Am J Physiol Lung Cell Mol Physiol 283: L170–L179, 2002.
First published February 22, 2002; 10.1152/ajplung.00402.2001.

Treatment of established asthma in a murine model using CpG oligodeoxynucleotides

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Received 15 October 2001; accepted in final form 14 February 2002

Kline, Joel N., Kunihiko Kitagaki, Thomas R. Businga, and Vipul V. Jain. Treatment of established asthma in a murine model using CpG oligodeoxynucleotides. *Am J Physiol Lung Cell Mol Physiol* 283: L170–L179, 2002. First published February 22, 2002; 10.1152/ajplung.00402.2001.—Allergen immunotherapy is an effective but underutilized treatment for atopic asthma. We have previously demonstrated that CpG oligodeoxynucleotides (CpG ODN) can prevent the development of a murine model of asthma. In the current study, we evaluated the role of CpG ODN in the treatment of established eosinophilic airway inflammation and bronchial hyperreactivity in a murine model of asthma. In this model, mice with established ovalbumin (OVA)-induced airway disease were given a course of immunotherapy (using low doses of OVA) in the presence or absence of CpG ODN. All mice then were rechallenged with experimental allergen. Untreated mice developed marked airway eosinophilia and bronchial hyperresponsiveness, which were significantly reduced by treatment with OVA and CpG. CpG ODN leads to induction of antigen-induced Th1 cytokine responses; successful therapy was associated with induction of the chemokines interferon-γ-inducible protein-10 and RANTES and suppression of eotaxin. Unlike previous studies, these data demonstrate that the combination of CpG ODN and allergen can effectively reverse established atopic eosinophilic airway disease, at least partially through redirecting a Th2 to a Th1 response.

allergy; cytokines; Th1/Th2; lung; immunomodulators

ONCE CONSIDERED A DISEASE of bronchospasm, asthma is now recognized to be an inflammatory disorder of the airways, which is associated with bronchial hyperreactivity and bronchospasm. The expression and release of T helper (Th) 2-like cytokines, frequently prompted by response to allergen, promote this inflammatory response. These cytokines, notably interleukin (IL)-4, IL-5, and IL-13, induce eosinophil chemotaxis and activation, mast cell stimulation, and IgE production in atopic as well as in nonatopic asthma. Although the molecular mechanisms of inflammation are similar in both types of asthma, the role of the antigen in inducing these inflammatory responses is central in atopic asthma. Moreover, although not all atopic individuals develop asthma, atopy is the single most important predictor for the development of asthma. Thus prevention and reversal of allergen-induced inflammation could have a significant impact on the morbidity of asthma.

Great strides have been made in recent years towards understanding the important role of inflammation in asthma, yet this has translated into relatively modest changes in therapeutic options for severe asthma. Despite the potentially serious adverse effects of high-dose inhaled steroids, including adrenal axis suppression, altered glucose metabolism, and cataract formation, corticosteroids remain the mainstay of therapy. Allergen immunotherapy is the only currently used treatment modality that potentially redirects the inflammatory response to allergen to an alternate, less disruptive pathway. Accepted for the treatment of atopic conditions such as rhinitis and conjunctivitis, immunotherapy has not been widely used in asthma. There is a risk of significant adverse reactions (e.g., status asthmaticus) in severe asthmatic patients, the group most likely to benefit from this therapy, which imposes dose limitations on the use of this potentially curative treatment. The addition of immunostimulatory adjuvants to immunotherapy may enhance the efficacy of this treatment, reducing the required dose and potentially reducing the risk of significant adverse effects.

In previous studies, we demonstrated that CpG oligodeoxynucleotides (CpG ODN, ODN centered on the dinucleotide CG), administered at the time of initial sensitization to antigen, are capable of preventing the subsequent development of eosinophilic airway inflammation in a murine model of asthma (13). CpG ODN are thought to interact with the Toll-like receptor-9 (16) to induce a cascade of cellular events (reviewed in Ref. 14) that lead to a myriad of stimulatory effects on B cells, NK cells, and antigen-presenting cells (APC) such as dendritic cells (15). Although CpG ODN do not directly stimulate T cells, they induce costimulatory signals on APC and produce a Th1 environment [e.g., through interferon (IFN)-γ release by NK cells] and thus induce the elaboration of Th1 cytokines. The protection offered by CpG ODN against the development of airway inflammation is associated with local and systemic induction of the Th1 cytokines IFN-γ and IL-12, although neither cytokine appears to be abso-

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lutely required (12). In addition Th2 cytokines and serum IgE levels are suppressed.

On the basis of these findings, we proposed to examine the role of CpG ODN as an immunotherapy adjuvant. For these studies, we used a murine model of asthma in which mice were initially sensitized to ovalbumin (OVA) and subsequently stimulated by inhalation of aerosolized OVA. The immunotherapy (low doses of OVA alone or in combination with CpG or control ODN) consisted of 8 wk of biweekly subcutaneous injections, and all mice were again stimulated with inhaled OVA at the end of the treatment. We hypothesized that administration of CpG ODN and allergen would deviate the sensitized mice from a Th2 response to allergen reexposure towards a Th1 response.

MATERIALS AND METHODS

Murine Models

OVA model of asthma. Six- to eight-week-old female C57BL/6 mice (Jackson Laboratories) were sensitized to OVA (10 μg with 1 mg alum) on *day 0* and challenged with inhaled OVA (6% solution, 30 min daily, 5 days/wk) on *weeks 2* and *3*. Some mice received ODN at the time of sensitization to confirm the results of previous studies.

OVA immunotherapy. Immunotherapy consisted of four biweekly subcutaneous injections (*weeks 4, 6, 8,* and *10*) of antigen (OVA, 10 μg in 100 μl saline) in the presence or absence of ODN (CpG or control ODN, 30 μg) or saline alone as a negative control; other mice received CpG ODN alone. After 8 wk of immunotherapy, mice were rechallenged with OVA (6% solution, 30 min daily, 5 days/wk) on *weeks 11* and *12*. All mice were killed 24 h after the final exposure to OVA (Fig. 1).

Schistosome egg model of asthma. To confirm the effect of allergen-specific immunotherapy, we used the schistosome egg model of asthma as previously described (1); mice were sensitized to schistosome eggs by intraperitoneal injection of 5,000 eggs on *day 0* and then received intranasal soluble egg antigen from schistosome eggs (SEA; 10 μg) on *days 7* and *14*.

SEA immunotherapy. For the immunotherapy studies, mice received four biweekly injections of SEA (2 μg sc), SEA plus CpG ODN (10 μg), or SEA plus control ODN, followed by two repeated rechallenges with intranasal SEA (*days 77* and *104*) before death.

General murine treatment. All mice were euthanized with pentobarbital sodium (150 mg/kg ip; Abbot Laboratories). At the time of euthanasia, phlebotomy was performed by retroorbital puncture. After euthanasia, the trachea of each mouse was cannulated, and saline washings were collected; lavages were processed for cell counts and differential analysis. All animal care and housing requirements of the National Institutes of Health Committee on Care and Use of Laboratory Animals were followed. Mice were housed in barrier facilities and were allowed access to food and water ad libitum. All protocols were reviewed and approved by the University of Iowa Animal Care and Use Committee.

ODN

ODN were provided by the Coley Pharmaceutical Group (Wellesley, MA) and had undetectable levels of lipopolysaccharide by the Limulus amebocyte lysate assay. CpG ODN: TCCATGACGTTCCTGACGTT; control ODN: TCCATGA*GC*TTCCTGA*GTC*T (CG dinucleotides underlined; substituted nucleotides italicized).

Physiology

Airway hyperreactivity was measured by methacholine-induced airflow obstruction, using a whole-body plethysmograph (Buxco Electronics, Troy, NY) as previously described (13); airway resistance was expressed as enhanced pause (Penh). Penh index is derived by dividing the Penh measured after exposure to a given concentration of inhaled methacholine by the Penh measured after inhalation of nebulized saline and thus represents a fold increase relative to baseline airway resistance.

Histopathological Examination

At the time of death, lungs were fixed, and sections were stained with hematoxylin and eosin stain for examination by light microscopy.

Splenocyte Culture

Single-cell suspensions of splenocytes were cultured in 24-well tissue culture plates at a final concentration of 5×10^6 cells/ml in RPMI-1640 supplemented with 2 mM glutamine, 10% heat-inactivated fetal calf serum, 25 mM HEPES, 50 mM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were stimulated with OVA at a final concentration of 100 μg/ml. Some cells were stim-

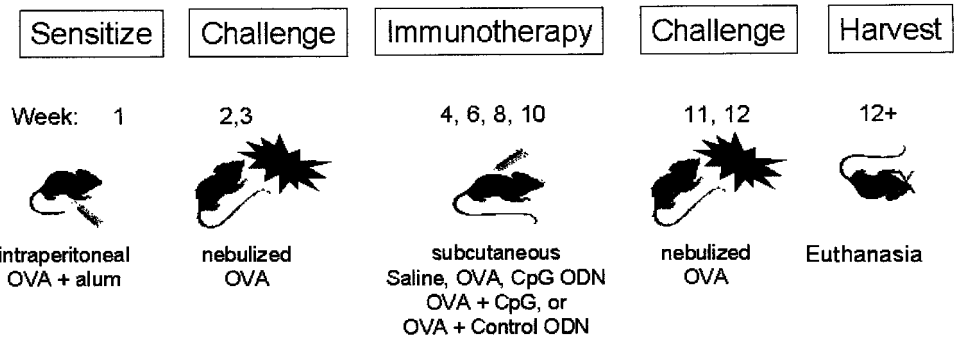

Fig. 1. Ovalbumin (OVA) murine model of asthma and immunotherapy protocol. C57BL/6 mice were sensitized to OVA by intraperitoneal injection and subsequently challenged with OVA by inhalation on *weeks 2* and *3*. For the immunotherapy model, 4 groups of mice underwent immunotherapy starting at *week 4*: 4 biweekly injections of OVA either alone or in the presence of control or CpG oligodeoxynucleotide (ODN); control mice received saline alone during the period of immunotherapy, and other mice received CpG ODN alone. All 4 groups of mice were rechallenged with OVA on *weeks 11* and *12*.

ulated with ODN (either CpG or control) at a final concentration of 0.01, 0.1, or 1.0 μg/ml. For other studies, cells were stimulated with Con A (Sigma) at a final concentration of 5 μg/ml. The supernatants were harvested 72 h after the culture, immediately frozen at −70°C, and subsequently batch processed.

Serum IgE

OVA-specific IgE was measured by ELISA in which plates were coated with OVA and biotinylated rat anti-mouse-IgE antibody (PharMingen, San Diego, CA) was the detection antibody. Units are optical density readings at 405 nm.

Cytokines

Murine IL-5 and IFN-γ were measured using a sandwich ELISA (OptEIA, PharMingen) according to the manufacturer's instructions. The lower limit of detection for IL-5 was 15.6 pg/ml and for IFN-γ was 31.3 pg/ml.

Detection and Quantitation of mRNA

Total RNA was extracted from lung flash-frozen in RNA STAT-60 (Tel-Test B, Friendswood, TX). The composition of RNA STAT-60 includes phenol and guanidinium thiocyanate in a monophase solution. The lung tissue was homogenized in the RNA STAT-60 using a tissue homogenizer. Chloroform was added, and the total RNA was subsequently precipitated by addition of isopropanol. The yield and purity of RNA were quantified by measuring the ratio and absorbances at 260 and 280 nm. RNase protection assays were performed using RiboQuant Multi-Probe RNase Protection Assay System (PharMingen). Multi-Probe Template Sets [mouse cytokine (mCK)-1 and mCK-5, PharMingen] were used, which include templates for IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-15, IFN-γ, macrophage inflammatory protein (MIP)-1α, MIP-1β, MIP-2, monocyte chemoattractant protein-1, T-cell activation protein (TCA-3), interferon-inducible protein-10 (IP)-10, eotaxin, regulated upon activation normal T cell-expressed and secreted (RANTES), L32, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Briefly, target RNA was hybridized with a ^{32}P-labeled antisense RNA probe in a hybridization buffer solution. Free probe and other single-stranded RNA were then digested with RNases. The protected hybridized RNA were purified and resolved on a denaturing polyacrylamide gel. The gel was dried on a vacuum gel drier and exposed to X-ray film at −70°C. The quantity of each mRNA species was determined based on the intensity of the appropriately sized, protected probe fragment. Gel images were obtained using Fluor-S MultiImager with Quantity One 4.1.0 software (Bio-Rad, Hercules, CA) to perform densitometry; signals were normalized to GAPDH signal to correct for any inadvertent gel-loading errors.

Statistics

Statistical significance was evaluated using the program SPSS 8.0 for Windows. Wilcoxon's signed-rank and Mann-Whitney *U*-tests were employed for comparing the means of related and unrelated samples, respectively.

RESULTS

On the basis of the results of our previous studies, we first examined the effect of CpG ODN administered at the time of sensitization to OVA. For these studies, we utilized a standard OVA murine model of asthma, and we confirmed that the administration of CpG ODN at the time of sensitization to OVA led to a significant reduction in lavage eosinophils (from $1.6 \pm 0.5 \times 10^6$ in untreated to $0.2 \pm 0.2 \times 10^6$ eosinophils in CpG ODN-treated mice, $n = 8$/group, $P < 0.01$) in this model. These mice were also substantially protected against the development of bronchial hyperreactivity to inhaled methacholine (Penh ratio at 50 mg/ml of methacholine decreased from 4.2 to 1.9, $P < 0.01$).

To determine whether CpG ODN are effective when administered after sensitization to allergen, we next evaluated their effect when given after sensitization but before airway exposure to OVA. For these studies, all mice were sensitized with OVA by intraperitoneal injection; on *day 7*, the treated mice then received CpG ODN along with a second administration of OVA, and control mice received the OVA alone or OVA and control ODN. All mice in this study then were exposed to OVA by inhalation on *days 14–16*. We found that the mice who received CpG ODN were also protected against the development of airway eosinophilia (although to a lesser degree than the mice pretreated with CpG ODN) but that bronchial hyperreactivity to inhaled methacholine was not significantly reduced (Fig. 2*A*: airway eosinophilia decreased from $2.3 \pm 0.7 \times 10^6$ eosinophils in untreated mice to $0.9 \pm 0.4 \times 10^6$ eosinophils in mice treated with OVA and CpG, $n = 8$/group, $P < 0.05$; Fig. 2*B*: Penh index at 50 mg/ml of methacholine decreased from 5.8 to 4.1, $P = 0.14$).

To model persistent asthma in humans, who, by current standards of treatment, require intensive anti-inflammatory therapy, we next treated mice with established airway eosinophilia. We first examined the effect of a single dose of CpG ODN (30 μg sc) in the presence or absence of OVA (10 μg). All mice were then re-exposed to OVA by inhalation 1 wk after the treatment. We found that this single treatment did not significantly reduce the subsequent development of airway eosinophilia (untreated mice: $1.9 \pm 0.4 \times 10^6$ eosinophils; CpG-treated mice: $1.6 \pm 0.4 \times 10^6$ eosinophils; CpG plus OVA-treated mice $1.4 \pm 0.4 \times 10^6$ eosinophils, $n = 4$/group, $P =$ not significant) or bronchial hyperreactivity to inhaled methacholine (Penh index at 50 mg/ml: untreated mice, 5.3 ± 0.8; CpG-treated mice, 4.9 ± 0.4; CpG plus OVA-treated mice, 6.1 ± 0.6).

Because a single treatment of CpG ODN alone was ineffective in reducing the manifestations consistent with asthma in this model, we speculated that a more prolonged treatment course and treatment with a combination of CpG ODN and OVA may be required to alter the established Th2-mediated responses; indeed, we have previously found that airway eosinophilia in this model persists for >7 days in untreated mice ($2.8 \pm 0.37 \times 10^6$ eosinophils at 6 h; $1.29 \pm 0.54 \times 10^6$ eosinophils at 24 h; $0.48 \pm 0.57 \times 10^6$ eosinophils at 7 days; $0.01 \pm 0.01 \times 10^6$ eosinophils at 14 days), supporting the need for a longer duration of therapy. Therefore, we next developed a model of allergen (OVA) immunotherapy in which mice with established airway disease were treated with four biweekly subcutaneous injections of saline (as an untreated control),

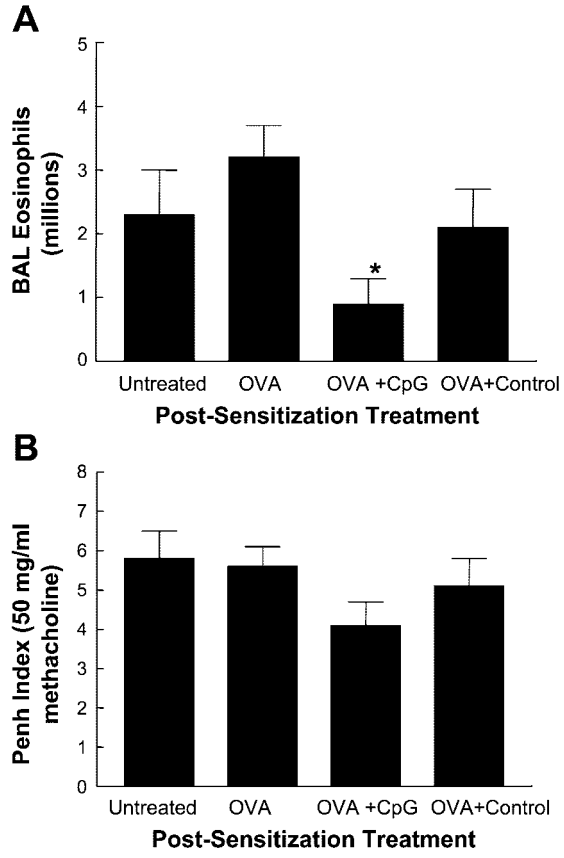

Fig. 2. Effect of CpG treatment between sensitization and airway challenge in prevention of airway eosinophilia and bronchial hyperreactivity in murine model of OVA-induced asthma. Mice were sensitized to OVA as previously described and then received treatment with CpG or control ODN or no treatment, before airway challenge with OVA. Administration of CpG but not control ODN prevented the subsequent development of bronchoalveolar lavage (BAL) eosinophilic inflammation (*A*), but not bronchial hyperreactivity (*B*). n = 8/group, $^*P < 0.05$ vs. untreated and control ODN-treated groups. Penh, enhanced pause.

OVA alone (10 μg), CpG ODN (30 μg), OVA plus CpG ODN, or OVA plus control ODN (30 μg); all mice were rechallenged with aerosolized OVA at the end of the protocol.

Untreated (control) mice developed marked eosinophilic pulmonary inflammation ($1.57 \pm 0.29 \times 10^6$ eosinophils, Fig. 3*A*). Mice treated with CpG ODN alone developed a significant decrease in eosinophilia as a percentage of the total cells [from 69.5 ± 6.6% in untreated mice to 41.7 ± 9.2% of bronchoalveolar lavage (BAL) cells, $P < 0.05$; macrophages increased concomitantly from 5.5 ± 0.38 to 25.2 ± 1.5%] but no significant reduction in total numbers of eosinophils ($1.23 \pm 0.40 \times 10^6$ eosinophils). No significant reduction was noted in either percent or total BAL eosinophils among the groups of mice treated with OVA alone ($1.57 \pm 0.29 \times 10^6$ eosinophils) or OVA plus control ODN ($1.07 \pm 0.42 \times 10^6$ eosinophils). Only those mice treated with OVA plus CpG ODN were found to have significant reductions in both BAL percent eosinophilia (5.67 ± 2.97%, $P < 0.005$) and total eosinophils ($0.11 \pm 0.04 \times 10^6$ eosinophils, $P < 0.005$).

Bronchial responsiveness to inhaled methacholine was assessed on all of these mice at three time points: before the first administration of OVA, after the first course of inhaled OVA, and at the end of the study before death. There were no significant differences among the groups of mice at baseline; after the first course of inhaled OVA, all mice developed bronchial hyperresponsiveness to inhaled methacholine (data not shown). At the end of the treatment period, however, the response of mice treated with CpG ODN plus OVA was significantly lower than that of the other groups of mice (Fig. 3*B*). Penh index at 50 mg/ml of methacholine was 6.24 for the saline-treated mice, 4.92 for the CpG-treated mice, 5.80 for the OVA-treated mice, 6.54 for the OVA plus control ODN-treated mice, and 2.62 for the OVA plus CpG ODN-treated mice. ($P < 0.01$ OVA plus CpG ODN vs. all other groups).

Evaluation of histopathological sections (Fig. 4) confirmed the effect of CpG ODN on inflammatory responses in the OVA murine model of asthma. A dense peribronchial cellular infiltrate was seen in untreated mice (Fig. 4*A*) as well as those treated with OVA (Fig. 4*C*) or OVA plus control ODN (Fig. *4E*). These re-

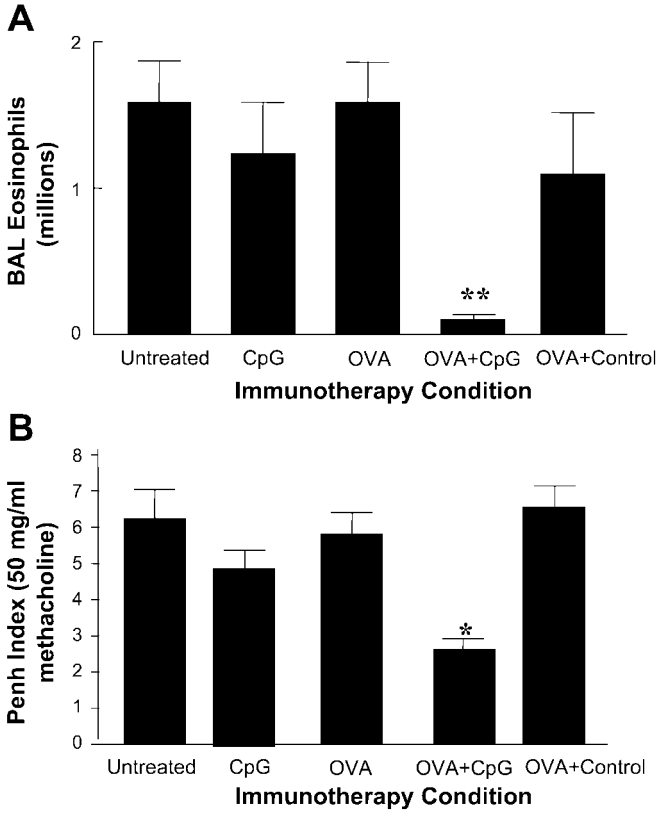

Fig. 3. Effect of antigen-based immunotherapy and CpG ODN on murine model of OVA-induced asthma. Mice were sensitized to and challenged with OVA as described in Fig. 1. Mice then were given saline injections (Untreated) or were treated with allergen immunotherapy in the absence (OVA) or presence of CpG (OVA + CpG) or control (OVA + Control) ODN or received CpG ODN alone (CpG). Treatment with OVA + CpG ODN resulted in significantly lower BAL eosinophilia (*A*) and bronchial hyperreactivity (*B*) compared with all other groups. n = 8/group, $^*P < 0.05$ and $^{**}P < 0.005$ vs. all other groups.

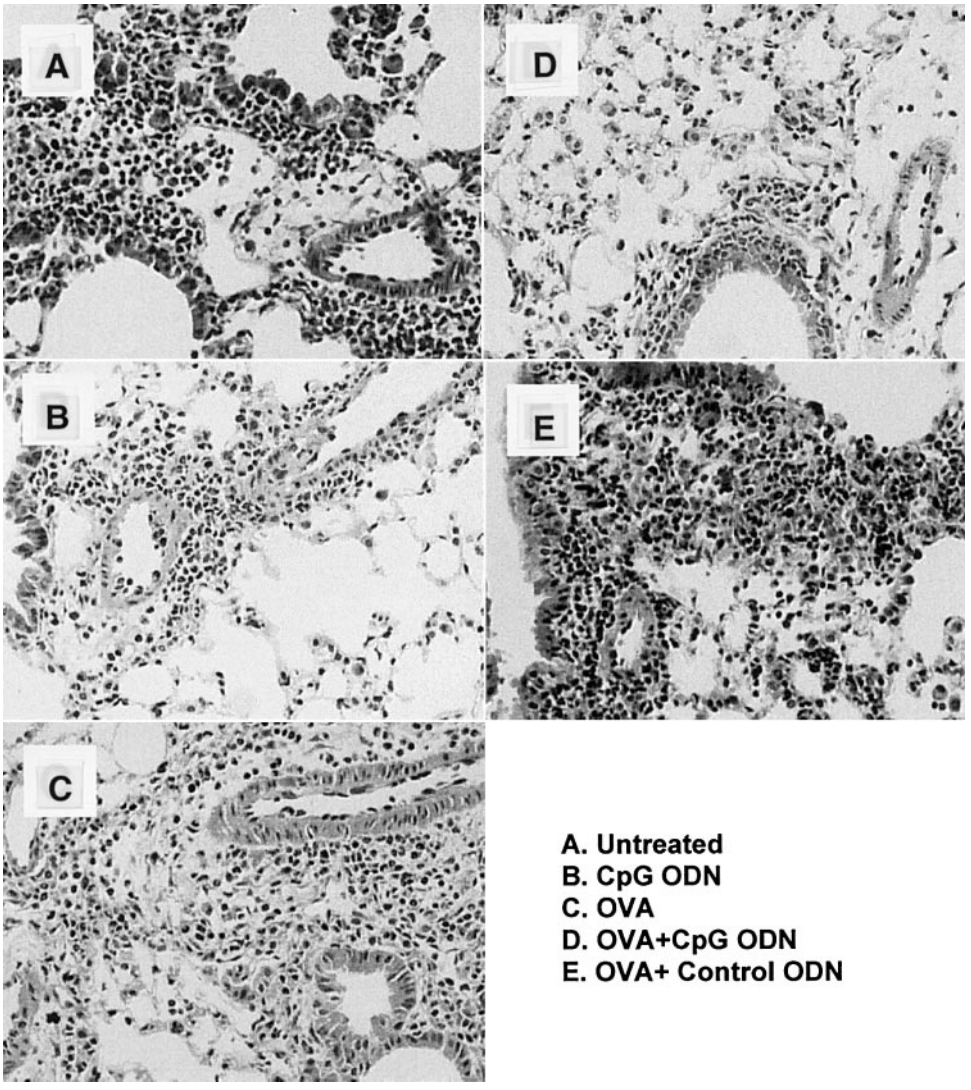

Fig. 4. Effect of immunotherapy on peribronchial eosinophilic inflammation in murine model of OVA-induced asthma. Mice were sensitized to and challenged with OVA before a course of immunotherapy. Compared with untreated mice (*A*), mice treated with CpG ODN alone (*B*) showed a moderate reduction in eosinophilic inflammation, and mice treated with OVA + CpG ODN (*D*) showed a substantial reduction in these responses. No marked improvement in inflammation was noted in those mice treated with OVA alone (*C*) or OVA + Control ODN (*E*). Sections photographed at ×40.

sponses were moderately reduced by treatment with CpG ODN alone (Fig. 4*B*) and substantially reduced in mice treated with OVA plus CpG ODN (Fig. 4*D*).

In previous studies (13), we demonstrated that CpG ODN can prevent the induction of IgE when administered before sensitization. We next evaluated the effect of immunotherapy on the induction of OVA-specific IgE. We found that IgE levels of mice treated with OVA plus CpG ODN were significantly lower (0.312 ± 0.079) than those in mice that remained untreated (0.945 ± 0.19), as well as mice treated with CpG ODN alone (0.713 ± 0.138), OVA alone (0.806 ± 0.143), or OVA plus control ODN (0.872 ± 0.122) (n = 8/group, $P <$ 0.01 vs. untreated, OVA-treated, and OVA plus control ODN-treated mice, Fig. 5).

To confirm whether the therapeutic effect of CpG ODN in immunotherapy was confined to OVA, a relatively weak allergen, we repeated these studies using the schistosome egg model of asthma, a much stronger allergen (13). In this model, we previously have determined that all sensitized and exposed mice develop significant airway eosinophilia and bronchial hyperresponsiveness by *day 14* (13). After biweekly immunotherapy with SEA, SEA plus CpG, or SEA plus control ODN, mice were rechallenged with two exposures to SEA by inhalation before death. Despite use of a stronger allergen and lower doses of CpG ODN, treatment with CpG plus SEA resulted in significantly reduced airway eosinophilia (Fig. 6). This was also associated with a significant reduction in bronchial hyperresponsiveness to inhaled methacholine only for the mice treated with SEA plus CpG ODN ($P < 0.05$). Penh index at 50 mg/ml of methacholine was 4.78 ± 0.9 for the saline-treated mice, 4.35 ± 1.3 for the SEA-treated mice, 2.28 ± 0.5 for the SEA plus CpG-treated mice, and 6.15 ± 1.4 for the SEA plus control ODN-treated mice.

We next sought to examine the role of altered Th1/Th2 balance in the protection against eosinophilic inflammatory responses offered by CpG ODN. For these studies, RNA extracted from the lungs of experimental mice was analyzed by RNase protection assay. These studies demonstrated a significant increase in transcripts for IP-10 and RANTES and a significant reduc-

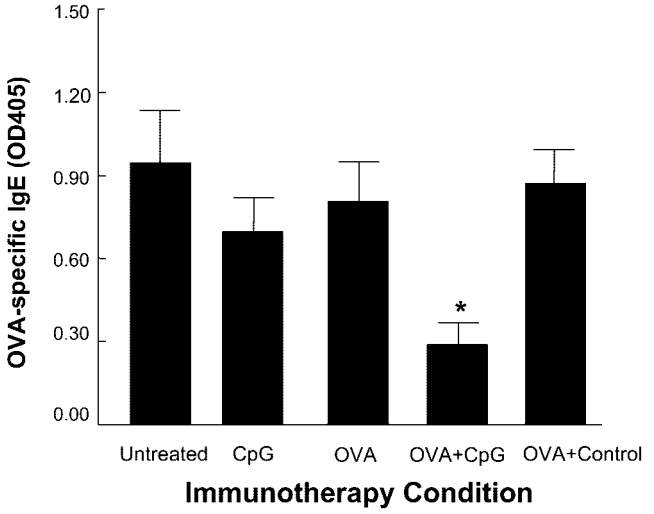

Fig. 5. OVA-specific IgE is reduced in mice that received CpG ODN. Immediately before death, mice underwent phlebotomy. Serum was separated and frozen and subsequently analyzed for OVA-specific IgE levels. Optical density (405 λ) readings of sera from mice treated with OVA + CpG ODN were significantly lower (0.312 ± 0.079) than those receiving saline (0.945 ± 0.19), OVA alone (0.806 ± 0.143), or OVA + Control ODN (0.872 ± 0.122). *$P < 0.01$ vs. untreated, OVA, OVA + Control ODN groups.

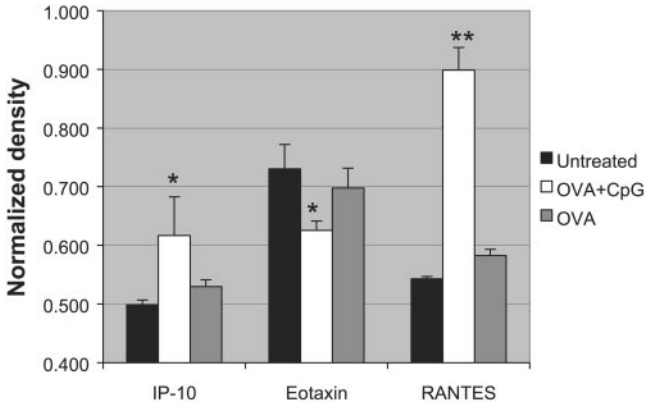

Fig. 7. RNase protection assay for detection of whole lung mRNA expression of chemokines showed induction of interferon-γ-inducible protein (IP)-10 and regulated upon activation normal T cell expressed and secreted (RANTES) and suppression of eotaxin in mice treated with OVA ± CpG immunotherapy. RNA was extracted from the lungs of mice killed after final airway challenge as shown in Fig. 1. RNAse protection assays were performed, and the density of signals was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to ensure equal loading of all lanes. Induction of IP-10 and RANTES and suppression of eotaxin were observed in samples from those mice that underwent immunotherapy with OVA + CpG ODN compared with untreated mice or with mice treated with OVA alone *$P < 0.05$, **$P < 0.01$ all comparisons, vs. untreated mice, $n = 4$.

tion in transcripts for eotaxin, in specimens from mice treated with OVA plus CpG compared with untreated mice or mice treated with OVA alone (Fig. 7). No significant changes were noted in the transcripts of the other mediators at the time point when the mice were killed (24 h after the final challenge).

To further examine the effect of CpG ODN on the Th1/Th2 balance, we next examined the effect of in vivo administration of CpG ODN on the in vitro release of the Th1 cytokine IFN-γ and the Th2 cytokine IL-5 by antigen-stimulated splenocytes. For these studies, mice were sensitized to OVA (by three weekly intraperitoneal injections) in the presence or absence of CpG ODN (30 μg). Splenocytes were then harvested and cultured for 72 h in the presence or absence of OVA (100 μg/ml). We found essentially no detectable IL-5 present in the supernatants of cells cultured in the absence of OVA; splenocytes from OVA-sensitized mice released significantly greater amounts of IL-5 than those from mice treated with CpG ODN at the time of sensitization [CpG (−) 609 ± 334 pg/ml; CpG (+) 68 ± 39 pg/ml, $n = 5$, $P < 0.01$]. Likewise, treatment with CpG ODN led to antigen-stimulated release of IFN-γ [CpG (−) 478 ± 85 pg/ml; CpG (+) 4,449 ± 751 pg/ml, $n = 4$, $P < 0.01$]. CpG alone, in the absence of antigen restimulation, did not lead to substantial induction of IFN-γ release in these studies, indicating that these responses were antigen specific. We next evaluated whether CpG ODN could convert established antigen-specific Th2 responses to antigen-specific Th1 responses. For these studies, we used splenocytes isolated from OVA-sensitized mice. These cells were stimulated with OVA (100 μg/ml) in vitro in the presence or absence of CpG ODN. We found that the response to stimulation with antigen in the absence of CpG ODN included release of both IL-5 (Fig. 8*A*, 140 ± 53 pg/ml) and IFN-γ (Fig. 8*B*, 686 ± 210 pg/ml); stimulation with increasing amounts of CpG ODN resulted in a significant decrease in OVA-induced IL-5 release (Fig. 8*A*, to 22 ± 12 pg/ml at CpG = 1.0 μg/ml, $P < 0.05$ vs. CpG = 0) and a significant increase in IFN-γ release, maximal at CpG of 0.1 μg/ml (Fig. 8*B*, 9,632 ± 335 pg/ml, $P < 0.001$ vs. CpG = 0). The discordance between maximal antigen-specific release of IFN-γ and maximal suppression of antigen-specific release of IL-5 was consistent and reproducible in multiple studies.

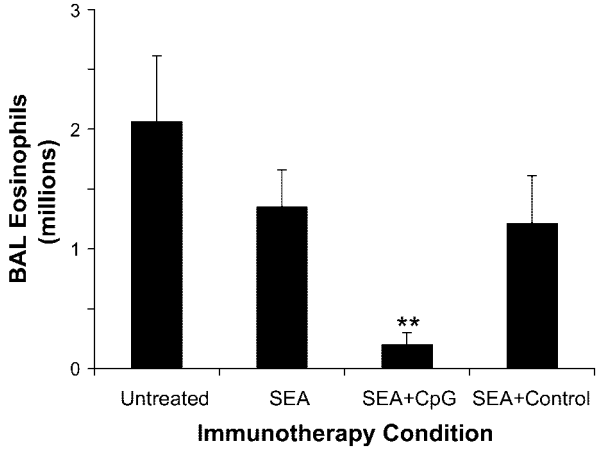

Fig. 6. Airway eosinophilia is reduced by immunotherapy with CpG using a schistosome egg murine model of asthma. Mice were sensitized to schistosome eggs and challenged with transnasal schistosome egg antigens (SEA); subsequently they received 4 biweekly treatments of SEA, SEA + CpG, SEA + Control ODN, or saline as a negative control. After rechallenge in the airway with SEA, mice were killed and evaluated for airway eosinophilia. **$P < 0.005$ vs. untreated, SEA, SEA+Control ODN groups.

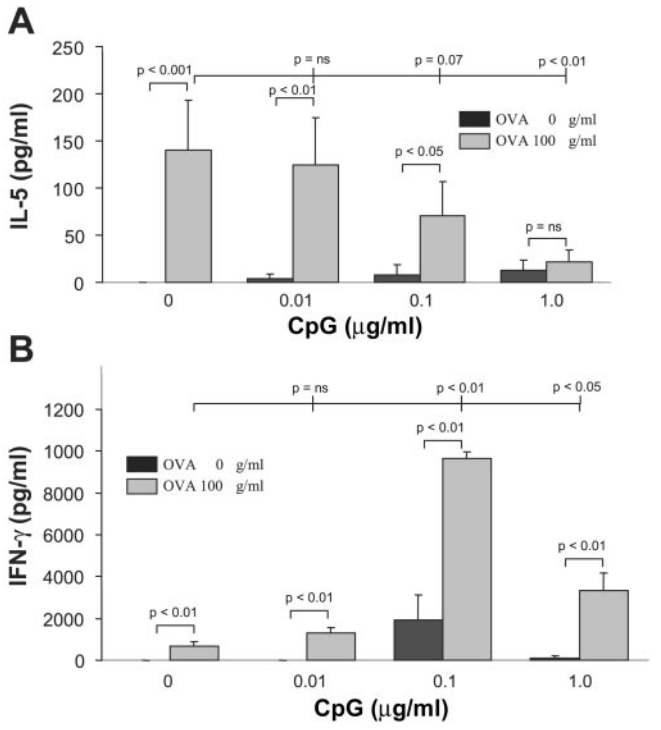

Fig. 8. Antigen-stimulated release of cytokines by splenocytes exposed to CpG ODN in vitro. Mice were sensitized to OVA (10 μg ip, weekly × 3 doses). One week after the third sensitizing dose of OVA, mice were killed; splenocytes were isolated from each mouse and cultured in the presence or absence of OVA (100 μg/ml) and CpG ODN (0–1.0 μg/ml). Supernatants were harvested after 72 h of culture. *A*: splenocytes cultured in the absence of OVA released minimal detectable interleukin (IL)-5. Splenocytes cultured in the presence of OVA released substantial amounts of IL-5; increasing concentrations of CpG ODN led to a dose-dependent reduction in release of this Th2 cytokine. *B*: splenocytes cultured in the absence of OVA released detectable concentrations of interferon (IFN)-γ when stimulated with CpG ODN at 0.1 μg/ml; this was reduced at the highest concentration of CpG ODN stimulation. Splenocytes cultured in the presence of OVA released significantly greater amounts of IFN-γ at all concentrations of CpG ODN; release of IFN-γ peaked at 0.1 μg/ml of CpG ODN. ns, Not significant.

To evaluate whether these observed changes were antigen-specific effects of CpG ODN, we carried out similar studies using splenocytes isolated from naïve mice. Naïve cells unstimulated or stimulated with increasing concentrations of CpG ODN released undetectable amounts of IFN-γ except after stimulation with 0.1 μg/ml CpG ODN (10,999 ± 1,571 pg/ml). In the presence of OVA, similar results were seen (except when stimulated with 0–1 μg/ml CpG ODN). These cells released very low concentrations of IFN-γ (538 ± 126 pg/ml unstimulated; 760 ± 143 pg/ml with 0.01 μg/ml CpG; 26,950 ± 4,083 pg/ml with 0.1 μg/ml CpG; 729 ± 295 pg/ml with 1.0 μg/ml CpG). Stimulation with Con A led to markedly elevated release of IFN-γ, which was nevertheless significantly increased by concurrent CpG ODN at the appropriate concentration (51,652 ± 3,561 pg/ml unstimulated; 54,813 ± 2,944 pg/ml with 0.01 μg/ml CpG; 65,965 ± 933 pg/ml with 0.1 μg/ml CpG; 52,284 ± 1,194 pg/ml with 1.0 μg/ml CpG). Concentrations of IFN-γ were similar when OVA was added to the culture conditions (data not shown). Essentially no detectable amounts of IL-5 were released from the naïve splenocytes in the presence or absence of OVA and CpG ODN. Stimulation with Con A did result in the release of modest levels of IL-5, which was partially suppressed by costimulation with CpG ODN (106 ± 15 pg/ml no CpG; 108 ± 15 pg/ml with 0.01 μg/ml CpG; 77 ± 13 pg/ml with 0.1 μg/ml CpG; 59 ± 10 pg/ml with 1.0 μg/ml CpG). Results were similar when OVA was added to the culture conditions (data not shown). From these data we concluded that *1*) CpG ODN at the ideal concentration of 0.1 μg/ml is capable of inducing significant levels of IFN-γ in the absence of an antigen-specific response; *2*) CpG ODN can suppress nonspecific (e.g., Con A induced) release of IL-5, although not to the same degree as it suppresses antigen-specific IL-5 release; and *3*) suppression of the Th2-driven responses seen in the recall response of cells from OVA-sensitized mice most likely has aspects of both antigen-specific and nonspecific effects of CpG ODN; it cannot be fully explained by induction of Th1 responses, such as IFN-γ release.

DISCUSSION

This report provides evidence, for the first time, that CpG ODN are capable of redirecting the immune response to antigen in animals with established airway eosinophilia. Mice who have been sensitized to OVA/alum have a Th2-type response when challenged with OVA by inhalation. Eosinophilic inflammation of the airways, nonspecific bronchial hyperreactivity, and induction of antigen-specific IgE antibody characterize this response. In vitro, antigen challenge to isolated splenocytes leads to release of Th2-type cytokines. Previously, we demonstrated that CpG ODN, administered at the time of sensitization to antigen, are capable of preventing the development of eosinophilic airway inflammation and bronchial hyperreactivity in a murine model of asthma. In this study we extend those earlier studies by showing that established atopic inflammation can be overcome by a combination of allergen and CpG ODN.

Although we intended to model allergen rush immunotherapy, using a short course of relatively high-dose allergen, we found that administration of the allergen alone was only moderately effective at diminishing airway eosinophilia, and we were unable to detect any reduction in OVA-specific IgE release. In addition, splenocytes from OVA-treated mice did not develop an antigen-specific Th1 phenotype. Mice treated with CpG ODN and OVA, on the other hand, had a marked shift toward a Th1 response to antigen, as well as reduction in airway eosinophilia, serum IgE, and bronchial hyperreactivity. The CpG ODN, then, may be efficacious through induction of Th1-specific lymphocytes that block, rather than promote, Th2-mediated eosinophilic inflammation. These findings suggest a potentially protective role for Th1-deviated lymphocytes in atopic airway disease. This is in contradistinction to the reports by Hansen and others (11) that Th1-mediated

responses in the lung can lead to substantial inflammation. In that study, however, adoptive transfer of highly activated Th1-specific lymphocytes was carried out, and the relevance of that model to immune responses seen in vivo is uncertain. In our current study, the degree of Th1 responses may be less than in the adoptive transfer model; indeed, the levels of Th1 cytokine production, although significantly increased from untreated mice, were not markedly elevated, nor were serum levels of these cytokines increased.

Others have confirmed that CpG DNA has a substantial and prolonged protective effect against the development of atopic inflammation. Broide et al. (2) found that CpG ODN, administered after sensitization but before airway challenge, could prevent the development of airway eosinophilia as effectively as 7 days of corticosteroids; this was associated with induction of a Th1 and inhibition of a Th2 cytokine response. Our results in this study concur. Sur et al. (37), using a murine model of ragweed-induced asthma, found that CpG ODN, given 48 h before allergen challenge, increased the ratio of IFN-γ- to IL-4-secreting cells and decreased allergen-specific Th2 responses. This protection lasted for at least 6 wk after treatment with CpG.

Shirota et al. (33) examined the role of CpG ODN conjugated to antigen and found that these conjugated compounds were effective in preventing the developing of antigen-specific inflammatory responses and that this effect was long lasting. In that study, the investigators noted that the conjugated ODN were more effective at preventing eosinophilic inflammation than a mixture of the antigen with CpG ODN. We elected to avoid conjugating the ODN, out of concern that this may allow the antigen to act as a hapten and lead to anti-DNA antibody formation. We agree with Shirota et al. that the combination of CpG ODN with an antigen powerfully deviates a Th2-type antigen-specific response to a Th1-type response. The combination is more effective at this than is the administration of CpG ODN alone. In earlier studies, we first showed that CpG is effective in preventing the development of inflammation when administered at the time of sensitization as well as subsequent to sensitization but before airway challenge (13). Our current study extends the observations noted by Shirota et al. in that reversal of well-established Th2-mediated eosinophilic lung inflammation is far more difficult, in an experimental setting, than prevention of its development.

More recently, Serebrisky et al. (31) found that CpG ODN can reverse allergic airway responses. In that study, the investigators administered CpG ODN only 24 h after each of two antigen challenges. That study differs from our model in that, in our current study, the eosinophilic inflammation was well established (10 days of inhaled OVA over 2 wk) before the initiation of therapy. Indeed, as in the Shirota study, it is likely that CpG ODN were administered in the absence of pulmonary eosinophilia, since in vivo recall antigen responses in the lung require at least two pulmonary exposures to develop eosinophilic infiltrates. Moreover, in our current study, we strongly rechallenged the treated mice (with an additional 2 wk of antigen inhalation) before death and analysis. Nevertheless, our studies and those of Serebrisky et al. are in agreement in that, even after the establishment of sensitization to allergen, CpG ODN can reverse the allergen-specific Th2 responses.

The role of IgE in allergic responses is in activating mast cells and basophils through cross-linking on their surface, which leads to degranulation and release of important inflammatory mediators. Peng et al. (26) recently reported that vaccination with CpG ODN can prevent the induction of IgE production by B cells, but not the suppression of previously induced IgE. Our current study shows that administration of CpG ODN and antigen, although not CpG ODN alone, leads to a diminished specific IgE response. One reason for the discrepant findings between our study and that of Peng et al. may be the dose and timing of the sensitizing antigen. In their model, mosquito saliva allergen was administered to mice by intradermal injection twice weekly for 10 doses; in the current study, a single dose of OVA in alum was used. Additionally, the immunotherapy arm in our current study was more vigorous, utilizing four injections of OVA, CpG ODN, or both. As in their study, however, we also found that treatment with CpG alone did not reverse the induction of IgE. Interestingly, transient skin responses were reported after the administration of intradermal CpG ODN to BALB/c mice by Peng et al. (26); we did not note any skin reactions in our protocol, which utilized C57BL/6 mice exclusively. Other studies have demonstrated that CpG ODN can induce IgG2a (4) as well as IgA, when administered via the mucosal route (8); we did not investigate other isotypes in this current study.

We (13) and others (2) have previously demonstrated that CpG ODN leads to the induction of Th1-type cytokines when used in conjunction with allergic murine models of asthma. In these current studies, we have described both antigen-specific and nonspecific effects of CpG ODN on reduction of Th2 and induction of Th1 responses. For the first time, we have demonstrated the lung-specific induction of the chemokines IP-10 and RANTES and suppression of eotaxin. CpG has previously been noted to induce the production of IP-10 (16, 38), which appears to be released by plasmacytoid dendritic cells and is associated with the induction of IL-12 (16) and IFN-γ (42). IP-10 appears to play a significant role in the control of Th1 responses; it drives the inflammatory response in Th1-type model disorders such as experimental autoimmune encephalomyelitis (7), is elevated in patients with Th1-mediated diseases, such as type I diabetes (32), and sarcoidosis (19), and promotes antigen-induced Th1 over Th2 responses in human in vitro assays (9). Using adenoviral-mediated gene transfer techniques, Wiley et al. (42) demonstrated that expression of IP-10 in the airways of Th2-polarized mice inhibits eosinophilic airway inflammation, reduces IL-4, and enhances IFN-γ expression. RANTES has been associated with allergic airway inflammatory changes in several settings: on the one hand, RANTES has been associated with asth-

matic eosinophilia (18), and blockade of RANTES receptors leads to significant reductions in lymphocytic and eosinophilic infiltration in OVA-induced airway inflammation (10), suggesting that induction of RANTES by CpG ODN might not aid in reducing inflammatory responses. On the other hand, RANTES is also expressed in Th1-mediated responses such as hypersensitivity pneumonitis, is induced after adoptive transfer of Th1 cells (10), and is thought to be a chemotactic for Th1 cells (35). This report of lung-specific RANTES induction by allergen immunotherapy supports the complex role of RANTES in modulation of allergic responses; RANTES has also been associated with the recruitment of neutrophils to the lung (25), and this may be responsible for the induction of neutrophils previously reported after pulmonary administration of CpG ODN (29, 43). The chemokine eotaxin is well linked with the asthmatic phenotype and has recently (27) been found to be central to IL-13-induced eosinophil recruitment.

To put these current studies in perspective, it is important to recognize the strengths and limitations inherent in utilizing a murine model of asthma. First, OVA is rarely an allergen in human disease. To enhance the generalizability of these findings, we elected to repeat some of the studies with the potent Th2-inducing antigens in schistosome eggs. Second, the Th1/Th2 dichotomy was first demonstrated in a murine system (20) and remains less clearly defined in humans. Indeed, the relevance of this balance is more evident in murine models of asthma than in human disease, and studies have demonstrated that Th1 cytokines, such as IL-12, are effective in reducing eosinophilic inflammation but not bronchial hyperresponsiveness (3). This may be a manifestation of human variability, as a dissociation between these aspects of phenotypic asthma appears to be common in patients (5). In mice, although some manipulations reduce eosinophilia but not airway responsiveness, other studies have demonstrated concordance among Th1/Th2 cytokine levels, airway eosinophilia, and airway hyperreactivity (30, 39, 40). In our previous studies, while we have demonstrated an association between CpG ODN-induced protection against cellular and physiological manifestations of asthma (13), we have also demonstrated that neither IFN-γ nor IL-12 is required for these effects (12). Finally, although mice demonstrate neither spontaneous bronchospasm nor allergen-induced degranulation of eosinophils (36), the OVA and other murine models of asthma are valuable for providing paradigms for the pathogenesis and treatment of allergic airway disease, which must then be proven useful in humans.

Our observations that CpG ODN were most effective in preventing aspects of atopic inflammation when administered in conjunction with antigen, rather than alone, suggested that CpG ODN may be an effective adjuvant for immunotherapy. Immunotherapy has been used to redirect patients' immune responses to allergen for decades; indeed, the first report of antigen-specific immunotherapy was administration of pollen to patients with hay fever, in 1911 (22). Despite continued reports of its success in treating allergic rhinitis (6) and other IgE-mediated disorders, immunotherapy as a treatment for asthma has waned. One reason for this decline has been the concern for the serious, potentially fatal, adverse effects, most notably anaphylaxis, that have been reported (17) to occur most commonly in asthmatic patients (17, 28, 41). The introduction of epitope-specific peptide immunotherapy (23, 24) has led to the promise of improved safety, with the elimination of epitopes that cause systemic responses, but the efficacy of these agents has been questioned (34).

Current thought on the management of asthma, as epitomized in the recent National Heart, Lung, and Blood Institute (NHLBI) asthma guidelines (21), has stressed anti-inflammatory therapy as the cornerstone of asthma treatment. The genesis of this approach is that reduction of inflammation should lead to diminished airway remodeling and thus less long-term asthma sequelae. Whether immunotherapy leads to the induction of blocking antibodies or to a shift from a Th2 to a Th1 milieu (1), when effective it clearly reduces allergen-induced inflammation. Moreover, immunotherapy (unlike currently available steroid and nonsteroidal anti-inflammatory regimens) offers the potential for long-term relief of symptoms and reduced adverse effects of atopic disease (6). The addition of CpG DNA as an adjuvant for immunotherapy may substantially enhance its usefulness for the management of allergic disease.

This study was funded by grants from the American Lung Association, NHLBI (HL-59324), and the Coley Pharmaceutical Group.

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