

## The Effect of CpG-Oligodeoxynucleotides with Different Backbone Structures and 3' Hexameric Deoxyriboguanosine Run Conjugation on the Treatment of Asthma in Mice

CpG-Oligodeoxynucleotide (ODN) has two backbones. Phosphorothioate backbone (PS) shows a strong immunostimulating effect while phosphodiester (PE) shows little in vivo. 3' hexameric deoxyriboguanosine-run (3' dG<sub>6</sub>-run) conjugation to PE CpG-ODN has been reported to enhance immunostimulation and to protect against asthma when injected at the time of sensitization in mice. We evaluated the treatment effects of PE and PS CpG-ODN with or without 3' dG<sub>6</sub>-run on asthma in pre-sensitized mice. BALB/c mice sensitized with ovalbumin and alum were challenged with 1% ovalbumin on three days. CpG-ODNs (100 μg) or PBS were injected 4 times; 27 hr before challenge and 3 hr before each challenge (CpG-dG<sub>6</sub>: CpG-ODN with 3' dG<sub>6</sub>-run, PE\*-CpG-dG<sub>6</sub>; PE-CpG-dG<sub>6</sub> with two PS backbones at the 5' terminus). PE-CpG showed no treatment effect. PE-CpG-dG<sub>6</sub> only increased ovalbumin-specific IgG2a. PE\*-CpG-dG<sub>6</sub> increased ovalbumin-specific IgG2a but also reduced BAL fluid eosinophils and airway hyperresponsiveness. PS-CpG increased ovalbumin-specific IgG2a, reduced airway inflammation and airway hyperresponsiveness. PS-CpG-dG<sub>6</sub> was less effective than PS-CpG on airway inflammation and airway hyperresponsiveness. In pre-sensitized mice, PE-CpG required not only 3' dG<sub>6</sub>-run but also the modification of two PS linkages at 5' terminus to inhibit features of asthma. PS-CpG was strong enough to inhibit asthma but PS-CpG-dG<sub>6</sub> was less effective.

**Key Words :** Asthma; Models, Animal; Allergy; Immunotherapy; CpG-ODN; Poly G

Yoon-Seok Chang<sup>1,2,3</sup>, Yoon-Keun Kim<sup>2,3</sup>,  
Hyuk-Soo Kwon<sup>2,3</sup>, Heung-Woo Park<sup>2,3</sup>,  
Kyung-Up Min<sup>2,3</sup>, You-Young Kim<sup>2,3</sup>,  
and Sang-Heon Cho<sup>2,3</sup>

Department of Internal Medicine<sup>1</sup>, Seoul National University College of Medicine, Institute of Allergy and Clinical Immunology<sup>2</sup>, Seoul National University Medical Research Center, Seoul; Department of Internal Medicine<sup>3</sup>, Seoul National University Bundang Hospital, Seongnam, Korea

Kim YK is now at the Division of Molecular and Life Sciences, Pohang University of Science and Technology, Pohang, Korea.

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### Address for correspondence

Sang-Heon Cho, M.D.  
Department of Internal Medicine, Seoul National University College of Medicine, 28 Yeongeon-dong, Jongno-gu, Seoul 110-744, Korea  
Tel : +82-2-760-2971, Fax : +82-2-764-3954  
E-mail : shcho@plaza.snu.ac.kr

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## INTRODUCTION

CpG-Oligodeoxynucleotides (ODN) is an oligodeoxynucleotides that contain CpG motifs, 5'-purine-purine-unmethylated deoxycytosine-deoxyguanosine-pyrimidine-pyrimidine-3' (1). CpG-ODN, a well-known TLR9 agonist, stimulates antigen-presenting cells (APCs) like plasmacytoid dendritic cells to produce Th1-promoting cytokines, such as type I IFN and IL-12. CpG-ODN is also involved in the maturation of plasmacytoid dendritic cells to potent antigen-presenting cells, the transition of monocytes into functional dendritic cells, NK cell stimulation, B cell activation, and in the production of Th1 type antibodies (2).

CpG-ODN is considered to be a potential new therapeutic modality for allergic diseases, cancer, and infectious diseases (2). Recently, a clinical trial of CpG-ODN conjugated with ragweed allergen *Amb a 1* was conducted in allergic rhinitis (3). Patients received only six weekly injections of

*Amb a 1*-CpG-ODN conjugate or placebo vaccine before the first ragweed season and were monitored during the next two ragweed seasons, and this 6-week regimen of allergen-CpG ODN vaccinations appeared to offer long-term clinical efficacy for the treatment of ragweed induced allergic rhinitis.

CpG-ODN can be classified into two forms of different backbone structures according to its chemical properties (4, 5). One of these has a phosphodiester backbone (PE) whereas the other has a phosphorothioate backbone (PS). The PE is the natural form of bacterial DNA which contains many CpG-motifs (20 times more than vertebrates) (1). This form serves as a danger signal, which is detected by TLR9, a pattern-recognition receptor, when bacteria invade the host (2). However, the PE form has no or little immunostimulatory effect in vivo because it is easily attacked by exonucleases. On the other hand the PS backbone is a synthetic form that is resistant to exonucleases, and which has a strong immunostimulatory effect even in vivo (4). This is why most exper-

iments on CpG-ODN have been performed with PS CpG-ODN in vivo. Moreover, the immunostimulatory effects of CpG-ODNs depend on flanking sequences and the optimal sequences are species-specific (2). Concerns about the possible side effects of PS CpG-ODN, such as, granuloma formation at high dosages and splenomegaly, drove the developments of new strategies to enhance the immunostimulatory potential of PE CpG-ODN (4-6). Some strategies now available; the conjugation of CpG-ODN with a 3' hexameric deoxyriboguanosine run (3' dG<sub>6</sub> run) (4, 5) and packing CpG-ODN into virus-like particles (7). We have previously demonstrated that the conjugation of CpG-ODN with 3' dG<sub>6</sub> run augmented the immunostimulatory effect of PE CpG-ODN and inhibited the development of asthma when it was delivered with allergen at the time of sensitization in mice (4). Moreover, the 3' dG<sub>6</sub> run was observed to have an enhancing effect in both PE and PS CpG-ODNs (4). Interestingly, PE CpG-ODN with a 3' dG<sub>6</sub> run induced less splenomegaly but had an inhibitory effect on allergen-specific IgE production similar to that of PS CpG-ODN (5). In this study, we evaluated the effects of PE or PS CpG-ODN with or without a 3' dG<sub>6</sub> run conjugate treatments at the time of allergen challenge.

## MATERIALS AND METHODS

### ODNs and mice

All CpG ODNs were purchased from GenoTech (Daejeon, Korea). Their sequences and modifications are presented in Fig. 1A. PS-CpG is used to designate PS CpG-ODN, and its PE form is represented by PE-CpG. PS-CpG-dG<sub>6</sub> is used to designate PS-CpG with six deoxyriboguanosine residues at the 3' terminus, and its PE form is designed as PE-CpG-dG<sub>6</sub>. PE\*-CpG-dG<sub>6</sub> represents PE-CpG-dG<sub>6</sub> with two PS backbones at the 5' terminus.

Six-week old female BALB/*c* mice were purchased from DBL (Daejeon, Korea). BALB/*c* mice were maintained in the clinical research institute at Seoul National University Hospital. All animal experiments were approved by the committee on animal experimentation at our institution.

### Immunization and induction of airway hyperresponsiveness

As Fig. 1B illustrates, six mice in each group were sensitized by injection of 20  $\mu$ g ovalbumin (OVA) (Grade V; Sigma, St. Louis, MO, U.S.A.) emulsified in 2 mg aluminum hydroxide intraperitoneally (i.p.). Animals received an identical booster immunization 14 days later. On days 21, 22, and 23 after the initial sensitization, mice were challenged for 30 min with an aerosol of 1% (w/v) OVA in PBS in a Plexiglas chamber using an ultrasonic nebulizer (NE-U12, Omron, Japan). Mice were given four injections of 100  $\mu$ g

of CpG ODNs or PBS i.p. in total volume of 100  $\mu$ L PBS, at 24 hr intervals from 27 hr before the first OVA inhalation challenge, i.e., 3 hr before each challenge.

Twenty-four hours after the final OVA challenge, airway hyperresponsiveness (AHR) was assessed by determining methacholine-induced airflow obstruction (*Penb*) using one chamber whole body plethysmography (Allmedicus, Anyang, Korea), as previously described (4, 8-10). Increasing doses of methacholine (ranging from 2.5-50 mg/mL; Sigma) were administered by nebulization for 3 min, and *Penh* values were calculated over the subsequent 3 min. Results were presented as PC<sub>200</sub> values, which are defined as the concentration of methacholine required to increase baseline *Penb* by 200%.

### Bronchoalveolar lavage

Forty-eight hours after the last OVA challenge, tracheae were cannulated and lungs were lavaged with five 0.4 mL aliquots of pyrogen-free saline. After Diff-quick<sup>R</sup> staining lung lavage cells in cytospin preparations, two investigators counted blindly more than 300 inflammatory cells under a light microscope and classified these as macrophages, lym-

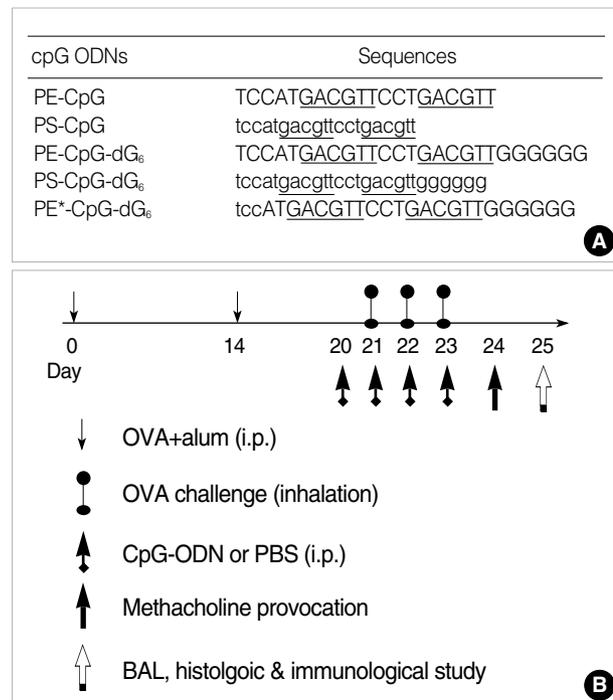


Fig. 1. Experimental protocols of sensitization and challenge. (A) Sequences and modifications of CpG ODNs used in this study. Immunostimulatory CpG motifs are underlined. Capital letters represent phosphodiester backbones and small letters phosphorothioate backbones. (B) BALB/*c* mice were sensitized by injecting OVA/alum intraperitoneally (i.p.) and later challenged with OVA on the indicated days (six mice per group). Mice were administered four injections of 100  $\mu$ g of CpG ODNs or PBS i.p. in a total volume of 100  $\mu$ L PBS at 24 hr intervals from 27 hr before the first OVA inhalation challenge.

phocytes, neutrophils, or eosinophils (4, 8).

### Lung histology

Following BAL, lungs were infused with 10% formalin and embedded in paraffin. Lung sections were stained with hematoxylin and eosin and assessed by light microscopy. Inflammation scores were graded as previously described (4, 8). Briefly, degree of peribronchial and perivascular inflammation was evaluated using a subjective scale of 0-3. A value of 0 was assigned when no inflammation was detectable, a value of 1 for occasional cuffing by inflammatory cells, a value of 2 when most bronchi or vessels were surrounded by a thin layer (one to five cells) of inflammatory cells, and a value of 3 when most bronchi or vessels were surrounded by a thick layer (more than five cells) of inflammatory cells. The total lung inflammation was defined as the average of peribronchial and perivascular inflammation score.

### Antibody responses

Forty-eight hours after the last OVA challenge, blood samples were obtained by cardiac puncture. Antibody titers were measured as previously described (4, 8). Briefly, microtiter plates (Dynex Technologies, Chantilly, VA, U.S.A.) were coated overnight with 2  $\mu\text{g}/\text{mL}$  of OVA in a 50 mM carbonate buffer (pH 9.6) at 4°C. Nonspecific binding was blocked with 2% bovine serum albumin for 1 hr at 20°C. After incubating with test sera for 2 hr, plates were incubated with horse radish peroxidase-labeled goat anti-mouse IgE or IgG2a (PharMingen, San Diego, CA, U.S.A.) for 1 hr at 20°C. The reaction was developed using a tetramethylbenzidine (Sigma, St. Louis, MO, U.S.A.) substrate and then stopped by adding 2 N  $\text{H}_2\text{SO}_4$ . Subsequently, optical density was measured at 450 nm. A high titer of anti-OVA IgE or IgG2a was used as a

standard, and linear standard curves were obtained by serially diluting standard serum. The results are expressed in arbitrary units (A.U.) according to measured O.D. values.

### Evaluation of splenomegaly

Mouse and spleen weights, the latter of which were cautiously separated without bleeding, were measured as previously reported (5). Spleen to body weight ratio were compared.

### Cytokine production by splenocytes

Cytokine production by splenocytes was evaluated as previously described (5). Briefly, spleens were homogenized using a 94- $\mu\text{m}$  screen (Bellco Glass Inc. Vineland, NJ, U.S.A.) to obtain single cell suspensions. Splenocytes ( $2 \times 10^6$ ) were then cultured with OVA (100  $\mu\text{g}/\text{mL}$ ) or PBS control in 12-well plates. After 2 days, IL-4, IL-5, IFN- $\gamma$ , and IL-12 production levels were quantified in culture supernatants by sandwich ELISA using specific monoclonal antibody pairs.

### Statistical analysis

Statistical analyses were performed using the Kruskal-Wallis and Mann-Whitney tests using SPSS version 12.0 (SPSS Inc, Chicago, IL, U.S.A.). Values for all measurements are expressed as means and standard errors of means.

## RESULTS

### Airway hyperresponsiveness

To investigate the treatment effects of CpG ODNs with

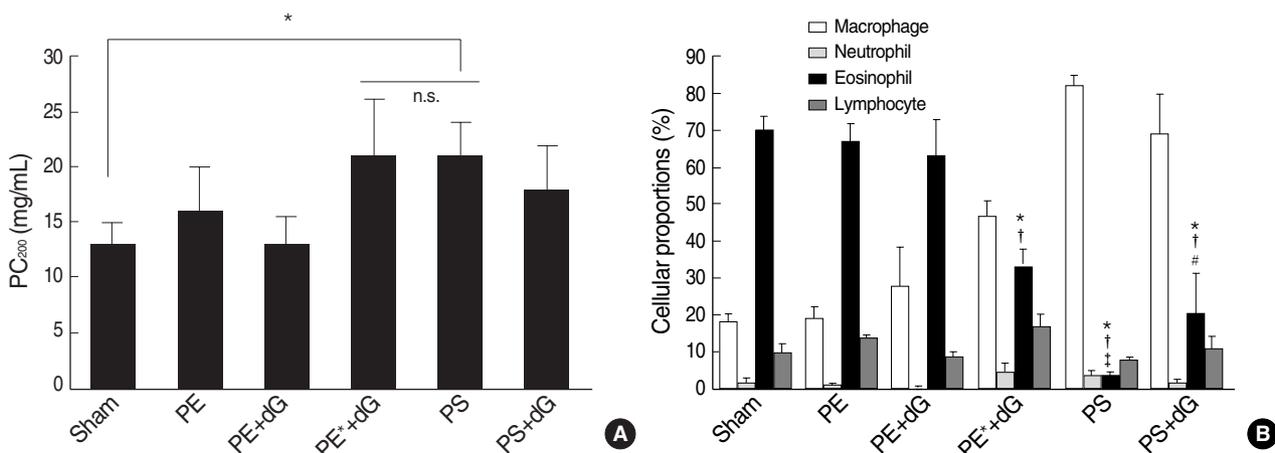


Fig. 2. Effects of the 3' dG<sub>6</sub> run conjugated to CpG ODNs on the development of airway hyperresponsiveness (AHR) (A) (\*:  $P < 0.05$ , ns: no significant difference), and cellular proportions in bronchoalveolar lavage (BAL) fluid (B) ( $P < 0.05$ , eosinophil proportions, \*: vs. PBS, †: vs. PE-CpG-dG<sub>6</sub>, ‡: vs. PE<sup>+</sup>-CpG-dG<sub>6</sub>, #: vs. PS-CpG). AHR is represented by PC<sub>200</sub>, and cellular proportions are expressed as relative percentages of cell counts. Representative data of two independent experiments are shown. Bars indicate mean values  $\pm$  SEM of six mice.

different backbones and with or without a 3' dG<sub>6</sub>-run in pre-sensitized mice against the development of allergic asthma, we measured AHR to inhaled methacholine after OVA challenge (Fig. 2A). As was expected, the administration of PS-CpG, but not of PE-CpG, effectively inhibited AHR. However, PE-CpG-dG<sub>6</sub>, which had shown a significant inhibitory effect when delivered at the time of sensitization (4), did not inhibit AHR as well as PE-CpG.

PE\*-CpG-dG<sub>6</sub> reduced AHR and this effect was comparable to that of PS-CpG ( $P>0.05$ ). Interestingly, the inhibitory effect of PS-CpG on airway hyperresponsiveness reduced when it was conjugated with a dG<sub>6</sub>-run at its 3' terminus (PS-CpG-dG<sub>6</sub>).

### Bronchoalveolar lavage fluid and airway inflammation

Eosinophil proportions in BAL fluid were similar in PBS ( $70.7 \pm 2.8\%$ ), PE-CpG ( $67.0 \pm 3.6\%$ ), and PE-CpG-dG<sub>6</sub> ( $62.9 \pm 10.8\%$ ) treated mice ( $P>0.05$ ). However, PE\*-CpG-dG<sub>6</sub> significantly decreased BAL eosinophil counts ( $33.1 \pm 3.8\%$ ) vs. PE-CpG treated mice ( $P<0.05$ ), as shown in Fig. 2B. The administration of PS-CpG more significantly inhibited airway eosinophilia ( $4.2 \pm 1.3\%$ ) than PE\*-CpG-dG<sub>6</sub> ( $P<0.05$ ). The administration of PS-CpG-dG<sub>6</sub> also significantly inhibited airway eosinophilia (by  $20.1 \pm 10.5\%$ ) but this was less than that achieved by PS-CpG ( $P<0.05$ ). No significant difference was observed between PS-CpG-dG<sub>6</sub> and PE\*-CpG-dG<sub>6</sub> treated mice in terms of BAL eosinophilia ( $P>0.05$ ).

Concerning lung histology, PE-CpG, PE-CpG-dG<sub>6</sub>, PE\*-CpG-dG<sub>6</sub>, and PS-CpG-dG<sub>6</sub> treated mice showed peribronchial and perivascular inflammation at the same level as that observed in PBS treated mice (peribronchial/total inflamma-

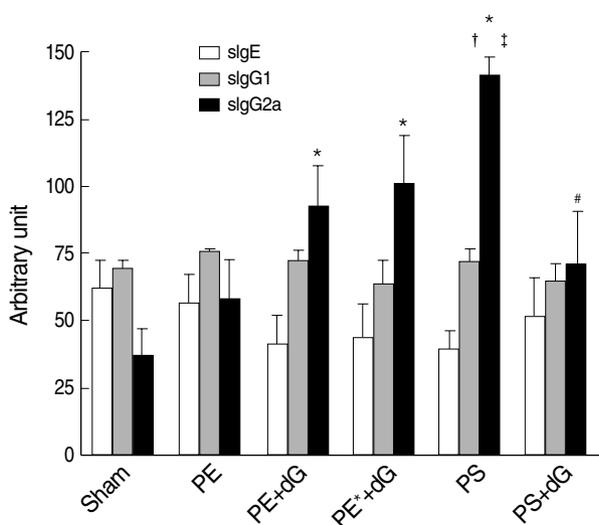


Fig. 3. Antibody responses in the sera of OVA-challenged BALB/c mice. Representative data of two independent experiments are shown. Bars indicate the mean values  $\pm$  SEM of six mice. ( $P<0.05$ , \*: vs. PBS, †: vs. PE-CpG-dG<sub>6</sub>, ‡: vs. PE\*-CpG-dG<sub>6</sub>, #: vs. PS-CpG).

tion scores:  $2.3 \pm 0.2/2.6 \pm 0.1$ ,  $2.3 \pm 0.2/2.6 \pm 0.2$ ,  $2.2 \pm 0.3/2.5 \pm 0.2$ ,  $2.1 \pm 0.1/2.3 \pm 0.1$  vs.  $2.2 \pm 0.2/2.5 \pm 0.1$ , respectively,  $P>0.05$ ). Only PS-CpG treated mice showed significant inhibitions of peribronchial, perivascular, and total lung inflammation vs. PE-CpG treated mice (peribronchial/total inflammation scores:  $1.4 \pm 0.4/1.6 \pm 0.6$  vs.  $2.3 \pm 0.2/2.6 \pm 0.1$ ,  $P<0.05$ ).

### Serum OVA specific antibodies

Interestingly, serum OVA specific IgE levels, which depend on IL-4 and IL-13, were not changed by any form of CpG backbone or by the presence of the 3' dG<sub>6</sub>-run, as shown in Fig. 3. Serum OVA specific IgG1 levels were not changed either.

IgG2a levels that depend on IL-12 and may reflect Th1 immune response were not changed by PE-CpG only, but increased after 3' dG<sub>6</sub>-run (PE-CpG-dG<sub>6</sub>) conjugation. PE\*-CpG-dG<sub>6</sub> also showed higher IgG2a levels that were comparable to those of PE-CpG-dG<sub>6</sub>. PS-CpG and PS-CpG-dG<sub>6</sub> treated mice also showed higher IgG2a levels than PE-CpG or PBS treated mice. However, IgG2a production was significantly lower for PS-CpG with a 3' dG<sub>6</sub>-run than for PS-CpG ( $P<0.05$ ).

### Evaluation of splenomegaly

The ratio of spleen to body weight was highest in the group treated with PS-CpG:  $17.88 \pm 0.49$  ( $P<0.05$ ), followed by PS-CpG-dG<sub>6</sub> ( $10.37 \pm 0.74$ ) and PE\*-CpG-dG<sub>6</sub> ( $7.77 \pm 0.78$ ). This ratio was higher for PS-CpG-dG<sub>6</sub> than for PE\*-CpG-dG<sub>6</sub>, and these two ratios were higher than those of

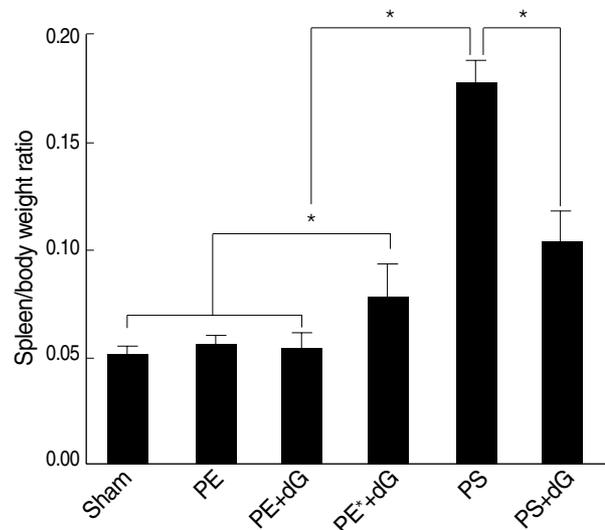


Fig. 4. Spleen to body weight ratios were the highest in animals treated with PS-CpG, followed by those treated with PS-CpG-dG<sub>6</sub> or PE\*-CpG-dG<sub>6</sub>. Mean spleen to body ratio was higher for PS-CpG-dG<sub>6</sub> than for PE\*-CpG-dG<sub>6</sub>, and these ratios were higher than those of PBS, PE-CpG, or PE-CpG-dG<sub>6</sub>. \*:  $P<0.05$ .

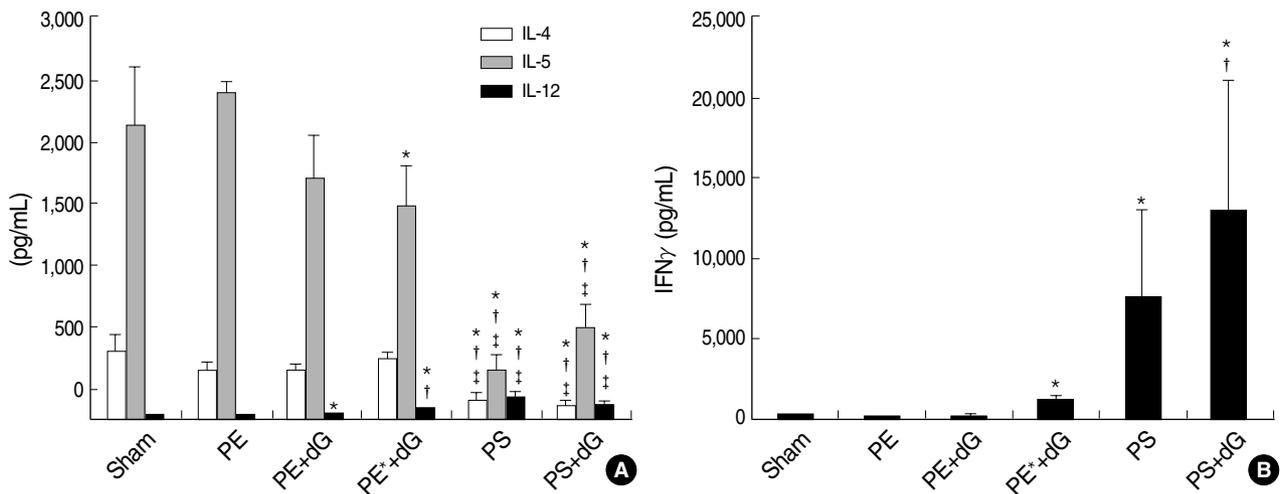


Fig. 5. Cytokine production by splenocytes. Repeat experiments produced similar results. Bars represent means value  $\pm$  SEM of experiments performed in triplicate. ( $P < 0.05$ , \*; vs. PBS, †; vs. PE-CpG-dG<sub>6</sub>, ‡; vs. PE\*-CpG-dG<sub>6</sub>).

PBS ( $5.10 \pm 0.18$ ), PE-CpG ( $5.53 \pm 0.23$ ), and PE-CpG-dG<sub>6</sub> ( $5.37 \pm 0.38$ ) ( $P < 0.05$ ) (Fig. 4).

#### Cytokine production by splenocytes

To understand the mechanisms underlying the prevention of allergic lung inflammation by the 3' dG<sub>6</sub>-run containing CpG ODNs, we examined cytokine production from splenocytes stimulated with OVA (Fig. 5). IL-4 production was significantly decreased by PS-CpG and PS-CpG-dG<sub>6</sub> ( $156.5 \pm 36.0$ ,  $115 \pm 17.8$  vs. PE-CpG  $372 \pm 41.7$  pg/mL,  $P < 0.05$ ). No significant change in IL-4 production was observed in PBS, PE-CpG, PE-CpG-dG<sub>6</sub> and PE\*-CpG-dG<sub>6</sub> treated mice ( $511.2 \pm 111.6$ ,  $372 \pm 41.7$ ,  $370.8 \pm 34.4$ ,  $453.7 \pm 55.3$  pg/mL,  $P > 0.05$ ).

IL-5 production was significantly decreased by PE\*-CpG-dG<sub>6</sub>, PS-CpG and PS-CpG-dG<sub>6</sub> ( $1,587.8 \pm 296.9$ ,  $363.9 \pm 116.7$ ,  $681.3 \pm 172.5$  vs. PE-CpG  $2,440.5 \pm 63.9$  pg/mL,  $P < 0.05$ ). No significant change in IL-5 production was observed in PBS, PE-CpG, and PE-CpG-dG<sub>6</sub> treated mice ( $2,189.1 \pm 432$ ,  $2,440.5 \pm 63.9$ ,  $1,798.3 \pm 319.9$  pg/mL,  $P > 0.05$ ).

IL-12 production was significantly increased by PE-CpG-dG<sub>6</sub>, PE\*-CpG-dG<sub>6</sub>, PS-CpG, and PS-CpG-dG<sub>6</sub> ( $45.2 \pm 2.3$ ,  $77.7 \pm 1.7$ ,  $173.9 \pm 29.9$ ,  $114.3 \pm 15.6$  vs. PE-CpG  $29.6 \pm 3.4$  pg/mL,  $P < 0.05$ ). No change in IL-12 production was observed for PBS, and PE-CpG treated mice ( $28.9 \pm 4.7$ ,  $29.6 \pm 3.4$  pg/mL  $P > 0.05$ ).

IFN- $\gamma$  production was significantly increased by PE\*-CpG-dG<sub>6</sub>, PS-CpG, and PS-CpG-dG<sub>6</sub> ( $1,233.7 \pm 154.6$ ,  $7,581.5 \pm 5,446.1$ ,  $13,019.4 \pm 8,009.9$  vs. PE-CpG  $151.6 \pm 76.8$  pg/mL,  $P < 0.05$ ). No significant change in IFN- $\gamma$  production was observed in PBS, PE-CpG, and PE-CpG-dG<sub>6</sub> treated mice ( $280.7 \pm 44.9$ ,  $151.6 \pm 76.8$ ,  $222.5 \pm 80.4$  pg/mL,  $P > 0.05$ ).

PE-CpG-dG<sub>6</sub> produced more amount of IL-12 than PE-CpG ( $P < 0.05$ ) which was correlated with increased production of serum OVA specific IgG2a. PE\*-CpG-dG<sub>6</sub> produced more amount of IL-12 than PE-CpG-dG<sub>6</sub> ( $P < 0.05$ ). PE\*-CpG-dG<sub>6</sub> produced less amount of IL-12 and more amount of IL-4 and IL-5 than PS-CpG and PS-CpG-dG<sub>6</sub> ( $P < 0.05$ ). IFN- $\gamma$  production by PE\*-CpG-dG<sub>6</sub>, PS-CpG, and PS-CpG-dG<sub>6</sub> was not different ( $P > 0.05$ ). The levels of IL-4, IL-5, IL-12, and IFN- $\gamma$  produced by PS-CpG and PS-CpG-dG<sub>6</sub> were not different ( $P > 0.05$ ).

## DISCUSSION

Treating CpG-ODNs with or without a 3' dG<sub>6</sub>-run at the time of allergen challenge produced results that differed from those obtained by treating CpG-ODNs at the time of sensitization (4). 3' dG<sub>6</sub>-run conjugation to CpG ODNs changed their asthma inhibiting effects in a backbone type dependent manner. PS-CpG administered at the time of challenge was strong enough to inhibit asthma phenotypes as delivered at the time of sensitization, while PE-CpG required not only 3' dG<sub>6</sub>-run but also the modification of two PE linkages at its 5' terminus to PS linkages to produce an inhibitory effect. PS-CpG-dG<sub>6</sub> was less effective and could not inhibit airway inflammation and airway hyperresponsiveness as PS-CpG.

PE-CpG has little immunostimulatory effect in vivo because its phosphodiester backbone is easily degraded by exonucleases (4, 5). We have shown that the failure of PE-CpG to inhibit asthmatic responses could be overcome by 3' dG<sub>6</sub>-run conjugation when PE-CpG-dG<sub>6</sub> is injected at the time of sensitization (4). Runs of dG residues can form a four stranded conformation, which is called the G-quartet, which confer nuclease resistance and facilitate binding to the scavenger receptors of macrophages (11, 12). Accordingly, 3' dG<sub>6</sub>-

run may extend the half-life of PE-CpG and allow the efficient targeting of PE-CpG by APCs, presumably via a scavenger receptor (4). In this study, however, PE-CpG-dG<sub>6</sub> had little immunostimulatory effect when it was delivered at the time of allergen challenges in pre-sensitized mice; in fact, it only increased IgG2a and mild IL-12 production in the present study.

PE\*-CpG-dG<sub>6</sub>, which has two PS linkages at the 5' terminus of PE-CpG-dG<sub>6</sub>, had a better immunostimulatory effect than PE-CpG or PE-CpG-dG<sub>6</sub>. PE\*-CpG-dG<sub>6</sub> inhibited airway hyperresponsiveness like PS-CpG, decreased BAL fluid eosinophilia, increased serum allergen specific IgG2a and Th1 cytokine production by splenocytes. Two PS bonds located at the 5' terminus can contribute to resistance against exonuclease and do not affect the G-quartet structure (12). PE\*-CpG-dG<sub>6</sub> was also more effective at preventing murine allergic asthma than PE-CpG-dG<sub>6</sub> in previous report (4). Thus, two 5' terminal PS linkages may synergize with 3' dG<sub>6</sub>-run in PE backbone CpG-ODN, and thus, further enhance immunomodulatory properties.

PS-CpG was more potent, and alone was found to decrease airway inflammation as determined by lung histology. This study suggests that a stronger immunomodulatory effect is required to reverse Th2 response after sensitization than at the time of sensitization. However, there is concern about the possible side effects of PS-CpG such as long-lasting effects of lymphadenopathy, splenomegaly, as well as sustained local IFN- $\gamma$  and IL-12 production after the administration (5, 6). Splenomegaly, a possible side effect of CpG-ODNs-extramedullary hematopoiesis, seemed to be more related to the presence of the phosphorothioate backbone, as we have previously reported (5). PS-CpG showed splenomegaly and splenomegaly was reduced by PS-CpG-dG<sub>6</sub>, which failed to inhibit airway hyperresponsiveness. PE\*-CpG-dG<sub>6</sub> inhibited airway hyperresponsiveness with less splenomegaly than PS-CpG or PS-CpG-dG<sub>6</sub>. It may be a possible advantage of PE\*-CpG-dG<sub>6</sub> that it could deliver immunomodulatory effects without increasing risk of splenomegaly.

PS-CpG inhibited both airway inflammation and airway hyperresponsiveness. PE\*-CpG-dG<sub>6</sub>, which inhibited airway hyperresponsiveness, inhibited eosinophilic airway inflammation in BAL fluid less than PS-CpG but could not inhibit airway inflammation in lung tissue. Dissociation of airway inflammation and airway hyperresponsiveness has been reported, and L-selectin could be one of possible factors (8, 13). PS-CpG may inhibit airway hyperresponsiveness by the increased production of IL-12 and IFN- $\gamma$  with decreased IL-4 and IL-5 production. For PE\*-CpG-dG<sub>6</sub>, increased production of IL-12 with decreased IL-5, which can decrease eosinophilia in BAL fluid, may be important mediators on the inhibition of airway hyperresponsiveness. IL-4, IL-5, IL-13, IL-10, and other factors could be involved in the complex mechanism of airway hyperresponsiveness (8, 14, 15). CpG-ODN is known to produce IL-10 which can show immunomodulatory effect

with suboptimal concentrations of Th1-type cytokines for IL-5 suppression (16). CpG-ODN can induce a much more vigorous Th1 in the absence of IL-10 (16). CpG-ODN can also regulate activity of indoleamine 2,3-dioxygenase, the rate-limiting enzyme in catalysis of tryptophan to kynurenines; kynurenines are potent immunomodulatory molecules that regulate T cell function (16). We failed to detect the regulatory function of CpG-ODN, which is the limitation in this study.

Discrepancy affected the immunomodulatory potential and the inhibition of airway hyperresponsiveness, possibly due to the combined effects of multiple mechanisms of airway hyperresponsiveness. PS-CpG-dG<sub>6</sub> inhibited eosinophilic airway inflammation in BAL fluid less than PS-CpG (but as much as that of PE\*-CpG-dG<sub>6</sub>) and could not inhibit airway hyperresponsiveness while cytokine levels measured showed no difference to those of PS-CpG. This finding contradicts that of our previous in vivo study, which found that 3' dG<sub>6</sub>-run conjugation showed an enhanced immunostimulatory effect for both PE and PS CpG when delivered at the time of sensitization (4). However, it concurs with those findings of previous studies (6, 11). In in vitro studies, it has been reported that the conjugation of poly-G to PS-CpG may act like a neutralizing motif (CpG-N) and the conjugation of a 3' dG<sub>6</sub>-run to PS-CpG was not found to potentiate TNF- $\alpha$  and IL-12 production by splenic dendritic cells; rather it had an inhibitory effect (6, 11, 17). Recently, characterization of suppressive or inhibitory oligonucleotides has been reported that inhibit TLR9 mediated activation of innate immunity (18). The inhibitory motif is a synthetic form of PS but not PE backbone, which is especially short, 11-15 base long oligonucleotides with pyrimidin-rich triplets followed by a GGG sequence, e.g. CCTN<sub>3-5</sub>GGG sequence (18-20). There are two forms; *trans* and *cis*-form. It was reported that *trans*-form showed stronger inhibitory effect than *cis*-form (18). In this study, the sequence of PS-CpG-dG<sub>6</sub> contained that of *cis*-form inhibitory sequence (cctgacgttggggggg) and that could be why PS 3' dG<sub>6</sub> showed less effect on PS-CpG in this study. However, the levels of cytokines such as IL-4, IL-5, IL-12 and IFN- $\gamma$  produced by PS-CpG and PS-CpG-dG<sub>6</sub> were not significantly different in this study. The exact mechanism involved remains to be elucidated. Measurement of suppressive cytokines such as IL-10 or TGF- $\beta$  might have given some clue for this discrepancy and it would be the limitation of this study.

Treating CpG-ODNs with or without a 3' dG<sub>6</sub>-run at the time of allergen challenge produced results that differed from those obtained by treating CpG-ODNs at the time of sensitization. PS-CpG was found to be the most effective form of CpG-ODN but induced splenomegaly. PE-CpG required not only 3' dG<sub>6</sub>-run but also the additional modification of two 5' linkages to PS to be some effective one with less splenomegaly. Conjugation of a 3' dG<sub>6</sub> run into PS-CpG, which contained *cis*-form of inhibitory motif, showed a negative

effect in this setting.

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