Eat Dirt

CpG DNA and Immunomodulation of Asthma

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Asthma is a disorder of increasing prevalence and severity that has been linked with reduced early-life exposure to microbes and microbial products. Populations with increased environmental exposures to pathogen-associated molecular patterns (e.g., children who have large numbers of older siblings, who were raised on farms, and who have earlier out-of-home day-care attendance) have fewer and less severe atopic disorders. The mechanism(s) responsible for these observations remain uncertain, but modulation by pathogen-associated molecular patterns of the inflammatory milieu (and thus the setting in which allergens may be encountered) has received strong support. One microbial product with marked immunostimulatory properties is bacterial DNA, which differs from mammalian DNA in the frequency of cytosine-guanine (CpG) dinucleotides; many of the effects of bacterial DNA can be recapitulated by oligodeoxynucleotides (ODNs) containing CpG in specific base sequence motifs (CpG ODNs). Because CpG ODNs induce Th1-type cytokines (which can suppress the Th2-type responses that cause many of the manifestations of allergic disease), we speculated that they may be useful in preventing or reversing the eosinophilic inflammation of atopic asthma. We found this to be the case, using murine models of incipient and established allergic asthma, but learned that the Th1-type cytokines were not critical for efficacy. Subsequent work has suggested that induction of regulatory-type responses (from T cells and antigen-presenting cells) is involved in the protection provided by CpG ODNs. Ongoing clinical trials are examining the utility of CpG ODNs alone and as an adjuvant for immunotherapy in human populations with atopic disease.

Keywords: asthma; hygiene hypothesis; immunotherapy; CpG oligodeoxynucleotides; T-regulatory cells

The prevalence and severity of asthma have markedly increased since the mid-20th century (1). The reasons for this rise remain unsettled; although air pollution is clearly linked to asthma exacerbations and morbidity (2), it seems to play a minor role, if any, in disease susceptibility (3). Important epidemiologic studies have provided strong evidence inversely linking the prevalence of atopic disorders, including asthma, with early-life exposures in a variety of settings. Children raised on farms (especially those who are exposed to the barnyard environment *in utero* and who drink unpasteurized milk) (4, 5), who have larger numbers of older siblings (6), or who attend group day care at an earlier age (7, 8) seem to be relatively protected against the current asthma epidemic that has been occurring, especially in developed nations, over the past several decades.

The “hygiene hypothesis” (9) links an increased risk of atopic disease with reduced microbial exposures during childhood. On

its face, this relationship is counterintuitive because respiratory viral infections can exacerbate and may even cause asthma and because bacterial infections are associated with worsening of airflow obstruction in chronic obstructive pulmonary disease, cystic fibrosis, and other clinical settings. How, then, could exposure to microbes be protective? Current understanding suggests that activation of the innate immune system by microbial products may be the answer to this question.

The innate immune system has evolved as the first-line protection against potentially harmful encounters with “alien” life forms. This ancient arm of the immune system provides rapid, though nonspecific, responses to potential pathogens (requiring no prior exposure) through a series of highly conserved pattern recognition receptors that bind microbial germline-encoded ligands. These ligands, pathogen/microbe-associated molecular patterns (PAMPs/MAMPs), are typically polysaccharides and polynucleotides that differ little within groups of organisms but are not found in the host. Engagement of the receptors generally leads to a rapid host response that activates innate and adaptive immune mechanisms, leading to elimination of the pathogen. Examples of these pattern recognition receptors include mannose receptors (which bind to terminal mannose groups on microbial glycoproteins, facilitating their endocytosis) (10), nucleotide-binding oligomerization domain proteins (which promote intracellular recognition of microbial peptidoglycans) (11), and the Toll-like receptors (TLRs). TLRs are highly conserved pattern recognition receptors that are related to the *Drosophila Toll* gene, which is important in defense against fungal infections. TLRs (10 of which are known in humans) are found on the cell surface and within the cell, where they facilitate recognition of and response to microbes and their components, such as endotoxin, bacterial flagellin, viral RNA, and bacterial DNA. Although engagement of each TLR activates a different specific molecular cascade, many induce the production of Th1-type cytokines, such as IL-12 and IFN- γ (12).

Induction of Th1 responses by microbial products was the first mechanistic explanation for the hygiene hypothesis. Atopic disorders are associated with a Th2 pattern of immune responses, characterized by elaboration of the cytokines IL-4, IL-5, and IL-13, among others (13, 14). These cytokines directly induce many of the manifestations of atopic inflammation, such as B-cell isotype switching to IgE production, eosinophil chemotaxis and activation, and airway-specific responses such as increased bronchial hyperreactivity. Because Th1 and Th2 responses are counterregulatory (15, 16), it was proposed that microbial products, such as endotoxin or bacterial DNA, that can induce Th1 responses may protect against unopposed or dysregulated Th2 responses and that a reduction of these exposures might lead to increased susceptibility to atopic asthma.

The likelihood that the beneficial effects of microbial exposures are due to “protective” Th1 responses has been reduced by several lines of evidence. First, epidemiologic studies have demonstrated that disorders mediated by Th1-type responses (e.g., type 1 diabetes, inflammatory bowel disease, and multiple sclerosis) have been increasing in much the same pattern, time-course, and geographic settings as Th2-driven atopic disease (17, 18); if

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a decline in Th1 responses to environmental agents were responsible for increased atopy and asthma, one would expect a reduction (or at least no increase) in Th1-linked diseases. Second, asthmatic inflammation is characterized by an increase in Th2 but not necessarily by a concomitant reduction in Th1 responses. Some studies have found increased IFN- γ expression during asthma exacerbations (19). Finally, animal studies suggest that airway and pulmonary inflammation induced by experimentally induced Th1 responses to antigen is at least as harmful as that engendered by Th2-driven atopic responses (20, 21) (and human Th1-associated lung disease can be seen in sarcoidosis and other granulomatous disorders), and normal nondiseased lungs and airways are characterized by a lack of inflammation, not a Th1 state. Thus, the protective results of hygiene-hypothesis-linked exposures are unlikely to result from a skewing of the Th1/Th2 balance.

An alternate mechanistic explanation for the hygiene hypothesis (22, 23) accounts for the parallel increases in Th1- and Th2-mediated disorders by linking reduced childhood exposure to microorganisms with a reduction in regulatory-type responses that are able to suppress Th1- and Th2-type inflammation. Several lines of evidence support the existence of constituent (naturally occurring) and inducible (adaptive) regulatory T cells, which have been classified on the basis of their expression of cell surface markers, cytokine production, and ontogeny (Th3, TR1, CD4⁺CD25⁺Foxp3⁺, etc.); regulatory effects are generally linked to the elaboration of IL-10 and/or TGF- β , although many of the identified cell groups also release other cytokines whose members may be Th1 or Th2 like (24, 25). Regulatory effects of non-T cells (e.g., dendritic cells [DCs]) have also been identified (26). These cells may be antigen specific in some cases, and their presence (or induction) has been linked with protection against the manifestations of atopic disorders. If early-life exposures to "nonhygienic" environments are required to generate a stable of effect regulatory cells, an absence of appropriate exposure may predispose to an imbalance between regulatory and effector (Th1 or Th2) cells, increasing disease susceptibility.

CYTOSINE-GUANINE DINUCLEOTIDE DNA: DISCOVERY AND POTENTIAL FOR THE TREATMENT OF ATOPIC DISEASE

More than a decade ago, Krieg (27) reported that bacterial DNA and synthetic oligodeoxynucleotides containing unmethylated cytosine-guanine (CpG) dinucleotides (found within bacterial and viral genomes in much greater frequency than in vertebrates) were strongly immunoactive, inducing proliferation and activation of B cells. Work that stemmed from this exciting discovery has identified many additional specific immune responses to CpG dinucleotide DNA and has elucidated the molecular pathways that link recognition of the CpG ligand with its effects.

TLR-9, the receptor for CpG DNA, is constitutively expressed by a limited number of immune cells in humans, most notably plasmacytoid DCs and B cells. CpG DNA enters cells by endocytosis (or may be released intracellularly after phagocytosis of a pathogen), binds TLR-9, and is translocated to the nucleus where it induces the activation of nuclear factor- κ B (12, 28). Early effects primarily lead to increased innate immune responses: B cells and plasmacytoid DCs are activated to release IL-10, type-I IFNs, IL-12, IFN-inducible protein-10, and other cytokines and chemokines, inducing a regulatory/Th1-oriented inflammatory milieu. Downstream responders to these signals include natural killer cells, T cells, and other cells, which amplify and modulate the immune response. Later effects include the induction of costimulatory receptors, immunoglobulin isotype switching by

B-cells, and the activation of a cascade of cellular responses promoting adaptive immune responses.

PREVENTION

Based on observations that CpG promoted Th1-type immune responses, we hypothesized that the administration of CpG DNA at the time of antigen sensitization would prevent subsequent atopic recall responses to the antigen. Using a schistosome egg antigen murine model of atopic asthma, we evaluated the effect of antigen inhalation in mice sensitized to schistosome eggs in the presence and absence of coadministered CpG oligodeoxynucleotides (CpG ODNs). We found that prophylactic vaccination of mice using CpG ODNs and allergen markedly suppressed subsequent development of allergen-induced eosinophilic airway inflammation, bronchial hyperresponsiveness, and serum IgE elevation (29). These findings have been confirmed by other groups (30, 31) and have resulted in efforts to develop CpG ODNs as a novel therapeutic agent for asthma. In addition, these studies serve to model how environmental exposures may modulate disease susceptibility, supporting the epidemiologic studies linking increased early-life exposure to "nonhygienic" environments with reduced prevalence and severity of atopic disease.

Because the effects of allergen exposure by patients with asthma are cumulative and because airway remodeling is an important contributor to asthma morbidity in nontreated and undertreated individuals, we examined the effect of CpG ODNs at the time of sensitization on changes consistent with airway remodeling in a chronic model of atopic asthma. For this study, mice were sensitized (in the presence or absence of CpG ODNs) to ovalbumin (OVA)/alum (by intraperitoneal injection) and subjected to thrice-weekly antigen inhalation exposure for 16 weeks. Mice pretreated with CpG ODNs developed significantly less subepithelial fibrosis, total lung collagen content, and goblet cell hyperplasia after chronic allergen exposure, consistent with a protection against airway remodeling (32). In addition, this was associated with reduced airway eosinophilia, airway hyperresponsiveness, and antigen-specific serum IgE levels to the inhaled antigen. Similar results have been shown in an allergic aspergillosis model of airway inflammation (33) and with chronic asthma induced by sensitization and repeated challenge with dust mite allergen (34).

THERAPY

Although demonstration of the ability to prevent sensitization in atopic asthma is an important step toward the development of a novel approach to asthma therapeutics, treatment of disease would be of greater potential clinical significance. We first evaluated whether CpG ODNs, administered by subcutaneous injection, could reverse established atopic asthma (35). After sensitization to and challenge with OVA, mice were treated with four biweekly administrations of OVA, CpG ODNs, or a combination of the two and then rechallenged with aerosolized OVA. Although neither OVA nor CpG ODNs alone significantly reduced the inflammatory or physiologic effects of the rechallenge, treatment with the combination markedly reduced airway eosinophilic inflammation, bronchial hyperreactivity, serum levels of OVA-specific IgE, and Th2 cytokine responses after *in vitro* allergen challenge of splenocytes. These protective effects were associated with induction of IFN-inducible protein-10 and RANTES (regulated upon activation, normal T-cell expressed and secreted) (and suppression of eotaxin) mRNA in whole lung extracts.

Because most asthma-relevant allergens are encountered by inhalation and because mucosal immunity has been speculatively

linked to protection from and susceptibility to allergic airway responses, we studied the effects of treatment with CpG ODNs and/or allergen immunotherapy administered via the airway mucosa (36). As with the systemic immunotherapy model, mice were sensitized to and challenged with OVA; subsequently, they received three biweekly treatments and were rechallenged with inhaled allergen. We compared administration of OVA, CpG ODNs, and the combination and evaluated these conditions in the presence or absence of "ambient" (spontaneously inhaled in a chamber) exposure to the allergen. In this case, although the administration of the combination immunotherapy most potentially reduced inflammation and physiologic changes, administration of CpG ODNs alone, in conjunction with spontaneous allergen exposure, was effective, whereas the allergen exposure alone worsened the same parameters. This suggested that airway mucosal administration of CpG ODNs in the setting of environmental allergen exposure may provide effective immunomodulatory treatment of atopic airway disease.

To explore whether this treatment induced allergen-specific responses or offered more generalized protection, we modified the model so that mice were sensitized to two allergens (OVA and either schistosome eggs or house dust mite). They were treated with OVA/CpG immunotherapy (or remained untreated) and challenged with the second allergen. We found that treatment designed to reverse OVA-induced allergic disease was effective in suppressing responses to a second, unrelated, allergen. Although in the case of the schistosome-sensitized animals eosinophilia was not significantly decreased (most likely because the eggs remain intraperitoneally, continually resensitizing the animals), dust mite-induced eosinophilia was reversed, and airway responses were improved in both cases. Thus, we found that although allergen is required for maximal CpG-induced protection, it induces nonspecific protective responses, suggesting an "active" regulatory or suppressive effect. These effects may be unique to the mucosal route of administration; others have reported that CpG ODNs function as strong mucosal adjuvants (37) and that CpG ODN-facilitated immunotherapy is significantly more effective via the intranasal rather than the intradermal route (38). We have also examined the efficacy of oral administration of CpG ODNs in established asthma (39) and found that although the dose of CpG ODNs required for prevention or therapy is an order of magnitude greater than for mucosal or systemic routes (with no formulation change), the oral route is also effective.

MECHANISMS

We initially hypothesized that CpG ODNs prevent atopic asthma by inducing a Th1 response that counterbalances and suppresses the Th2 inflammation responsible for most of its manifestations. Considerable evidence links TLR-9 ligation with enhanced Th1 responses, including IFN- γ production by natural killer cells and induction of type I interferons and IL-12 by plasmacytoid DCs. We found that, in concert with suppressed airway eosinophilia and airway hyperreactivity, bronchoalveolar lavage levels of IL-4 were suppressed with a modest reciprocal increase in IL-12 and IFN- γ (29). In subsequent studies (using gene knockout mice and blocking antibodies), however, we found that CpG ODNs required neither IFN- γ nor IL-12 to prevent asthmatic inflammation, and inhibition of allergic responses was only modestly suppressed in the absence of both key Th1 cytokines (40). This suggested that conversion from a Th2-type to a Th1-type response to antigen was not an adequate explanation for the protective effects of CpG ODNs.

Using an *in vitro* allergen rechallenge model of atopic responses, we found that CpG ODNs inhibited OVA-induced IL-5

release by splenocytes from OVA-sensitized mice in a concentration-dependent manner (41). Although IFN- γ and IL-12 were released by CpG-stimulated splenocytes, maximal induction of these Th1-type cytokines occurred at suboptimal concentrations for IL-5 suppression, and Th2 inhibition occurred in splenocytes from IFN- γ /IL-12 knockout mice. In contrast, IL-10 release from OVA-sensitized splenocytes was also induced by stimulation with CpG ODNs, and, in this case, in a concentration-dependent manner. There was a clear inverse correlation between IL-10 release and IL-5 suppression, suggesting the importance of this regulatory cytokine in mediating the effects of CpG-ODNs. In the absence of IL-10, CpG ODNs induced a much more vigorous Th1 response than otherwise seen, highlighting the complexity and interrelated immune effects of cytokine networks in the modulation of atopic inflammation.

In addition to the importance of IL-10 in mediating the protective effects of CpG DNA, other evidence points to a role for regulatory cells in preventing manifestations of atopic asthma. CpG DNA seems to modulate allergic responses by regulating activity of indoleamine 2,3-dioxygenase (IDO), the rate-limiting enzyme in catalysis of tryptophan to kynurenines; kynurenines are potent immunomodulatory molecules that regulate T-cell function. Although expression of IDO has been associated with suppression of Th1 responses by eosinophils (42), when induced by CpG DNA (or by the TLR-4 ligand, LPS) in DCs, it enhances T-regulatory (Treg) cell numbers and function (43) in a Th1-dependent manner. Induction of IDO in the lung, in response to TLR ligands, suppresses eosinophilic airway inflammation and Th1-driven pulmonary responses in a SCID/Th1 transfer model (44).

Tolerance to allergen in atopic airway disease has been linked to induction of regulatory cells. Regulatory cells have been demonstrably induced by CpG DNA in a number of different experimental settings. Stimulation with CpG DNA of human plasmacytoid DC/T-cell cocultures in the setting of mumps virus induces virus-specific memory CD4⁺ T cells that seem to be Th1-like regulatory cells (45). CD4⁺CD25⁺ Treg cells can be induced *in vitro* by coculture of human plasmacytoid DCs and naive T cells; these cells are hyporeactive to antigen rechallenge and inhibit proliferation of naive T cells in an antigen-nonspecific manner (46). In mice, induction of tolerance to transcutaneous administration of proteins is dependent on CD4⁺CD25⁺ regulatory T cells that are induced by CpG DNA (47). Suppression of experimental (dextran sulfate-induced) colitis by CpG ODNs is transferable by CD4⁺CD62L⁺ regulatory cells; this protection is not seen in TLR-9-deficient mice (48). We have found antigen-specific and nonspecific responses to immunotherapy with CpG and allergen in murine models of atopic asthma (36) and speculate that the induction of an active regulatory population is responsible for the latter effects.

Studies examining how CpG DNA affect the sequelae of chronic asthma have identified a number of mechanisms that may prevent airway remodeling. Mice treated with CpG DNA before repeated allergen inhalation challenge demonstrate reduced peribronchial angiogenesis, which is associated with reduced vascular endothelial growth factor (VEGF) expression by airway cells and lower VEGF levels in the airway fluid (49); this may be secondary to inhibited expression of IL-4 and IL-13, which promote VEGF production (50). The TLR-4 agonist endotoxin has been shown to up-regulate VEGF production by macrophages (51). The bronchial fibrosis that develops in chronic asthma is most likely due to enhanced expression of transforming growth factor (TGF)- β in the airway; TGF- β promotes collagen deposition by fibroblasts by increasing the production of extracellular matrix components and by suppressing their degradation, inhibiting collagenase expression and enhancing expression

of tissue inhibitor of metalloproteinase (52). CpG DNA reduces the expression of TGF- β 1 (possibly secondary to suppressed Th2 responses) and reduces the number of airway myofibroblasts (53). CpG DNA also directly inhibits airway epithelial expression of the matrix metalloproteinase (MMP)-9 in the setting of repeated allergen challenge and *in vitro* macrophage production of MMP-9, which probably contributes to its suppression of airway remodeling (54). Finally, reduced goblet cell metaplasia/hyperplasia (32, 53) may be secondary to inhibited Th2 responses, with suppression of IL-9 release (55), a strong inducer of *Muc5* (Figure 1).

HUMAN EXPERIENCE

Early data from clinical trials of CpG ODNs in atopic disorders have been promising; most of the available literature has focused on ragweed-induced allergic disease. In allergic rhinitis, conjugation of ragweed pollen allergen (Amb a1) with a CpG ODN (AIC, Amb a1/immunostimulatory DNA conjugate) induces an *in vitro* Th1 response from peripheral blood mononuclear cells of ragweed-allergic patients, in contrast with the Th2 response to stimulation with Amb a1 alone (56). Protective antibody responses are directly proportional to the number of oligonucleotides conjugated to the allergen (57). Treatment of ragweed-allergic subjects with escalating doses of AIC reduced *ex vivo* ragweed-specific Th2 responses (IL-5, CCL17, and CCL22 but

not IL-13) and transient increases in IFN- γ , CXCL9, and IL-10 (58). In a separate study, a short course (six escalating doses) of immunotherapy with AIC moderately suppressed (compared with placebo) allergy symptoms but significantly suppressed postseasonal increases in nasal eosinophilia in ragweed-allergic subjects (59). More recently, a randomized, double-blind, placebo-controlled phase 2 trial of AIC in ragweed-allergic adults (six weekly injections of AIC or placebo) found that the treatment was safe and effective (60); significant improvement in peak-season rhinitis and daily nasal symptom scores and midseason overall quality-of-life scores were seen in the AIC-treated group in the first season. This brief course of therapy resulted in prolonged protection, with significant peak-season rhinitis daily nasal symptom diary scores also seen the following season, with no additional treatment. A transient increase in Amb a1-specific IgG antibody (first season) and a suppression of Amb a1-specific IgE antibody (both seasons) was also seen.

Inhalation therapy of patients with atopic asthma with a CpG ODN preparation led to a modulation in the airway immune milieu but not in airway physiologic responses (61). Subjects were randomized to receive placebo or CpG ODN for 4 weeks and then subjected to allergen inhalation challenge. The group treated with CpG demonstrated increased expression of IFN- γ and IFN- γ -associated genes (e.g., IFN- γ -inducible 10-kD protein, monokine induced by IFN- γ , IFN-stimulated gene 54, and monocyte chemotactic protein-1 and -2) from sputum and

Figure 1. Mediators of acute and chronic airway inflammation in atopic asthma and effect of CpG DNA. In this simplified illustration, environmental exposure of sensitized individual to aeroallergen induces IL-4 release from mast cells; antigen presentation by antigen-presenting cells (APCs) in the setting of IL-14 promotes proliferation and activation of Th2-committed cells. Th2 cytokine products activate eosinophils, basophils, and B cells and induce goblet cell hyperplasia/metaplasia. Products of mast cells, basophils, and eosinophils promote epithelial denudement, myofibroblasts hyperplasia, extracellular matrix/collagen production and organization, and angiogenesis. CpG DNA induces Th1 and T-regulatory (Treg) responses that are primarily (in humans) mediated via plasmacytoid dendritic cells and B cells. These cells suppress the activation of Th2 cells, preventing (or reversing) acute eosinophilic inflammation and subchronic/chronic remodeling of the airways. NK = natural killer; TGF = transforming growth factor; VEGF = vascular endothelial growth factor.

peripheral blood mononuclear cells after allergen challenge, but no reduction in airway hyperresponsiveness nor in allergen-induced airway eosinophilia was seen (61). The authors speculate that dosage and timing considerations may account for these divergent effects; other studies are ongoing to investigate therapy of allergic asthma by CpG ODNs alone and conjugated to allergen.

CONCLUSIONS AND FUTURE DIRECTIONS

Immunomodulatory therapies are the focus of considerable attention in the treatment of a variety of disorders, ranging from cancer to autoimmune diseases. The hygiene hypothesis suggests that the current epidemic of atopic disorders may be due to a reduction, in modern life, in exposure to microbes and their products. One such microbial product is bacterial DNA, the effects of which are recapitulated by oligonucleotides containing immunostimulatory CpG motifs. Preclinical and early clinical studies provide support for the use of CpG ODNs as a novel approach to therapy and prevention of atopic diseases. A number of important questions remain:

- What cell(s) is/are required for the therapeutic effects of CpG ODNs in atopic disease?
- Are regulatory effects induced by CpG ODNs confined to a specific class of cells, or are the effects mediated through antigen-presenting (e.g., B cells, DCs) and lymphocytes (regulatory T-cell populations)?
- Are the antigen-nonspecific effects sufficient to warrant the use of CpG ODNs as “monotherapy” for asthma, or would it be more practical to consider this class an adjuvant for immunotherapy or “vaccine” approaches to disease prevention?

Ongoing and future research is needed to address these questions.

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