

CpG-oligodeoxynucleotides inhibit airway remodeling in a murine model of chronic asthma

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Background: We have previously demonstrated that CpG oligodeoxynucleotides (CpG-ODNs) protect against eosinophilia and airway hyperresponsiveness in murine models of allergen-induced asthma. Acute inflammation is hypothesized to induce chronic airway responses, but no previous studies have evaluated the effects of CpG-ODNs on allergen-induced airway remodeling. Because remodeling is thought to be responsible for many of the long-term adverse effects on asthmatic patients, we evaluated whether CpG-ODNs might similarly prevent these changes using a murine model of recurrent allergen exposure. **Objective:** The purpose of this study was to evaluate the effect of CpG-ODNs on chronic inflammatory changes and airway remodeling by using a murine model of chronic allergen-induced asthma.

Methods: C57BL/6 mice were sensitized to ovalbumin (OVA) and subsequently exposed to nebulized OVA by means of inhalation 3 times weekly for 6 weeks. Some mice received CpG-ODNs by means of intraperitoneal injection at the time of sensitization. At the end of the exposure period, mice were evaluated for the development of airway inflammation, airway hyperresponsiveness, and airway remodeling.

Results: OVA-sensitized mice exposed to recurrent airway challenge with OVA have chronic inflammation, persistent airway hyperresponsiveness, and evidence of airway remodeling, including subepithelial collagen deposition and goblet cell hyperplasia-metaplasia. These changes are significantly reduced in mice treated with CpG-ODNs. Interestingly, mice treated with CpG-ODNs exhibit increased levels of bronchoalveolar lavage transforming growth factor β , suggesting that regulatory T cells might be responsible for some of these protective effects.

Conclusion: CpG-ODNs are effective not only in preventing acute inflammation but also appear to reduce markers of airway remodeling that develop after chronic allergen exposure. (*J Allergy Clin Immunol* 2002;110:867-72.)

Key words: Asthma, murine, CpG oligodeoxynucleotides, airway remodeling, collagen, subepithelial fibrosis, transforming growth factor β , regulatory T lymphocytes

Airway remodeling of the asthmatic airway, the result of persistent inflammation in the bronchial wall, is represented by structural and morphometric changes of the airway epithelium, prominent features of which include subepithelial fibrosis (excess deposition of collagen in the subepithelial layer)^{1,2} and goblet cell (GC) hyperplasia and metaplasia.^{3,4} The functional consequences of remodeling include loss of distensibility of asthmatic airways and bronchial hyperreactivity, which persist despite removal of antigens.^{5,6} Epidemiologic studies demonstrate an accelerated progressive decrease in FEV₁ among asthmatic patients. Furthermore, these changes might be significant determinants of airway hyperreactivity (AHR) and frequency of asthma attacks in chronic asthma, as well as status asthmaticus.^{2,3} These selective epithelial changes occur after repeated exposure to aerosolized allergen.^{7,8}

Current guidelines for asthma emphasize anti-inflammatory therapy. Glucocorticoids remain the first-line agents in asthma, and leukotriene pathway antagonists, the only new approach to treatment introduced in the past decade, provide significant benefits to only about one third of all patients. It is not clear whether the current therapeutic options prevent the structural changes in asthma. Chronic treatment with β_2 -agonists have been shown to have no effect on airway remodeling,⁹ and aspects of the response to glucocorticoid steroids might enhance collagen formation.¹⁰ Furthermore, very few asthmatic patients achieve a normal bronchial reactivity, even after years of steroid therapy. Thus a need for novel immunotherapeutic agents to prevent airway remodeling exists.

Previous reports of CpG-oligodeoxynucleotides (ODNs) in murine models of asthma have used limited episodes of exposure to aerosolized antigen, and the effects of CpG-ODNs on structural changes in the airway have not been characterized. We previously demonstrated that CpG-ODNs effectively prevent,¹¹ as well as reverse,¹² allergen-induced changes of acute inflammation. To investigate the influence of CpG-ODNs on airway remodeling and chronic airway hyperresponsiveness, we used a chronic model, in which remodeling

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Abbreviations used

ABPAS: Alcian Blue and periodic acid–Schiff
 AHR: Airway hyperreactivity
 BAL: Bronchoalveolar lavage
 GC: Goblet cell
 ODN: Oligodeoxynucleotide
 OVA: Ovalbumin
 Penh: Enhanced pause
 TGF- β : Transforming growth factor β

changes in the airway develop after repeated episodes of allergen challenges.

METHODS**Animals**

Six-week-old female C57BL/6 mice were purchased from The Jackson Laboratory. Protocols were reviewed and approved by and mice were maintained under the direct supervision of the Animal Care and Use Committee of the University of Iowa, according to the requirements of the National Institutes of Health Committee on the Care and Use of Laboratory Animals.

Antigen exposures

A 2.5% solution of ovalbumin (OVA, Fischer) in water was used for antigen exposures. The size distribution of the aerosol was determined by using a particle counter (Aerodynamic Particle Sizer, TSI). The aerosol sizes were distributed log-normally, with a count median aerodynamic diameter of 0.82 μ m and geometric SD of 1.46 μ m. From this distribution, 95% were less than 2.5 μ m in diameter and were likely to be deposited tracheo-bronchially. A mean OVA concentration of 3.8 ng/mL was measured during the exposures.

Murine model of chronic asthma

Mice were sensitized by means of intraperitoneal injection of OVA (10 μ g) precipitated with alum (100 μ g) on days 0 and 14, followed by OVA challenge (nebulized 2.5% solution 30 min/d, 3 d/wk for 6 weeks). Some mice also received 30 μ g of CpG oligonucleotides by means of intraperitoneal injection along with OVA (CpG+OVA). Mice were killed 48 hours after the last OVA inhalation. Control groups included negative (saline) control mice and mice not sensitized with intraperitoneal OVA but exposed to OVA by means of inhalation.

Oligodeoxynucleotides

The CpG-ODNs included 2 CpG motifs (TCCATGACGTT-CCTGACGTT). ODNs were supplied by the Coley Pharmaceutical Group and had undetectable levels of LPS, as determined by using the limulus amoebocyte lysate assay.

Airway physiology

AHR was measured by means of methacholine-induced airflow obstruction. Mice were placed into plethysmographs (WBP, Buxco Electronics) and interfaced with computers by using differential pressure transducers. Measurement was made of respiratory rate, tidal volume, and enhanced pause (Penh). Airway resistance is expressed as follows:

$$\text{Penh} = [(\text{Te}/0.3 \text{ Tr}) - 1] \times [2\text{Pef}/3\text{Pif}],$$

where Te is defined as expiratory time (in seconds), Tr is defined as relaxation time (in seconds), Pef is defined as peak expiratory flow (in milliliters per second), and Pif is defined as peak inspiratory

flow (in milliliters per second). Increasing doses of methacholine (0–50 mg/mL) were administered by means of nebulization (for 180 seconds), and Penh was calculated over the subsequent 3 minutes. Penh index is normalized to the baseline (after saline exposure) Penh as follows:

$$\text{Penh index} = \text{Measured Penh}[\text{methacholine}]/\text{Penh}[\text{saline}].$$

Whole lung lavage

After being killed, the mice were placed in the supine position with their head tilted back, and the trachea was cannulated; lungs were lavaged with 3.0 mL of saline. Total and differential counts were obtained.

Histopathology

At the time of death, lungs were fixed in paraformaldehyde, and tissue blocks were embedded in paraffin. Lung sections used for staining underwent deparaffinization and hydration. For enumeration of mucin-positive GCs, Alcian Blue and periodic acid–Schiff (ABPAS) stain was used. Sections were stained with Masson Trichrome stain to evaluate collagen deposition.

Morphometry

Images of airway sections were obtained randomly at 20 $\times$ magnification with an RT Color Digital camera (Diagnostics Instruments). Design-based protocols were adopted, as previously described, to quantify the inflammatory response in the airway epithelium.^{13,14} Briefly, 3 to 6 images (depending on length of intact epithelium) were analyzed per section by using Image Pro-Plus software (Media Cybernetics) calibrated with a reference micrometer slide. Numbers of cells (by nuclear profile) per unit surface of basement membrane were obtained. GCs were identified as cells staining purple-magenta (mucin positive) with ABPAS. Data were used to calculate cells per 100 μ m of basement membrane.

Cytokine, immunoglobulin, and collagen assays

OVA-specific antibodies were measured by means of ELISA, during which plates are coated with OVA (10 mg/mL). Biotinylated rat anti-mouse IgE (PharMingen) was used as the detection antibody. Units are OD readings at 405 nm. Transforming growth factor β (TGF- β), IL-5, and IL-10 were quantified by means of ELISA (R&D). Collagen levels were measured in homogenized lung samples by using the Sircol Collagen Assay (Biocolor, Ltd), which is a quantitative dye-based method that binds to the (Gly-X-Y)_n triple-helical sequences found in native collagen.

Statistics

Mann-Whitney *U* tests were used for comparing the means of samples. Ten mice were randomly assigned to each group. Statistical significance was defined as a *P* value of less than .05.

RESULTS**CpG-ODNs inhibit airway eosinophilia, chronic AHR, and serum IgE**

To determine whether the protective effects of CpG-ODNs previously seen in acute models of asthma would persist after repeated exposures to allergen, we first evaluated airway eosinophilia, AHR, and immunoglobulin production. We found a significant induction of airway eosinophilia elicited by repeated allergen exposures, which was almost completely prevented in mice treated with CpG-ODNs (saline, $0 \pm 0 \times 10^6$ eosinophils; inhaled

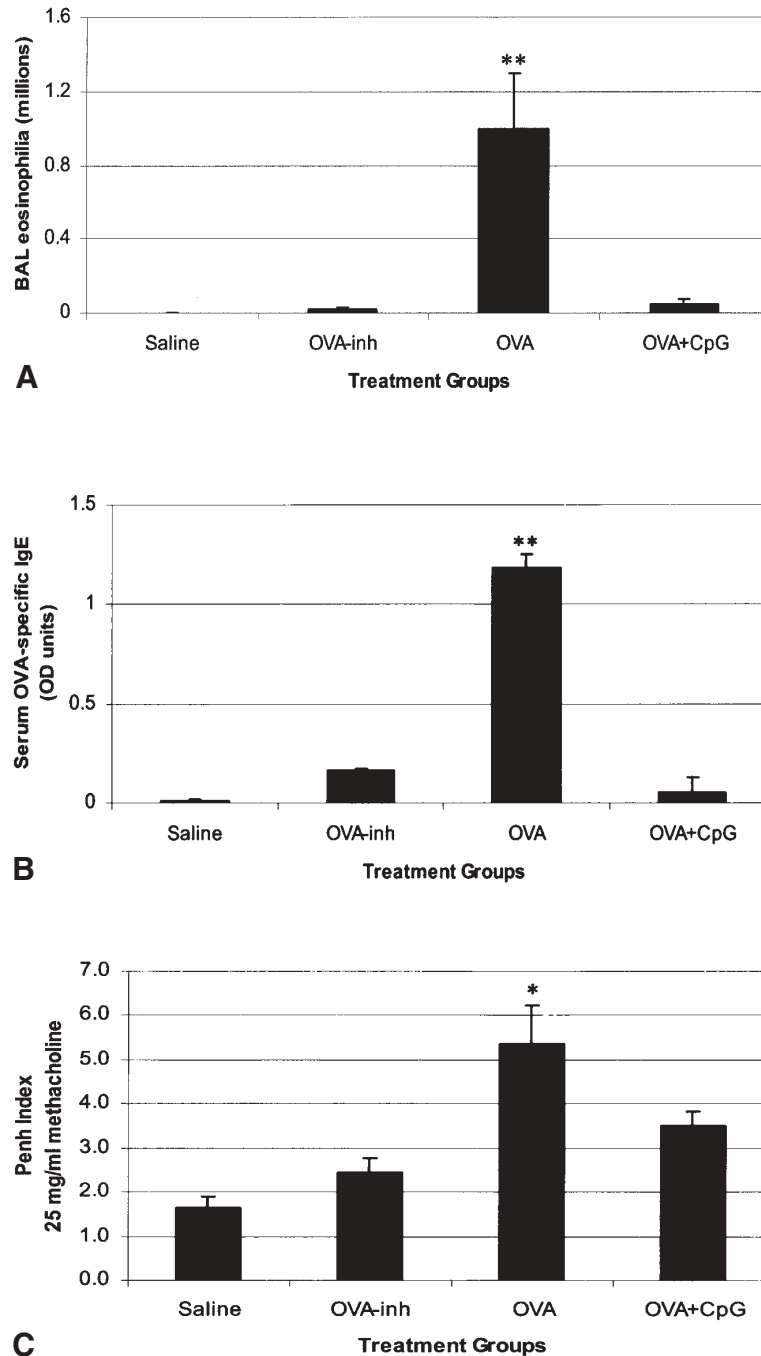


FIG 1. CpG-ODNs prevent eosinophilic airway inflammation, nonspecific bronchial hyperresponsiveness, and antigen-specific IgE induction in a murine model of chronic asthma. Repeated inhalation of OVA in sensitized mice leads to induction of significant BAL fluid eosinophilia (**A**), OVA-specific IgE (**B**), and bronchial hyperresponsiveness (**C**) to inhaled methacholine (25 mg/mL), all of which are significantly reduced in mice treated with CpG-ODNs at the time of sensitization. * $P < .05$ and ** $P < .001$, OVA versus all other groups.

OVA, $0.02 \pm 0.01 \times 10^6$ eosinophils; OVA, $1.0 \pm 0.3 \times 10^6$ eosinophils; OVA+CpG, $0.05 \pm 0.02 \times 10^6$ eosinophils; $P < .001$ OVA+CpG vs OVA; Fig 1, A). Bronchoalveolar lavage (BAL) fluid lymphocytes were significantly increased in the OVA group ($0.19 \pm 0.05 \times 10^6$) compared with those in all other groups (saline, 0.10

$\pm 0.04 \times 10^6$; inhaled OVA, $0.04 \pm 0.01 \times 10^6$; OVA+CpG, $0.09 \pm 0.01 \times 10^6$), and no differences in BAL fluid neutrophils were noted. We also found that untreated mice had significantly increased OVA-specific IgE, which was inhibited in CpG-treated mice (saline, 0.01 ± 0.01 OD units at 405 λ ; inhaled OVA, 0.16 ± 0.01

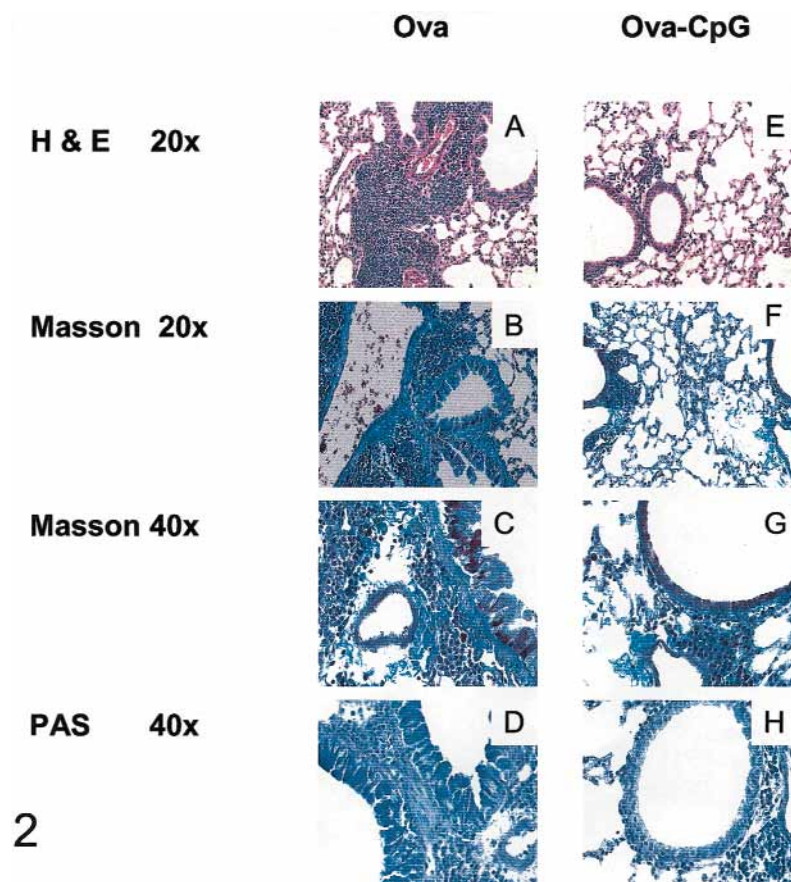


FIG 2. CpG-ODNs prevent peribronchial inflammation, subepithelial collagen deposition, and GC hyperplasia-metaplasia in a murine model of chronic asthma. Repeated inhalation of OVA in sensitized mice leads to significant chronic peribronchial inflammation characterized by eosinophil infiltration (**A**; hematoxylin and eosin staining) and airway remodeling characterized by subepithelial collagen deposition (**B** and **C**; Masson Trichrome staining) and GC hyperplasia-metaplasia (**D**; ABPAS staining), all of which are significantly reduced in mice treated with CpG-ODNs at the time of sensitization (**E-H**).

OD units at 405λ; OVA, 1.18 ± 0.07 OD units at 405λ; OVA+CpG, 0.05 ± 0.08 OD units at 405λ; $P < .001$; Fig 1, *B*). We next examined the development of AHR and found it significantly higher in untreated mice relative to mice treated with CpG-ODNs (Penh index at 25 mg/mL methacholine: saline, 1.64 ± 0.25 ; inhaled OVA, 2.43 ± 0.34 ; OVA, 5.34 ± 0.9 ; OVA+CpG, 3.51 ± 0.32 ; $P < .05$ OVA vs OVA+CpG; Fig 1, *C*); no changes were seen in baseline (premethacholine) Penh values. Tissue sections demonstrated marked peribronchial inflammatory infiltrate in untreated mice (completely absent in saline and inhaled OVA control mice, data not shown), which was almost completely abrogated in mice treated with CpG-ODNs (Fig 2, *A* and *E*).

CpG-ODNs inhibit subepithelial fibrosis

The effects of CpG-ODNs on airway remodeling have not been studied previously. We first evaluated subepithelial collagen with Masson Trichrome stain and found a marked induction of subepithelial collagen in mice sub-

jected to repeated exposures to OVA, which was inhibited in CpG-treated mice (Fig 2, *B*, *C*, *F*, and *G*). Furthermore, we conducted morphometric measurements to quantify these changes and found significant inhibition in the thickness of the collagen layer in CpG-treated mice (OVA, $1950 \pm 138 \mu\text{m}^2$; OVA+CpG, $1220 \pm 96 \mu\text{m}^2$; $P < .05$). These changes in the airway were associated with significant differences in total lung collagen content (OVA, $1463 \pm 268 \mu\text{g}$; OVA+CpG, $549 \pm 74 \mu\text{g}$; $P < .01$).

CpG-ODNs inhibit GC hyperplasia-metaplasia

To evaluate for GC hyperplasia-metaplasia, we stained the lung sections with ABPAS. We found a pronounced induction of GCs in untreated mice relative to CpG-treated mice (Fig 2, *D* and *H*). GCs, rarely present in normal murine airway epithelium, were significantly increased by means of morphometry in chronically exposed mice; this was inhibited in CpG-treated mice (OVA: 18.09 ± 2.41 vs OVA+CpG: 1.68 ± 0.87 GCs per 100 μm of basement membrane; $P < .005$).

CpG-ODN administration is associated with an increase in TGF- β levels in lavage fluid

To evaluate mechanisms through which CpG-ODNs might offer protection against chronic inflammation, we measured levels of IL-10 and TGF- β in lung lavage fluid. We found significantly increased levels of TGF- β in mice treated with CpG-ODNs compared with that seen in untreated mice (saline, 42.7 ± 38 pg/mL; OVA, 189.0 ± 44.9 pg/mL; OVA+CpG, 311.5 ± 40.4 pg/mL; $P < .05$); interestingly, mice receiving inhaled OVA, putatively tolerized to OVA, exhibited even greater concentrations of BAL fluid TGF- β (522.0 ± 130.9 pg/mL). No significant differences in BAL fluid IL-5 (saline, 12.2 ± 9.1 pg/mL; inhaled OVA, 41.5 ± 22.6 pg/mL; OVA, 25.0 ± 6.9 pg/mL; OVA+CpG, 36.8 ± 8.6 pg/mL) or IL-10 levels were noted (saline, 512.4 ± 169.8 pg/mL; inhaled OVA, 225.7 ± 183.6 pg/mL; OVA, 711.8 ± 290.0 pg/mL; OVA+CpG, 344.1 ± 115.4 pg/mL).

DISCUSSION

Several reports now confirm the protective effects of CpG-ODNs on acute inflammation in murine models of asthma. In this study we provide the first evidence that CpG DNA might prevent remodeling in a murine model of chronic asthma. Airflow obstruction in asthma is not always fully reversible and is a function of duration and severity of previous asthma.¹⁵ Structural changes in the airway might account for much of this irreversibility. Unlike sensitization to allergens, remodeling develops over a prolonged period of time, presumably because of repeated inflammation from environmental triggers. The possibility of preventing the airway structural changes therefore seems to be plausible, potentially reducing the development of irreversible airflow obstruction. Also, targeting airway remodeling early in the pathologic process might have greater therapeutic effect. Unfortunately, current modes of therapy seem unlikely to prevent these changes.^{9,10}

Subepithelial fibrosis is a key feature of airway remodeling in human asthma.¹ It has been reported previously by using a murine model that subepithelial fibrosis only develops after 4 to 6 weeks of allergen exposure.⁷ We therefore chose to administer inhalational allergen for 6 weeks in our current study. Our results suggest that repeated exposures induce significant subepithelial collagen deposition. Treatment with CpG-ODNs significantly inhibited this fibrosis, and we found a 40% decrease in the subepithelial collagen thickness in treated mice, which was also associated with reduced AHR. The reduction in collagen thickness is consistent with changes seen in asthmatic human airways, demonstrating 30% to 60% increase in thickness.^{16,17} In our study we examined subepithelial fibrosis and remodeling changes at the level of conducting (medium and small size) airways. Recent studies assessing airway wall thickness by using high-resolution computed tomography have reported an increase in thickness in the small airways, which is not always observed in the large airways.^{18,19} Further-

more, airway wall thickness is predominantly seen in the small airways in nonfatal asthma.²⁰

Although many studies of remodeling focus on collagen deposition, epithelial changes are also seen in chronic asthma. GC hyperplasia in the airway epithelium promotes excessive mucin secretion and is known to be a prominent feature of even mild asthma.^{3,21-23} CpG-ODNs can inhibit GC hyperplasia in short-term models^{24,25}; we show here that pretreatment with CpG-ODNs also prevents GC hyperplasia in a chronic model.

In our study all treated mice were subjected to 6 weeks of inhalational allergen exposures after administration of CpG-ODNs. This suggests that the effects of CpG on airway structural changes and chronic AHR persisted at least 6 weeks after its administration. This is consistent with a previous report²⁶ demonstrating long-term inhibition of airway eosinophilia and T_H2 cytokines by CpG DNA.

We found enhanced levels of TGF- β , but not IL-10, in the airways of CpG-treated mice. TGF- β has recently received considerable attention as a potential protective factor in asthma^{27,28} and is thought to be released by regulatory T cells.^{29,30} Because we have previously demonstrated that protection by CpG-ODNs is independent of T_H1 cytokines,³¹ an intriguing possibility is that induction of immunosuppressive regulatory T cells mediates some of these effects.

We conclude that although the mechanisms of airway remodeling remain to be fully elucidated, further investigation into remodeling processes and drugs targeting its prevention is required; this animal model will facilitate such studies. Recent reports from human clinical trials suggest the CpG-ODNs are well tolerated in human subjects.³² Although the protective effects of CpG DNA on acute inflammatory markers of atopic asthma have been reported in several murine models,^{11,26,33} our results suggest that CpG-ODNs might also provide protection against chronic inflammation and remodeling changes, possibly through induction of regulatory T cells. Thus CpG-ODNs might represent a disease-modifying form of therapy both by preventing the initial generation of atopic responses to allergens and by preventing chronic inflammatory structural changes in the airways.

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