISS-ODN alone group only. This discrepancy may be caused by the amount of ISS-ODN administered. Whereas in the previous study, 100 μ g of ISS-ODN was used, 50 μ g of ISS-ODN was used herein, and this may be the reason for the incomplete effectiveness.

It is reasonable to believe that intranasally delivered ISS-ODN alone can attenuate allergic rhinitis if the amount of ISS-ODN is increased. However, the adverse effects of ISS-ODN at high doses should be considered. The ISS-ODNs might enhance the immunogenicity of self-proteins, triggering the development of autoimmune disease.²¹ Hasegawa and

Hayashi²² showed that repeated administration of high doses of bacterial DNA can elicit the production of autoantibodies against double-stranded DNA in normal mice. By chemically conjugating ISS-ODN to CTB, the amount of ISS-ODN to attenuate allergic rhinitis can be reduced, lowering the potential risk of adverse reactions.

Although the specific IgE titer can be significantly reduced when treated with allergen conjugated to ISS-ODN during the immunization period,⁴ the results of this study show inhibition of immediate-phase hypersensitivity responses despite similar IgE titers. Therefore, it seems that ISS-ODN/CTB treatment may have compromised basophilic cells, the main providers of histamine, on their ability to contribute to the immediate-phase response irrespective of IgE downregulation. Because only trace amounts of IgE are sufficient to fully arm basophilic cells for allergen-induced degranulation,² we consider it likely that ISS-ODN/CTB inhibited the activities of basophilic cells in the nasal mucosa by additional mechanisms that remain to be determined. As proposed in a previous study¹⁰ with ISS-ODN, inhibition of histamine synthesis by basophils, inhibition of mast cell recruitment to the nasal mucosa, and inhibition of allergen-specific T_H2 cytokine production can be potential mechanisms.¹⁰

The IgG isotypes (IgG1 and IgG2a) were measured as an indirect way to determine T_H bias of the IgG response. In a previous study,¹⁰ it was noted that ISS-ODN treatment modestly inhibited allergen challenge-induced IgG1 production. In the present study, the levels of IgG1 did not show significant differences; on the other hand, treatment with ISS-ODN/CTB resulted in an increase in IgG2a, still suggesting a T_H1-biased response. The discrepancy with the previous study can be attributed to the different amount of ISS-ODN delivered or to the different immune mechanism of ISS-ODN conjugated to CTB that needs to be clarified with further study.

We attribute the antiallergic effects of the ISS-ODN/CTB conjugate to its enhancement of T_H1 immunity, as evidenced by the increase in serum IgG2a levels and the increase in the production of T_H1 cytokines. However, it remains obscure why although increased compared with control, ISS-ODN/CTB conjugate had no significant increase in the production of IFN- γ . Although T_H1 responses predominate, these responses are not critical for attenuating allergic responses. This has been shown in a previous study,²³ where ISS-ODN led to a decrease in IL-5 in sera of IFN- γ knockout mice. The proposed mechanisms for this antiallergic effect include induction of regulatory responses elicited by cytokines such as IL-10 and transforming growth factor β . The results of this study indicate a significant increase in IL-10 in spleen cells of mice treated with high-dose ISS-ODN/CTB conjugate, suggesting a regulatory response and involvement of multiple antiallergic mechanisms. Alternatively, the immunomodulatory effects may have been mediated by nasal lymphocytes and not by splenocytes, as suggested by Suzuki and colleagues.²⁴

Molecular studies point out that similar to ISS-ODN, ISS-ODN/CTB conjugate-induced immune responses depend on TLR-9 signaling. However, as opposed to ISS-ODN, ISS-ODN/CTB conjugate-induced immunity is not critically dependent on endosomal acidification and cannot be inhibited by inhibitory ODN.¹² These data point to shared and distinct intracellular pathways used by ISS-ODN and ISS-ODN/CTB conjugate for the transduction of activation signal.

Several mechanisms can be hypothesized for the enhanced immunostimulatory activity of the ISS-ODN/CTB conjugate. The CTB can increase the uptake of ISS-ODN not only across the nasal mucosal barrier but also by target cells, allowing more ISS-ODN to reach the endosomal compartments. Ganglioside GM1, the CTB receptor, is present on all antigen-presenting cells, including dendritic cells, macrophages, and B cells. Therefore, it is likely that the conjugation of CTB to ISS-ODN efficiently enhances the uptake of ISS-ODN by target cells. Alternatively, the conjugation of CTB to ISS-ODN results in aggregation and multimerization of ISS-ODN, which can lead to enhanced TLR-9 activation.²⁵

In consideration of future clinical applications, ISS-ODN/CTB conjugate can be delivered in nasal sprays to patients with allergic rhinitis, obviating the need for frequent physician visits and repeated injections. Also, the allergen-independent immunomodulatory effect may give way to treating patients sensitized to multiple aeroallergens. Further studies to maximize effectiveness and to elucidate the mechanism by which ISS-ODN/CTB conjugate enhances attenuation of allergic rhinitis are needed.

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Requests for reprints should be addressed to:
 Chul Hee Lee, MD, PhD
 Department of Otorhinolaryngology
 Seoul National University Hospital
 28 Yongon-dong
 Chongno-gu
 Seoul 110-744, Korea
 E-mail: chulhee@snu.ac.kr