

The enhanced effect of a hexameric deoxyriboguanosine run conjugation to CpG oligodeoxynucleotides on protection against allergic asthma

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Background: Oligodeoxynucleotides containing a CpG motif (CpG ODNs), as potent inducers of T_H1 immunity, are considered promising candidates for immune modulation in asthma. We have previously demonstrated that conjugation of a hexameric deoxyriboguanosine run to the 3' terminus (3' dG₆-run) of phosphodiester (PE) CpG ODNs enhanced their immunostimulatory activities in vitro.

Objective: This study aimed to evaluate the effect of a 3' dG₆-run conjugation to PE or phosphorothioate (PS) CpG ODNs on protection against murine allergic asthma in vivo.

Methods: Balb/c mice were sensitized to ovalbumin by intraperitoneal injection with or without CpG ODNs (PS CpG ODNs, PE CpG ODNs, and those with 3' dG₆-run) and subsequently challenged with ovalbumin. We evaluated airway hyperresponsiveness, eosinophil proportion in bronchoalveolar lavage fluid, airway inflammation, and ovalbumin-specific antibody responses.

Results: The conjugation of a 3' dG₆-run to PE CpG ODNs enhanced the production of IFN- γ from ovalbumin-specific T_H cells and prevented the development of asthma in terms of airway hyperresponsiveness, airway eosinophilia, and ovalbumin-specific IgE responses; these effects were comparable to those of PS CpG ODNs. Enhanced effects of the 3' dG₆-run were also observed in PS CpG ODNs, though they were lower than those in PE CpG ODNs.

Conclusion: This study suggests that conjugation of a 3' dG₆-run to CpG ODNs might provide an effective method for immune modulation of allergic asthma. (*J Allergy Clin Immunol* 2001;108:570-6.)

Key words: Asthma, CpG oligodeoxynucleotides, 3' dG₆-run, phosphodiester, T_H1, immune modulation

To date, many drugs have been developed for the treatment of allergic diseases, including corticosteroids, inflammatory mediator antagonists, and cytokine modulators.¹ However, these anti-inflammatory drugs can induce side effects and do not have prolonged therapeutic effects. Because aberrant T_H2 immune responses play a major role in allergic diseases, selective suppression of T_H2 responses is crucial for protection against allergic disorders. It has been known that bacterial DNA or oligodeoxynucleotides that contain CpG motifs (CpG ODNs) stimulate antigen-presenting cells (APCs) to produce IL-12 as well as induce natural killer cell activity.^{2,3} In addition, CpG ODN is one of the most powerful T_H1-skewing adjuvants in rodents.^{4,5} Subsequent experiments have shown that administration of CpG ODNs inhibits the development of allergic responses in murine models of asthma.⁶⁻⁸ Given the short half-life of phosphodiester (PE) ODNs in vivo, phosphorothioate (PS)-modified ODNs have been used because of their enhanced nuclease resistance.⁹

Polydeoxyriboguanosine sequences on the flanks of CpG ODNs have been reported to increase natural killer cell activity and IFN production.¹⁰ We previously demonstrated that the conjugation of a hexameric deoxyriboguanosine run at the 3' terminus (3' dG₆-run) to phosphodiester (PE) CpG ODNs significantly augmented TNF- α and IL-12 production in murine splenic dendritic cells in vitro.¹¹ On the basis of these findings, we hypothesized that PE CpG ODNs would effectively prevent allergic asthma when it was conjugated to a 3' dG₆-run in vivo.

In this study, we examined airway hyperresponsiveness (AHR), airway eosinophilia, and ovalbumin (OVA)-specific IgE responses to demonstrate the ability of PE CpG ODNs conjugated to a 3' dG₆-run as a factor in protection against allergic asthma. We also tested the effects of conjugation of a 3' dG₆-run to PS CpG ODNs in the prevention of allergic asthma.

METHODS

ODNs and mice

All CpG ODNs were purchased from GenoTech (Taejeon, Korea), and their sequences and modification are presented in Fig 1, A. PS-

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Abbreviations used

3' dG ₆ -run:	Hexameric deoxyriboguanosine run at 3' terminus
AHR:	Airway hyperresponsiveness
APC:	Antigen-presenting cell
BAL:	Bronchoalveolar lavage
ODN:	Oligodeoxynucleotide
OT-II:	Ovalbumin-specific, MHC class II-restricted α/β TCR transgenic [mice]
OVA:	Ovalbumin
PC ₂₀₀ :	Provocative concentration of methacholine required to increase the baseline enhanced pause by 200%
PE:	Phosphodiester
PE-CpG:	Phosphodiester CpG ODNs
PE*-CpG-dG ₆ :	PE-CpG-dG ₆ with 2 phosphorothioate backbones at the 5' terminus
Penh:	Enhanced pause
PS:	Phosphorothioate
PS-CpG:	Phosphorothioate CpG ODNs

CpG designates phosphorothioate (PS) CpG ODNs, and the phosphodiester (PE) form is designated by *PE-CpG*. *PS-CpG-dG₆* is PS-CpG with 6 deoxyriboguanosine residues at the 3' terminus; the PE form is *PE-CpG-dG₆*. *PE*-CpG-dG₆* represents PE-CpG-dG₆ with 2 phosphorothioate linkages at the 5' terminus.

Six-week-old male Balb/c mice were purchased from Japan SLC, Inc (Shizuoka, Japan). OVA-specific, MHC class II-restricted α/β TCR transgenic (OT-II) mice¹² were kindly provided by Dr W. R. Heath (of the Walter and Eliza Hall Institute of Medical Research, Victoria, Australia). Balb/c mice were maintained at the clinical research institute of Seoul National University Hospital, and OT-II mice were maintained at the Pohang University of Science and Technology. All animal experiments were approved by the appropriate committees on animal experimentation at our institution.

Immunization and induction of AHR

As Fig 1, B illustrates, 6 mice in each group were sensitized by intraperitoneal injection of 20 μg OVA (grade V; Sigma, St Louis, Mo) emulsified in 1 mg of aluminum hydroxide with or without 50 μg of CpG ODNs in a total volume of 200 μL PBS. They received an identical booster immunization 14 days later. On days 21, 22, and 23 after the initial sensitization, the mice were challenged for 30 minutes with an aerosol of 1% (w/v) OVA in PBS in a plexiglass chamber through use of an ultrasonic nebulizer (NE-U12, Omron, Japan). Twenty-four hours after the final OVA inhalation, AHR was assessed by methacholine-induced airflow obstruction through use of 1-chamber, whole-body plethysmography (Allmedicus, Anyang, Korea). As an index of airway resistance, increases in enhanced pause (Penh) were measured, as follows:

$$Penh = (Te/RT - 1) \times (PEF/PIF),$$

where *Penh* = enhanced pause, *Te* = expiratory time (in seconds), *RT* = relaxation time (in seconds), *PEF* = peak expiratory flow (in milliliters per second), and *PIF* = peak inspiratory flow (in milliliters per second).

Increasing doses of methacholine (ranging from 2.5 to 50 mg/mL; Sigma) were administered by nebulization for 3 minutes, and *Penh* values were calculated over the subsequent 3 minutes. Each result is presented as the concentration of methacholine required to increase the baseline *Penh* by 200% (PC₂₀₀).

CpG ODNs	Sequences
PE-CpG	TCCATGACGTTCTCTGACGTT
PS-CpG	tccatgacgttcctgacgtt
PE-CpG-dG ₆	TCCATGACGTTCTCTGACGTTGGGGGG
PS-CpG-dG ₆	tccatgacgttcctgacgttgggggg

A PE*-CpG-dG₆ tccATGACGTTCTCTGACGTTGGGGGG

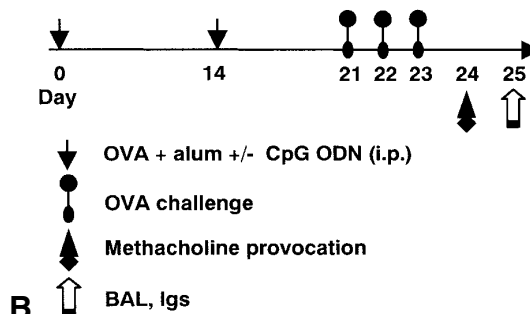


FIG 1. Experimental protocols for sensitization and challenge. **A**, Sequences and modifications of CpG ODNs used in this study are listed. Immunostimulatory CpG motifs are underlined. An *uppercase letter* represents phosphodiester backbone; a *lowercase letter* indicates phosphorothioate backbone. **B**, Balb/c mice were sensitized by intraperitoneal injection of OVA/alum with each of the CpG ODNs presented in Fig 1, A and challenged with OVA on the indicated days.

Bronchoalveolar lavage

Forty-eight hours after the last OVA challenge, the tracheae of the mice were cannulated and the lungs lavaged with five 0.4-mL aliquots of pyrogen-free saline solution. After Diff-Quik (Dade Behring Ag, Düringen, Switzerland) staining of lung lavage cells in cytospin preparation, 2 investigators counted blindly more than 300 inflammatory cells under a light microscope and classified them as macrophages, lymphocytes, neutrophils, and eosinophils.

Lung histologic analysis

After bronchoalveolar lavage (BAL), the lungs were infused with 10% formalin and embedded in paraffin. Lung sections were stained with hematoxylin and eosin and assessed through use of light microscopy. Inflammation scores were graded by 3 individuals who were unaware of the constitution of the treatment groups. The degree of peribronchial and perivascular inflammation was evaluated on a subjective scale of 0 to 3, as described elsewhere.¹³ Briefly, a value of 0 was assigned when no inflammation was detectable, a value of 1 when there was occasional cuffing with inflammatory cells, a value of 2 when most bronchi or vessels were surrounded by a thin layer (1 to 5 cells) of inflammatory cells, and a value of 3 when most bronchi or vessels were surrounded by a thick layer (>5 cells) of inflammatory cells. Total lung inflammation was defined as the average of the peribronchial and perivascular inflammation scores.

Antibody responses

Forty-eight hours after the last OVA challenge, blood samples were obtained from the mice via cardiac punctures. Briefly, microtiter plates (Dynex Technologies, Chantilly, Va) were coated overnight with 2 μg/mL OVA in a 50-mmol/L carbonate buffer (pH 9.6) at 4°C. Nonspecific binding was blocked with 2% bovine serum albumin for 1 hour at 20°C. After incubation of test sera for 2 hours,

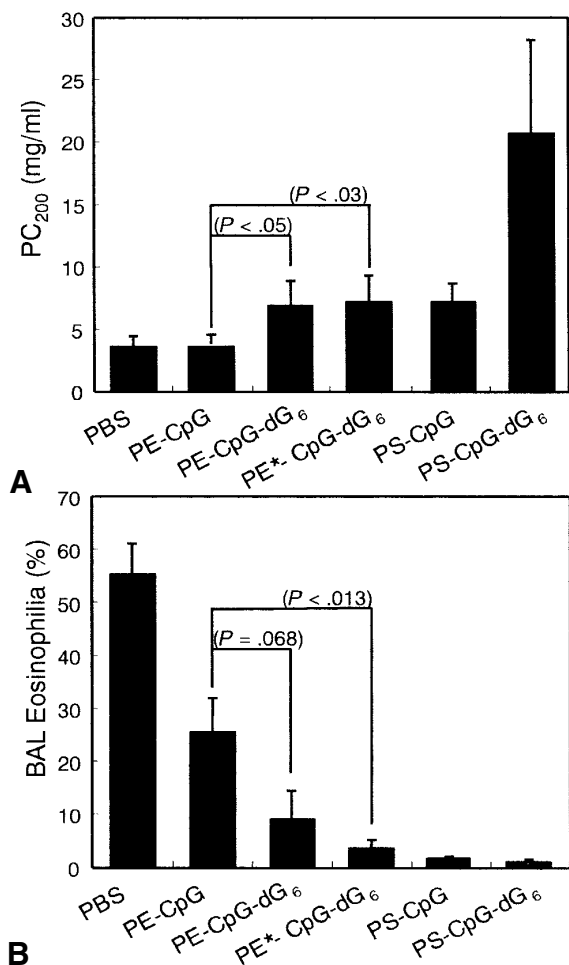


FIG 2. Effects of CpG ODNs with a 3' dG₆-run on the development of AHR and airway eosinophilia. After the ovalbumin challenge (Fig 1), AHR to inhaled methacholine (**A**) and eosinophil counts in BAL fluids (**B**) were measured. Each AHR value is represented as PC₂₀₀; eosinophil cell counts are expressed as the relative percentage of eosinophil counts. Representative data for 2 independent experiments are shown. Each bar indicates the mean value for 6 mice.

the plates were incubated with horseradish peroxidase-labeled goat antimouse IgE or IgG2a (PharMingen, San Diego, Calif) for 1 hour at 20°C. The reaction was developed with a tetramethylbenzidine (Sigma) substrate and then stopped by the addition of 2 N H₂SO₄. Subsequently, optical density was measured at 450 nm. A high titer of anti-OVA IgE or IgG2a was used as a standard, and the linear standard curves were obtained through use of a series of dilutions of standard serum. The results were expressed in arbitrary units according to each optical density value.

Stimulation of lymph node cells from OT-II in vitro

All of the lymph nodes from the OT-II mice were pooled and homogenized on a 94-μm screen (Bellco Glass, Inc, Vineland, NJ) to obtain a single cell suspension. A total of 2 × 10⁶ lymph node cells from the OT-II mice were cultured with each CpG ODN (10 μg/mL) and OVA (10 μg/mL) in 12-well plates. After 6 days, cells were harvested and restimulated with APCs in the presence of 500

μg/mL OVA. Mitomycin C (Sigma)-treated splenocytes were used as APCs. After 2 days, culture supernatants were quantitated for IFN-γ production by sandwich ELISA through use of specific mAb pairs for detection of IFN-γ, as previously described.¹¹

Statistical analysis

Statistical analysis was performed through use of the Student *t* test for cytokine production. Studies of AHR, eosinophilia, and antibody responses were compared through use of the Kruskal-Wallis and Mann-Whitney tests.

RESULTS

Effect of CpG ODNs with or without a 3' dG₆-run on the development of AHR in OVA-challenged mice

To investigate the effects of CpG ODNs with or without a 3' dG₆-run on protection against allergic asthma, we first measured AHR to inhaled methacholine after the allergen challenge. Administration of PS-CpG, but not of PE-CpG, effectively prevented AHR (Fig 2, A). Interestingly, PE-CpG-dG₆ also significantly inhibited AHR by 2.5-fold in comparison with PE-CpG ($P < .05$), indicating that the conjugation of a 3' dG₆-run significantly enhanced the effect of PE CpG ODNs on the inhibition of AHR. Administration of PE*-CpG-dG₆ also significantly reduced AHR by 2.6-fold relative to PE-CpG ($P < .03$). The effects on AHR of PE-CpG-dG₆ and PE*-CpG-dG₆ were comparable to that of PS-CpG ($P > .05$). In addition, the inhibitory effects of PS-CpG were further enhanced when it was conjugated with a dG₆-run at the 3' terminus (PS-CpG-dG₆).

Prevention of allergic lung inflammation by coadministration of various CpG ODNs

Forty-eight hours after the last OVA challenge, BAL was done to examine whether conjugation of a 3' dG₆-run to PE or PS CpG ODNs inhibited eosinophilic airway inflammation. The eosinophil proportion of BAL fluid was decreased by 57% in PE-CpG-dG₆-treated mice in comparison with PE-CpG-treated mice ($P = .06$), though this proportion was decreased by 54% in PE-CpG-treated mice in comparison with PBS-treated mice ($P < .01$), as shown in Fig 2, B. Moreover, administration of PE*-CpG-dG₆ further inhibited the BAL eosinophil counts by 86% in comparison with those of PE-CpG ($P = .01$). On the other hand, administration of PS-CpG almost completely inhibited airway eosinophilia ($P < .005$), so there was no further decrease of airway eosinophilia by the addition of dG₆ at the 3' terminus of PS-CpG.

With respect to lung histology, PBS-treated mice showed severe peribronchial and perivascular inflammation, as shown in Fig 3, A and Table I. PE-CpG-treated mice also showed prominent peribronchial and perivascular inflammation, as shown in Fig 3, B, though the degree of inflammation was slightly lower than that in PBS-treated mice (Table I). In contrast, PE-CpG-dG₆ significantly inhibited peribronchial ($P < .02$), perivascular ($P < .005$), and total lung (52% inhibition; $P < .001$) inflammation in comparison with PE-CpG, as shown in

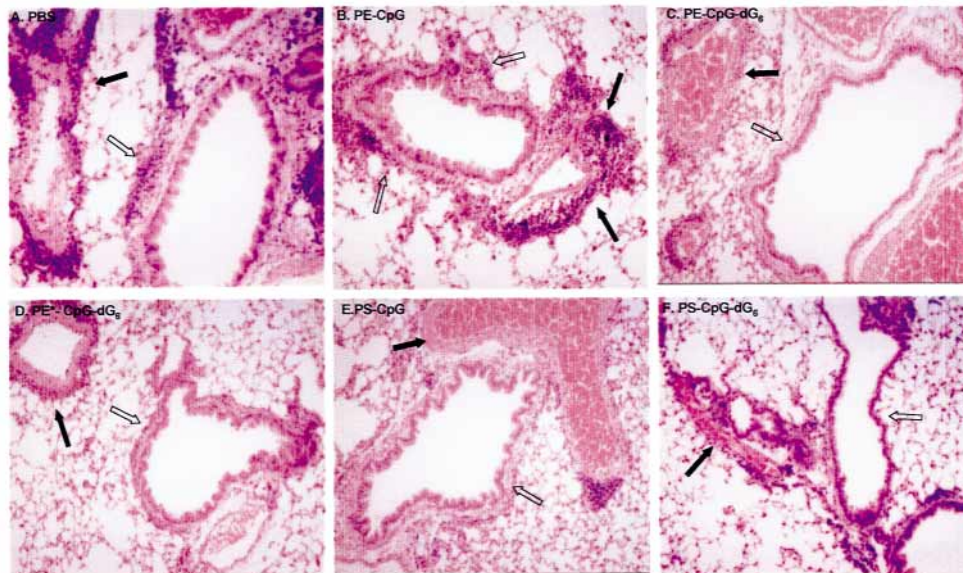


FIG 3. Representative figures of the lung tissue from each group. These show that with respect to the degree of peribronchial (open arrow) and perivascular (solid arrow) inflammation of the lung, PBS-treated (A) and PE-CpG-treated (B) mice were not so different. However, the inflammatory scores of PE-CpG-dG₆-treated (C), PE*-CpG-dG₆-treated (D), PS-CpG-treated (E), and PS-CpG-dG₆-treated (F) mice were significantly decreased in comparison with those of PE-CpG-treated mice (B; $\times 100$, hematoxylin-eosin stain). For more details, see Table I.

TABLE I. Effects of CpG ODN containing a 3' dG₆-run on peribronchial and perivascular lung inflammation

	Lung inflammation		
	Peribronchial	Perivascular	Total
PBS	2.63 ± 0.11	2.87 ± 0.04	2.75 ± 0.06
PE-CpG	2.16 ± 0.13	2.53 ± 0.18	2.35 ± 0.12
PE-CpG-dG ₆	$1.16 \pm 0.28^*$	$1.08 \pm 0.26^{**}$	$1.12 \pm 0.18^{***}$
PE*-CpG-dG ₆	$1.47 \pm 0.28^\#$	$1.43 \pm 0.43^{##}$	$1.45 \pm 0.25^{###}$
PS-CpG	1.60 ± 0.21	1.27 ± 0.28	1.43 ± 0.17
PS-CpG-dG ₆	$1.00 \pm 0.24^\dagger$	1.30 ± 0.24	1.15 ± 0.17

The degrees of inflammation were scored in blinded fashion twice each by 3 different individuals. Total lung inflammation was defined as the average of the peribronchial and perivascular inflammation scores. Intraobserver variation was $<5\%$. For details, see Methods section.

* $P < .02$; ** $P < .005$; *** $P < .00001$ for PE-CpG-dG₆ compared with PE-CpG.

$P < .03$; ### $P < .03$; ### $P < .003$ for PE*-CpG-dG₆ compared with PE-CpG.

† $P < .05$ for PS-CpG-dG₆ compared with PS-CpG.

Fig 3, C. Mice treated with PS-CpG and PS-CpG-dG₆ also showed reduction of peribronchial, perivascular, and total lung inflammation (Table I). It is worth mentioning that lung inflammation scores appeared to be slightly higher in mice treated with PE*-CpG-dG₆ than in mice treated with PE-CpG-dG₆ and slightly higher in mice treated with PS-CpG than in mice treated with PS-CpG-dG₆. In particular, peribronchial inflammation was significantly lower in PS-CpG-dG₆-treated mice than in PS-CpG-treated mice ($P < .05$).

Effects of CpG ODNs with or without a 3' dG₆-run on antibody production in OVA-challenged mice

Serum OVA-specific IgE levels were measured to assess the effects of CpG ODNs on the immunoglobulin

production that depends on IL-4 and IL-13 secreted by T_H2 cells.¹⁴ Treatment with PS-CpG or PS-CpG-dG₆ completely inhibited IgE production, whereas treatment with PBS induced a significant increase in IgE levels, as shown in Fig 4, A. PE-CpG-dG₆-treated mice showed an IgE titer that was 84% lower than that seen in PE-CpG-treated mice, though PE-CpG appeared to reduce the IgE titer by 46% in comparison with PBS. Moreover, IgE levels were almost abolished in PE*-CpG-dG₆-treated mice in comparison with PE-CpG-treated mice ($P < .02$); this was comparable to what was seen in mice treated with PS-CpG.

IgG2a levels in mice treated with various kinds of CpG ODNs are shown in Fig 4, B. The IgG2a levels were higher in PS-CpG- and PS-CpG-dG₆-treated mice than in PE-CpG- and PE-CpG-dG₆-treated mice. IgG2a production was also enhanced in mice treated with PE*-CpG-dG₆ in

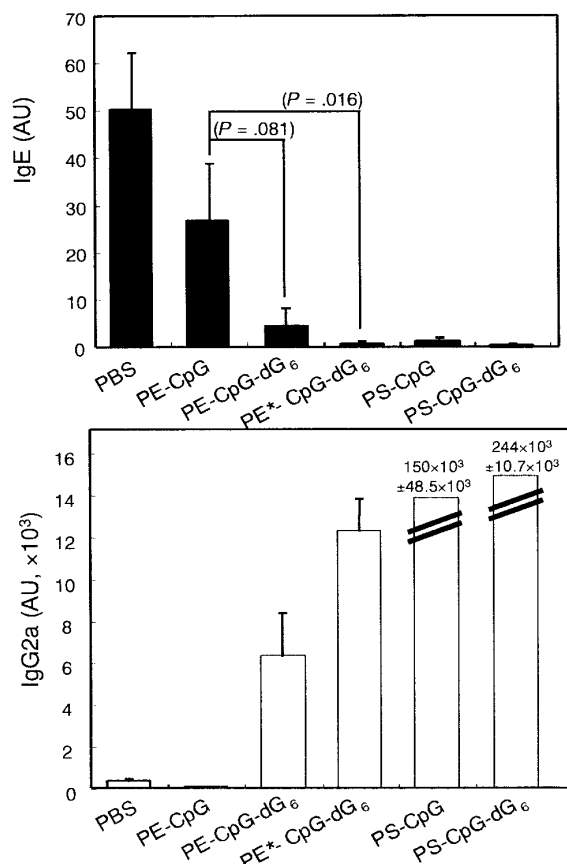


FIG 4. Antibody responses in sera of OVA-challenged Balb/c mice. Representative data for 2 independent experiments are shown; each bar indicates the mean ($\pm$ SEM) value for 6 mice.

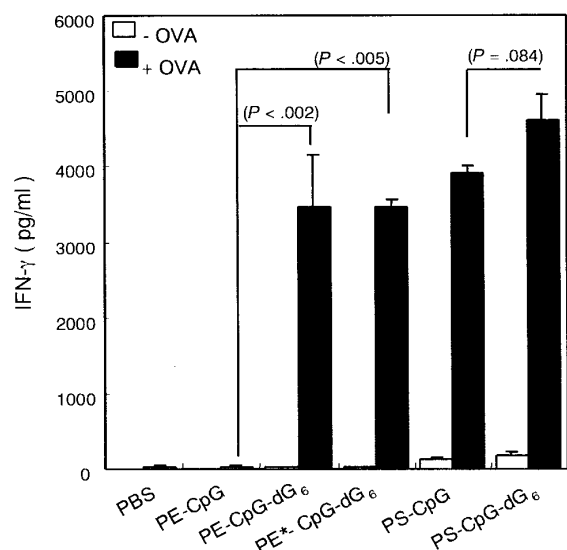


FIG 5. Antigen-specific IFN- γ production from OT-II. The experiments were reproduced with similar results; each bar represents the mean ($\pm$ SEM) value for triplicates.

comparison with mice treated with PE CpG ODNs with and without dG₆-run. The conjugation of a 3' dG₆-run to PE CpG ODNs also enhanced IgG2a production in comparison with PE CpG ODNs without dG₆-run. No significant differences were observed in total IgG and IgG1 responses between any groups of mice (data not shown).

Effect of CpG ODNs with or without a 3' dG₆-run on the development of antigen-specific T_H1 cells

To understand the mechanisms underlying the prevention of allergic lung inflammation by the 3' dG₆-run-containing CpG ODNs, we examined the effects of a 3' dG₆-run on the *in vitro* differentiation of OVA-specific T_H cells. Lymph node cells isolated from OT-II mice were activated with OVA in the presence of various CpG ODNs and then restimulated with OVA for IFN- γ production. PS CpG ODNs, but not PE CpG ODNs, slightly stimulated the lymph node cells to produce IFN- γ in the absence of costimulation with OVA (Fig 5, *open bar*). However, there were significant increases in the production of IFN- γ from OVA-specific T_H cells primed with OVA plus CpG ODNs, indicating that IFN- γ production is OVA-specific. PE CpG ODNs had poor activity in the induction of T_H1 cells because IFN- γ was not produced from T_H cells cultured with PE-CpG plus OVA. However, PE-CpG-dG₆ induced a significant amount of IFN- γ ($P < .002$), which was comparable to that of PS-CpG. PE*-CpG-dG₆ also promoted IFN- γ production ($P < .005$). When we tested the effect of PS CpG ODNs with or without a 3' dG₆-run on the T_H cell differentiation, PS-CpG-dG₆ induced more IFN- γ production than PS-CpG, though the value was not statistically significant ($P = .084$). IL-4 production was not detected from any of these T_H cells (data not shown).

DISCUSSION

In this study, we demonstrated that the conjugation of a 3' dG₆-run to CpG ODNs enhanced its protective activity against murine allergic asthma *in vivo*. This protective effect was observed when both PS-CpG and PE-CpG were conjugated with a 3' dG₆-run, though the effect was more prominent in the case of PE-CpG.

PE-CpG has poor immunostimulatory activity, presumably because of its susceptibility to nuclease digestion. PE-CpG had little or no effect on the generation of T_H1 cells or on protection against murine allergic asthma. However, the failure of PE-CpG to inhibit asthmatic responses could be overcome by a 3' dG₆-run conjugation. It is known that runs of dG residues, because of their potential to form 4-stranded conformations—referred to as a *G-quartet*—confer nuclease resistance and facilitate their binding to the scavenger receptors of macrophages.¹⁵ Accordingly, 3' dG₆-runs might extend the half-life of PE-CpG and efficiently target PE-CpG into APCs, presumably via a scavenger receptor, as shown in Fig 6.

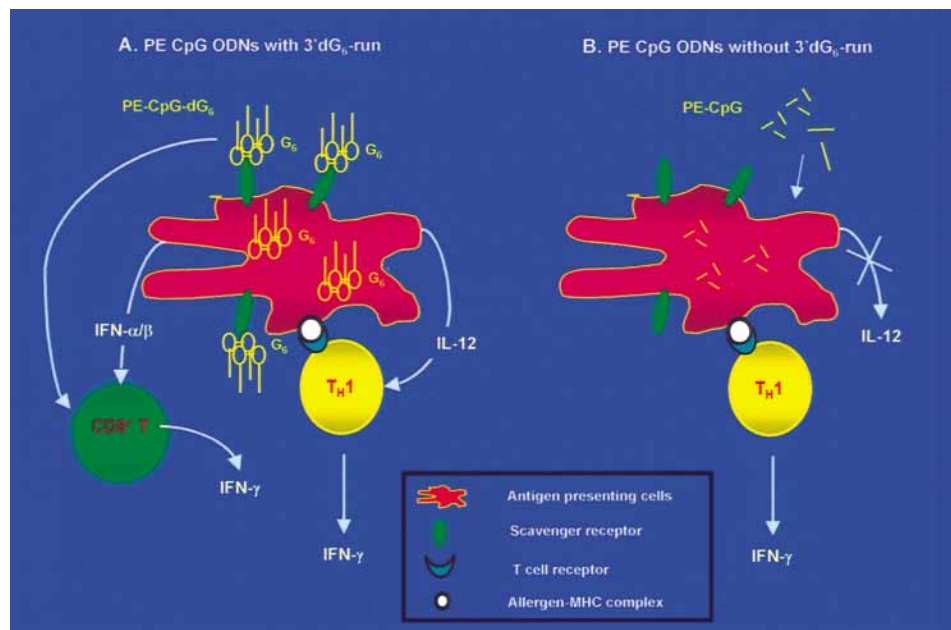


FIG 6. Proposed mechanism for the enhanced antiallergic activity of PE CpG ODNs containing a 3' dG₆-run. **A**, Runs of dG residues are known to form 4-stranded conformations—referred to as a *G-quartet*—that bind to macrophage scavenger receptors and confer nuclease resistance. Accordingly, 3' dG₆-runs might efficiently target PE CpG ODNs into APCs via scavenger receptors and extend the half-life of PE CpG ODNs. Increased intracellular concentration of PE CpG ODNs thus promotes activation of APCs to produce IL-12, which induces IFN- γ -producing T_H1 cell development. PE CpG ODNs also activate CD8⁺ T cells via APC-derived type I IFN; in addition, 3' dG₆-runs might directly costimulate CD8⁺ T cells. **B**, Most PE CpG ODNs were degraded by nucleases and were thus unable to activate APCs to produce cytokines such as IL-12. Therefore, allergen-specific T_H cells fail to differentiate into the T_H1 phenotype.

Because PS bonds located at the 5' terminus contribute to resistance against exonucleases and do not affect G-quartet structure,¹⁵ we synthesized PE*-CpG-dG₆, which has 2 PS linkages at the 5' terminus from PE-CpG-dG₆. In our preliminary study, PE*-CpG-dG₆ increased the production of both TNF- α and IL-12 from splenic adherent cells by a level 2-fold higher than that associated with PE-CpG-dG₆ (data not shown). The result of this study showed that PE*-CpG-dG₆ was more effective in the prevention of murine allergic asthma than PE-CpG-dG₆, implying that the effect of terminal PS linkages might synergize with that of a 3' dG₆-run and therefore further enhance its immunostimulatory properties.

In previous reports by us and by others, the conjugation of a 3' dG₆-run to PS-CpG did not potentiate TNF- α and IL-12 production from splenic dendritic cells; rather it showed inhibitory effects.^{11,16} It was recently demonstrated that poly dG motifs on the flanks of PS ODNs directly costimulated CD8⁺ T cells, whereas poly dG-containing PS CpG ODNs abolished the macrophage stimulatory potential.¹⁷ Because CD8⁺ T cells might also play an important role in the regulation of allergen-specific IgE responses,¹⁸ the 3' dG₆-run within PS-CpG-dG₆ might affect antiasthmatic effects in vivo. In this study, PS-CpG-dG₆ seemed to be more effective than PS-CpG without conjugation of a 3' dG₆-run in protection against asthma development, though the additive or synergistic effects

were not so prominent—mainly because PS-CpG themselves were highly immunostimulatory. This effect might be due to increased production of IFN- γ from OVA-specific T cells by poly dG-containing PS CpG ODNs. These findings suggest that poly dG-containing PS CpG ODNs potentiate in vivo antiasthmatic activity of PS CpG ODNs by production of IFN- γ from CD8⁺ T cells.

To date, the use of PE ODNs has been limited because of their short half-life in vivo. Consequently, PS-stabilized ODNs have been used in most applications.⁹ However, PS ODNs were reported to cause massive splenomegaly and other toxicities related to excessive immune stimulation, including the death of the immunized hosts, when they were used in large amounts.^{19,20} Furthermore, inflammatory responses triggered by PS ODNs have a profound effect on chronic inflammatory diseases such as asthma and other lung diseases.²¹ It was recently shown that allergen-conjugated PS CpG ODNs could reduce the therapeutic doses of PS CpG ODNs for allergic diseases.^{22,23} To the best of our knowledge, little is known about the toxic effects induced by PE ODNs. The present study shows the antiallergic activity of PE CpG ODNs with a 3' dG₆-run. Therefore, the use of 3' dG₆-run-containing PE CpG ODNs might be an alternative way to reduce the potential adverse effects triggered by PS CpG ODNs. The enhanced effects of PS CpG ODNs with a 3' dG₆-run also suggest the possibility of reducing the therapeutic doses of PS CpG ODNs.

In conclusion, the utility of CpG ODNs as immunomodulatory agents is promising in various diseases in addition to asthma. This study suggests that the use of the PE CpG ODNs with a 3' dG₆-run will provide a new approach in the prevention of allergic asthma in human beings.

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