Mucosal immunotherapy with CpG oligodeoxynucleotides reverses a murine model of chronic asthma induced by repeated antigen exposure

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Jain, Vipul V., Thomas R. Businga, Kunihiko Kitagaki, Caroline L. George, Patrick T. O’Shaughnessy, and Joel N. Kline. Mucosal immunotherapy with CpG oligodeoxynucleotides reverses a murine model of chronic asthma induced by repeated antigen exposure. Am J Physiol Lung Cell Mol Physiol 285: L1137–L1146, 2003.—Murine models of acute atopic asthma may be inadequate to study the effects of recurrent exposure to inhaled allergens, such as the epithelial changes seen in asthmatic patients. We developed a murine model in which chronic airway inflammation is maintained by repeated allergen [ovalbumin (OVA)] inhalation; using this model, we examined the response to mucosal administration of CpG DNA (oligonucleotides) and specific antigen immunotherapy. Mice repeatedly exposed to OVA developed significantly greater airway hyperresponsiveness and goblet cell hyperplasia, but not airway eosinophilia, compared with those exposed only twice. CpG-based immunotherapy significantly reversed both acute and chronic markers of inflammation as well as airway hyperresponsiveness. We further examined the effect of mucosal immunotherapy on the response to a second, unrelated antigen. Mice sensitized to both OVA and schistosome eggs, challenged with inhaled OVA, and then treated with OVA-directed immunotherapy demonstrated significant reduction of airway hyperresponsiveness and a moderate reduction in eosinophilia, after inhalation challenge with schistosome egg antigens. In this model, immunotherapy treatment reduced bronchoalveolar lavage (BAL) levels of Th2 cytokines (IL-4, IL-5, IL-13, and IL-10) without changing BAL IFN-γ. Antigen recall responses of splenocytes from these mice demonstrated an antigen-specific (OVA) enhanced release of IL-10 from splenocytes of treated mice. These results suggest that CpG DNA may provide the basis for a novel form of immunotherapy of allergic asthma. Both antigen-specific and, to a lesser extent, antigen-nonspecific responses to mucosal administration of CpG DNA are seen.

allergen immunotherapy; eosinophils; airway hyperresponsiveness; inflammation; CpG oligodeoxynucleotide

ATOPY IS AN IMMUNE DISORDER of hypersensitivity to environmental allergens and a major cause of asthma worldwide. The prevalence and morbidity of asthma have increased and are a growing health concern, especially in industrialized countries (35). This burden on health care resources demands novel intervention strategies against asthma.

Airway mucosal surfaces, the first defense against inhaled agents, form the most important interface between the immune system and the environment. The airway mucosa is constantly exposed to environmental allergens that trigger atopic responses only in some individuals. These responses are characterized by predominant expression of T helper (Th) 2 cytokines in the airway, which occurs after repeated cycles of allergen exposure, maintaining the Th2 polarity of naive CD4+ cells (17). Th2 cytokines also induce airway hyperresponsiveness (AHR) through direct effects on resident airway cells. It therefore seems plausible to hypothesize that modulation of local (mucosal) T cell responses may significantly impact airway inflammation and AHR.

Immunotherapy for allergy is the therapeutic administration of allergen to reduce atopic responses to environmental exposure; its efficacy is thought to be due to immune deviation, deletion, or anergy. Although recent meta-analyses demonstrate significant improvement in asthma control following immunotherapy (2), conventional immunotherapy is thought ineffective in the control of asthma by some (3), and its use in asthma remains controversial; nevertheless, this approach has been endorsed by the World Health Organization (7a). Even when effective, some patients, including many children, are reluctant to use this therapy from needle phobia. Mucosal administration of allergen immunotherapy has a number of potential advantages over traditional intradermal administration and may be the key to successful implementation of immunotherapy. Besides eliminating the need for injections (an important determinant of compliance, especially in children), it engenders lower costs and may reduce the risk of anaphylactic reactions relative to intradermal administration (31). In addition, studies investigating CpG DNA as an immunostimulatory

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adjuvant have shown that it confers more effective immune responses when administered mucosally rather than parenterally (6, 18), suggesting that mucosal administration of CpG oligodeoxynucleotides (ODN) may be an effective means of delivering enhanced allergen immunotherapy.

To simulate the effect of recurrent allergen inhalation, we developed a murine model of chronic airway inflammation using repeated ovalbumin (OVA) inhalation. Using this model, we addressed the hypothesis that mucosal administration of CpG-ODN alone and in combination with allergen is an effective route to deliver specific allergen immunotherapy; we further examined whether mucosal administration of CpG and specific allergen immunotherapy affects the response to an unrelated allergen, in this case schistosome eggs (SE). We found that CpG-ODN was effective in reversing established eosinophilic inflammation and hyper-responsiveness of the airways; moreover, immunotherapy against a single allergen improved AHR, but not airway inflammation, following exposure to a second allergen.

METHODS

Animals

Six-week-old C57BL/6 female mice were purchased from Jackson Laboratory. Mice were maintained under the supervision of the Animal Care and Use Committee of the University of Iowa in accordance with the requirements set forth by the National Institutes of Health Committee on Care and Use of Laboratory Animals; animals were housed in specific pathogen-free environments and were allowed access to food and water ad libitum.

OVA Exposure

A 1% solution of OVA in double-distilled water was used for antigen exposures. Filtered air was passed at 6 l/min through an Aero-Tech nebulizer (CIS-US) to generate an aerosol. The size distribution of the aerosol was determined using a particle counter (Aerodynamic Particle Sizer, TSI). The aerosol sizes were distributed log-normally with a count median aerodynamic diameter of 0.82 μm and geometric SD of 1.46 μm. A mean OVA concentration of 3.8 ng/ml was measured in the chamber during the exposures.

Murine Model of Asthma

**Immunotherapy model.** All mice were initially sensitized to and then challenged with OVA before the onset of immunotherapy: mice were given an intraperitoneal injection of OVA (10 μg with 1 mg alum.) on days 0 and 7, followed by inhalation of OVA (1% solution, 30 min) on days 14 and 16. Mucosal immunotherapy consisted of transnasal administration of CpG-ODN (1 μg) with (OVA+CpG) or without (CpG) OVA (5 μg) in saline (0.9% NaCl, 30 μl) on days 21, 35, and 49. Untreated mice received transnasal saline during the immunotherapy period. With the exception of one group of untreated control mice, all other mice underwent repeated allergen exposure (RAE) to OVA (1% solution 10 min before and after each therapy). After the last immunotherapy, all mice were rechallenged with OVA (days 56 and 58; 1% solution, 30 min) and then killed (day 59). (The protocol is outlined in Fig. 1A.)

**Double allergen model.** To investigate antigen specificity of immunotherapy, we induced allergic responses to SE.

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**Fig. 1.** Murine model of asthma and immunotherapy protocols. A: mice were sensitized to ovalbumin (OVA) by intraperitoneal (ip) injection (days 0, 7; solid vertical arrows) and challenged with aerosolized OVA (days 14, 16; dashed vertical arrows). Mucosal immunotherapy was administered transnasally (days 21, 35, 49; solid bold vertical arrows): CpG oligodeoxynucleotides (ODN) with or without OVA. All mice except some control mice received repeated inhalational allergen exposures (RAE, dotted vertical arrows) of OVA before and after each therapy dose. All mice were rechallenged with OVA (days 56, 58) and killed 24 h after the final challenge. B: mice were sensitized to OVA and to schistosome eggs (days 0, 7), challenged with aerosolized OVA (days 14, 16), and then treated with CpG+OVA immunotherapy or remained untreated (days 21, 35, 49); all mice received RAE of OVA. All mice were challenged with schistosome egg antigen (SEA) by intranasal instillation (days 56, 58) before death.
these studies, mice were sensitized to OVA (as above) as well as to SE (5,000 eggs, ip) on days 0 and 7, then subjected to inhalation of OVA (1% solution, 30 min, days 14 and 16). As with the preceding protocol, mice were treated with three doses of biweekly immunotherapy (days 21, 35, and 49) with OVA and CpG or remained untreated; both groups of mice underwent RAE. One week after the last dose of therapy, all mice were challenged with schistosome egg antigen (SEA, 10 μg transnasally) on days 56 and 58 and killed 24 h later. (The protocol is outlined in Fig. 1B.) We have previously shown that mice sensitized to SE and then challenged with SEA developed marked AHR, eosinophilia, and a rise in IgE immunoglobulins (25).

ODN

The CpG-ODN were constructed on a phosphothioate backbone and consisted of 20 bases containing two CpG motifs (TCCATGACGTTCCTGACGTT). Control ODN, used for some studies, contain no CpG motif (TCCATGAGCTTCTCGAGTCT). ODN were supplied by the Coley Pharmaceutical Group (Wellesley, MA) and had undetectable levels of lipopolysaccharide by the Limulus amebocyte assay.

Airway Physiology

AHR was measured by methacholine-induced airflow obstruction using plethysmography (Buxco Electronics), as previously described (27). Response to inhaled methacholine is noted as enhanced pause (Penh) index (the Penh, expressed as a ratio of the baseline postsaline inhalation Penh).

Lung Lavage

After death (150 mg ip pentobarbital sodium; Abbott Laboratories, North Chicago, IL), lungs were lavaged (27). Total and differential cell counts were obtained; bronchoalveolar lavage (BAL) fluid was stored at −70°C for later measurement of cytokine levels.

Histopathology and Morphometry

At the time of death, lungs were excised and fixed; sections were stained with Biebrich scarlet and Alcian blue-periodic acid Schiff stains. For morphometric analysis, images of airway sections were obtained at ×40 magnification and analyzed using design-based protocols (7, 37). Goblet cells and epithelial cells were separately enumerated, and the ratio of these cell types was calculated individually for at least eight airway sections for each mouse.

Splenocyte Studies

Single cell suspensions of spleen cells were plated in 24-well tissue culture plates at a final concentration of 5 × 10^6 cells/ml in RPMI 1640-based complete medium and cultured at 37°C in 5% CO2 (23). Some cells were cultured without stimulation, some were stimulated with OVA at a final concentration of 100 μg/ml, and others were stimulated with SEA at a final concentration of 10 μg/ml. Culture supernatants were harvested after 72 h of culture and stored at −70°C until the measurement of cytokine levels.

Cytokines and Antibodies

Murine IL-4, IL-5, IL-10, IFN-γ (OptEIA, PharMingen), and IL-13 (Duoset, R&D Systems) were measured by sandwich ELISA. OVA-specific antibodies were measured by ELISA (26).

Data Analysis

Statistical significance was evaluated with the program SPSS 10.0 for Windows. Student’s t-test and ANOVA were employed for comparing groups of samples, as appropriate. A P value of <0.05 was considered significant.

RESULTS

CpG-ODN Reverse Established Eosinophilic Airway Inflammation and Specific IgE

We have previously demonstrated that systemic administration of CpG-ODN and allergen but neither agent alone can readily reverse established eosinophilic airway inflammation and AHR in a murine model of atopic asthma (26). For these current studies, we first examined whether mucosal administration of allergen immunotherapy would induce similar therapeutic responses, especially in the setting of repeated exposure to inhaled allergen. OVA-sensitized mice, previously challenged with aerosolized OVA, were subjected to a mucosal immunotherapy protocol, as described in METHODS and outlined in Fig. 1A. We found that exposure to RAE did not significantly alter the induction of BAL eosinophilia [untreated, 1.76 ± 0.23 × 10^6; RAE, 1.58 ± 0.19 × 10^6 eosinophils; P = not significant (ns)] (Fig. 2). A significant reduction in BAL eosinophilia was noted both in mice that received immunotherapy with the mixture of OVA and CpG (OVA + CpG, 0.18 ± 0.03 × 10^6, P < 0.01 vs. RAE), as well as those treated with CpG alone (CpG, 0.44 ± 0.16 × 10^6, P < 0.01 vs. RAE); the difference in reduction between mice treated with CpG alone and CpG with OVA did not reach statistical significance. Mice that were treated with control ODN alone or with OVA did not demonstrate significant differences from untreated mice in these or in subsequent studies.

Immunotherapy condition

![Graph showing the effect of immunotherapy on lung eosinophils](http://ajplung.physiology.org/)

Fig. 2. Mucosal immunotherapy using CpG-ODN reverses established airway eosinophilia. According to the protocol (Fig. 1A), mice underwent mucosal immunotherapy with CpG-ODN in the presence or absence of OVA. Except for the untreated group (untreated), all mice received repeated inhalational allergen exposures including some mice that were untreated (RAE). Mucosal immunotherapy consisted of transnasal administration of CpG-ODN in the presence (OVA + CpG) or absence of OVA (CpG). Treatment with CpG-ODN resulted in significantly lower BAL eosinophilia compared with saline treated groups (**P < 0.01 vs. RAE, n = 8/group).
To investigate the effect of mucosal immunotherapy on antibody synthesis, we measured serum levels of Th2-associated OVA-specific IgE as well as Th1-induced IgG2a (Fig. 3). As with the airway eosinophilia, we found similar elevations of OVA-specific IgE in untreated mice (1.26 ± 0.24) and mice exposed to RAE in the absence of treatment (1.14 ± 0.14) and significant reductions in mice treated with CpG-ODN alone (0.66 ± 0.18; \( P < 0.05 \) vs. RAE) or with OVA and CpG-ODN (0.22 ± 0.06; \( P < 0.01 \) vs. RAE) (Fig. 3). This pattern was reversed in the case of the Th1-induced immunoglobulin OVA-specific IgG2a, which was significantly lower in untreated mice (0.16 ± 0.03) and mice exposed to RAE (0.49 ± 0.03) than in mice treated with CpG-ODN (1.55 ± 0.16; \( P < 0.01 \) vs. RAE) or OVA and CpG (1.26 ± 0.2; \( P < 0.01 \) vs. RAE).

**RAE Significantly Enhance AHR and Induce Chronic Epithelial Inflammatory Changes, Which Are Inhibited by CpG-ODN Immunotherapy**

We assessed AHR to inhaled methacholine 24 h after the final allergen challenge and immediately before death (Fig. 4). Compared with untreated control mice (Penh index at 50 mg/ml methacholine 8.91 ± 1.03), mice exposed to RAE developed significantly greater AHR (Penh index at 50 mg/ml methacholine 14.86 ± 2.58, \( P < 0.01 \) vs. untreated); AHR was significantly reduced in both the group of mice treated with CpG-ODN alone (Penh index at 50 mg/ml methacholine 4.97 ± 1.69, \( P < 0.05 \) vs. RAE) and the group treated with OVA and CpG allergen immunotherapy (Penh index at 50 mg/ml methacholine 1.87 ± 0.15, \( P < 0.01 \) vs. RAE). There was no statistically significant difference between the responses of the two CpG-treated groups of mice.

![Fig. 3. Effect of CpG-ODN on antigen-specific immunoglobulin synthesis. Immediately before death, mice underwent phlebotomy. Serum was separated and frozen and subsequently analyzed for OVA-specific immunoglobulin levels. Optical density (OD, 405nm) readings of OVA-specific serum IgE from treated mice (CpG and OVA+CpG) were significantly lower than untreated mice (untreated and RAE). A concomitant increase in the levels of OVA-specific serum IgG2a was seen in the treated mice (CpG and OVA+CpG) compared with the untreated RAE mice. (*) \( P < 0.05 \); **(*) \( P < 0.01 \), vs. RAE, n = 8/group.](image)

![Fig. 4. Mucosal immunotherapy using CpG-ODN protects against airway hyperresponsiveness (AHR) following antigen challenge. AHR to inhaled methacholine was assessed 24 h after the final allergen challenge, immediately before death. We found significantly elevated AHR in the untreated mice (untreated and RAE groups) compared with the treated mice (CpG and OVA+CpG groups). RAE mice demonstrated significantly greater AHR compared with untreated mice not repeatedly exposed to OVA. There was no significant difference in AHR between the treated groups (CpG vs. OVA+CpG), both of which were significantly reduced relative to RAE. (*) \( P < 0.05 \); **(*) \( P < 0.01 \), vs. RAE, n = 8/group. Penh, enhanced pause.](image)

We next examined the cellular changes induced by RAE as well as the mucosal treatment with CpG. Because the RAE group of mice demonstrated marked enhancement of AHR, we speculated that epithelial remodeling may have developed in response to RAE. We first evaluated the degree of hyperplasia of goblet cells (GC), rarely present in normal murine airway epithelium, and their production of airway mucins (Fig. 5). We conducted morphometric measurements to characterize the epithelial changes (Table 1) and found a significant elevation in the ratio of GC to epithelial cells in the mice exposed to RAE (GC ratio 3.92 ± 0.49) compared with untreated control mice (GC ratio 0.32 ± 0.15; \( P < 0.005 \)) and that this increase was significantly abrogated by the treatment with CpG-ODN (0.82 ± 0.12; \( P < 0.01 \) vs. RAE) or the combination of OVA and CpG-ODN (0.11 ± 0.12; \( P < 0.001 \) vs. RAE). Furthermore, the amount of stored mucin in the epithelium (measured by area fraction of mucin) was significantly higher in the RAE mice than in the untreated control mice (RAE, 10.2 ± 0.9 vs. untreated, 1.2 ± 0.4; \( P < 0.005 \)), and this storage of mucin was significantly reduced in the airway epithelial cells of mice treated with CpG alone (4.1 ± 0.9; \( P < 0.01 \) vs. RAE) or with OVA (0.4 ± 0.1; \( P < 0.005 \) vs. RAE). These results suggest that repeated exposure to inhaled allergen induces chronic epithelial inflammatory changes in this murine model of asthma and that treatment with CpG-ODN inhibits this epithelial remodeling. These changes do not correlate with the induction of BAL eosinophilia nor with peribronchial eosinophilic infiltration (by Biebrich scarlet stain, not shown).
Antigen-Specific Immunotherapy Using CpG-ODN Protects Against the Development of AHR, But Not Airway Eosinophilia, Following Exposure to an Unrelated Antigen

One potential mechanism to explain the suppression of allergen-induced responses is induction, by CpG-ODN, of inhibitory cells or mediators that could downregulate Th2-driven inflammation developed in response to all antigens and stimuli; to explore this question, we next investigated whether immunotherapy directed against a specific allergen would inhibit the response to a second, unrelated allergen. We first evaluated whether sensitization to two allergens altered the inflammatory response to inhalation of either allergen alone. We found similar degrees of airway eosinophilia in mice sensitized to both OVA and SE then challenged with aerosolized OVA (1.95 ± 0.43 × 10^6 eosinophils) and in mice sensitized and challenged with OVA alone (1.64 ± 0.36 × 10^6 eosinophils, n = 6/group, P ns).

We next modified the mucosal immunotherapy protocol to evaluate the effect of immunotherapy directed against OVA on schistosome-induced inflammation, as is described in METHODS and Fig. 1B. After treatment, all mice were challenged with SEA and then studied. Mice treated with OVA-specific immunotherapy demonstrated significant inhibition of AHR following challenge with SEA (Fig. 6; Penh index at 50 mg/ml of methacholine: OVA+CpG/SEA, 2.12 ± 0.39; RAE/SEA, 6.01 ± 0.89; P < 0.005); reduction of airway eosinophilia levels in the treated mice did not reach statistical significance (RAE/SEA, 1.78 ± 0.21 × 10^6 vs. OVA+CpG/SEA, 1.32 ± 0.14 × 10^6; P = 0.10). In separate studies, schistosome-sensitized mice treated...
with specific immunotherapy (SEA+CpG) demonstrated a significant reduction in both AHR and BAL eosinophilia (data not shown), similar to the protection seen in OVA-sensitized mice treated with OVA+CpG immunotherapy. Treatment was associated with a significant reduction of BAL IL-4, IL-5, IL-10, and IL-13, with no significant change in BAL IFN-γ (Fig. 7). Interestingly, in vitro recall studies demonstrated that splenocytes from immunotherapy-treated mice release less IL-5 and IFN-γ following stimulation with either SEA or OVA than do splenocytes from untreated control mice (Fig. 8). In contrast, IL-10 responses appear to be antigen specific; mice treated with immunotherapy directed against OVA release increased concentrations of IL-10 following in vitro rechallenge with OVA but reduced concentrations after rechallenge with SEA. This suggests the possibility that suppressive IL-10 responses may be antigen specific in this model, whereas suppression of other inflammatory mediators may be antigen nonspecific.

**DISCUSSION**

These studies clearly demonstrate that mucosal administration of CpG-ODN can reverse established eosinophilic airway inflammation and established AHR. We and others have recently shown that parenteral administration of CpG-ODN and antigen, either coadministered (26) or conjugated (38), is effective in reversing these changes. However, mucosal immunotherapy seems to hold promise as a successful alternative to the traditional systemic route of administration. In these current studies, we have extended our previous findings by developing a murine model in which airway inflammation is magnified or maintained by repeated inhalation of allergen and shown that the resulting epithelial changes are reversed with mucosal CpG-ODN-based immunotherapy. In addition, we have successfully used monotherapy (CpG alone) in the setting of spontaneous (like ambient environmental) exposure to the experimental allergen. Finally, we have demonstrated for the first time that antigen-specific mucosal immunotherapy using CpG-ODN may protect against unrelated allergenic triggers.
challenged with SEA. One criticism that has been leveled at the use of Penh response as a surrogate for bronchial resistance is that the variable integrates the nasal response, rather than isolating the intrathoracic component of AHR. In the case of this study, where we are examining the transnasal (and not the intratracheal) administration of CpG ODN, the Penh is a relevant variable to follow.

The majority of atopic asthma patients are sensitized to allergens early in life (36); once they develop established airway inflammation, this may be reinforced by naturally occurring recurrent exposure to environmental allergens (10). In experimental models, BAL eosinophilia does not increase significantly until 48 h after the first airway challenge (47), and the degree of eosinophil infiltration increases with each additional challenge. Hence, we elected to begin immunotherapy with CpG-ODN 1 wk after the first challenge, at a time when all mice had been exposed to aerosolized OVA twice. These mice thus had well-established eosinophilic airway inflammation before the institution of therapy, unlike previous studies in which therapy was started 1 (39) to 4 days (38) after a single airway exposure to allergen. Furthermore, we repeatedly exposed mice to allergen during the course of immunotherapy to model natural allergen challenges that asthmatic patients receive. Indeed, we found that RAEOA induced significantly higher AHR and GC hyperplasia compared with mice that did not receive repeated allergen challenges, although airway eosinophilia did not differ between these groups; these changes were significantly inhibited in CpG-treated mice. These results suggest that previous studies using murine models without ongoing exposure to inhaled allergen (38, 39, 42, 43) may not address the inflammatory responses present in chronic asthma. These therapeutic effects are consistent with our previously reported studies showing that CpG-ODN can prevent airway remodeling when administered at the time of sensitization (19).

Previous reports have demonstrated that mucosal administration of CpG-ODN effectively prevents development of antigen-specific inflammatory responses (42). Our findings are consistent with those reports and further demonstrate that both coadministration of CpG-ODN and OVA as well as monotherapy using CpG-ODN alone while mice are allowed to spontaneously inhale nebulized OVA are effective in reversing established disease. Sur et al. (45), using a murine model of ragweed-induced asthma, demonstrated that intratracheal administration of CpG-ODN, given after sensitization but 48 h before allergen challenge, increased the ratio of IFN-γ to IL-4-secreting cells and decreased allergen-specific Th2 responses. Shirota et al. (42) investigated the effect of mucosal coadministration as well as conjugated administration of CpG-ODN and antigen, given at the time of antigen challenge, and found that both therapeutic approaches could prevent the development of antigen-specific inflammatory responses. These previous studies, however, did not investigate the question of the effect of

### Fig. 8. Mucosal immunotherapy using CpG-ODN in dual-sensitized mice alters recall response of splenocytes to antigen challenge. Splenocytes were isolated from mice sensitized to both OVA and schistosome eggs and then treated with immunotherapy against OVA (OVA+CpG/SEA) or not treated (RAE/SEA); splenocytes were cultured alone (control) or in the presence of OVA or SEA for 72 h, then supernatants were harvested for later measurement of IL-5 (A), IFN-γ (B), and IL-10 (C). Splenocytes from OVA+CpG-treated mice released significantly less IL-5 and IFN-γ following stimulation with either OVA or SEA; IL-10 release was significantly increased from the OVA+CpG-treated splenocytes stimulated in vitro with OVA, but not those stimulated with SEA. *P < 0.05, RAE/SEA vs. OVA+CpG/SEA; n = 8/group.

Although the use of whole body plethysmography has recently been criticized as a measure of AHR (15, 16, 33), in previous studies we (24, 26, 28, 32) have demonstrated a strong correlation between induction or suppression of airway inflammation and alterations in methacholine-induced Penh in rodent models of asthma. Indeed, in the current study, OVA mucosal immunotherapy significantly reduced both airway eosinophilia and AHR in OVA-challenged mice and significantly reduced AHR (the reduction in airway eosinophilia did not reach statistical significance) in mice.
CpG-ODN on established airway eosinophilia. Our current studies significantly extend these findings by demonstrating the reversal of established chronic airway inflammation.

One potential result of the transnasal route is inadvertent oral administration, which could play a role in the therapeutic response. In this model, we do not believe that oral delivery of CpG ODN is very significant; although CpG ODN are immunogenic when administered enterally, in separate studies (data not shown) we have found that the concentrations required to demonstrate a therapeutic effect are approximately one log greater than those used for transnasal therapy.

Excessive mucus production is a common occurrence in asthma, particularly during asthma exacerbations, and it is associated with significant mortality and morbidity in asthma (4, 8). GC hyperplasia in the airway epithelium contributes to the excessive mucin secretion in the airway lumen and is known to be a prominent feature of severe asthma (41). Recently, it has been shown that even mild asthma is associated with increased amounts of stored mucin in the airway epithelium, as a result of GC hyperplasia (11). Those investigators also reported an increased amount of secreted mucin in moderate asthma vs. mild asthma, implicating the release of mucin from GCs as a potential pathogenic mechanism for exacerbations (11). We therefore characterized the effects of CpG-ODN on airway epithelium. In our model utilizing RAE, we found a significant induction of GC hyperplasia/metaplasia (quantified by the increased ratio of goblet cells to epithelial cells) in mice receiving RAE, which was inhibited by treatment with CpG-ODN. Moreover, we conducted morphometric measurements to analyze the amount of stored mucins in the airway cells and found that CpG-ODN treatment led to a significant decrease in the amount of stored mucin. Significant evidence demonstrating the importance of IL-4, IL-5, IL-9, and IL-13 in GC hyperplasia and mucin production (14, 30, 46, 50) suggests that CpG-ODN most likely function in this role by downregulating Th2-type responses.

It has been shown previously that AHR in murine models can be induced by as few as two inhalation challenges with experimental allergen. In our model, we found that mice receiving RAE developed significantly higher AHR compared with mice exposed less to antigen. This enhanced AHR was associated with the increased GC hyperplasia, although airway eosinophilia was unchanged. This is another example of dissociation between degree of eosinophilia and bronchial hyperresponsiveness that has been previously noted in murine models and may reflect a response to the epithelial changes. Treatment with CpG-ODN (alone or in combination with the allergen) was effective in blocking the AHR as well as the markers of inflammation. As we found in this study, others have also found a dissociation between airway eosinophilia and AHR (29).

To our knowledge, all previous studies of the effects of CpG-ODN on murine models of allergic airway inflammation have used a monosensitization model. However, the majority of atopic asthmatics are sensitized to more than one allergen, usually early in life (51). In an attempt to address this issue, we modified our immunotherapy model by sensitizing mice to two experimental allergens (OVA and SE) and then treating one group with OVA+CpG immunotherapy and leaving the others untreated. After challenging both groups of mice with SEA, we found that CpG-ODN-based immunotherapy was effective in protecting against AHR but not in preventing airway eosinophilia in this double allergen model.

How might allergen immunotherapy based on CpG-ODN reduce AHR without suppressing airway eosinophilia following challenge with an unrelated antigen? Among prominent effects of CpG-ODN in models of asthma is a suppression of Th2 cytokine responses, often associated with an induction of Th1 cytokines. In our current study, we found reductions in BAL concentrations of IL-4, IL-5, IL-13, and IL-10 and no change in IFN-γ; in vitro splenocyte recall challenges likewise showed reduced IL-5 and IFN-γ and a dichotomous IL-10 response. Certainly, reductions in IL-5 (in the airway or systemically) would be expected to suppress eosinophilia to a greater degree than AHR (9, 16). The lack of induction of IFN-γ is not surprising, as we have previously shown that Th1 cytokines are not critical (although they likely play a moderate contributing role) for the antiasthmatic effects of CpG-ODN (27). Other investigators have found that both IL-4 and IL-13 can mediate AHR independently of airway eosinophilia and IL-5 (9, 21); in addition, both of these cytokines have recently been reported to induce AHR through direct effects on resident airway cells (49). In our double allergen model, we found decreased levels of IL-13 and IL-4 in the BAL fluid of mice treated with OVA+CpG-ODN compared with levels in untreated mice, with by far the greatest reduction in IL-13. These results suggest that suppression of IL-13 may play a role in reversing established AHR, which is consistent with the findings of Wills-Karp et al. (50), demonstrating that blockade of IL-13 is sufficient to reverse established AHR, independently of eosinophils.

Another possible mechanism for the effects of CpG-ODN is regulation of IL-10. IL-10 is described as a Th2 cytokine (34), but its role in atopic asthma is complicated. Increased expression or exogenous administration of IL-10 augments induction of AHR despite inhibiting airway eosinophilia (20, 48). Interestingly, we found that levels of IL-10 in the airway were suppressed in treated mice vs. untreated mice, raising the possibility that this may contribute to their reduced AHR. In contrast to its local airway effects, IL-10 has systemic anti-inflammatory properties, suppressing both Th1 and Th2 cytokine responses, and it can induce anergy or tolerance when applied to CD4+ (12) or dendritic cells (44). It appears to be critical in mediating T cell tolerance and protection against allergen-induced AHR (5). We (23) have previously demonstrated that CpG-ODN strongly induce IL-10 from a variety of cells and that this induction at least partially mediates the protective effect of CpG-ODN against
atopic inflammation. The source of IL-10 induction remains incompletely understood. One possibility is regulatory T cells (CD25+CD4+, T<sub>R</sub>I cells), which are felt to play an important role in maintaining immunological tolerance (22, 40) by effects that include reduced antigen-presenting cell-induced proliferation of CD4<sup>+</sup> T cells, suppressed immunoglobulin production, and reduced antigen presentation (13). Induction of T<sub>R</sub>I cells might explain both the antigen-specific effects of CpG-ODN as well as (through the release of anti-inflammatory cytokines) limited nonspecific responses in the setting of atopic inflammation. In this current study, we found that splenocytes from mice treated with OVA+CpG-ODN released increased concentrations of IL-10 after challenge with OVA, but not after challenge with SEA; this supports an antigen-specific component to the IL-10 response. Thus depending on its location of expression, IL-10 can lead to both beneficial and adverse consequences in asthma.

In conclusion, our studies suggest that mucosal administration of CpG-ODN may reverse airway inflammation and AHR in chronic asthma. This treatment also provides protection against the induction of AHR following challenge with an unrelated antigen. Immunotherapy thus has the potential to be disease modifying in the pathogenesis of atopic asthma by preventing and reversing established airway inflammation and AHR and, more importantly, by inhibiting the development of sensitization at an early age. Our results support the need for further studies investigating the use of CpG-ODN in the immunotherapy of asthma.

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DISCLOSURES

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