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Immunotherapy of asthma using CpG oligodeoxynucleotides

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Abstract Asthma and other atopic disorders have increased in prevalence and severity over the past three decades. Reduced risk of atopic disease associated with early life exposure to infections and microbes has raised the possibility that pathogen-associated molecular patterns (PAMPs) may confer protection against allergic disorders, a concept that has been named the ''Hygiene Hypothesis''. This relationship is most likely mediated through the induction of specific patterns of anti-atopic immune responses that follow engagement of innate immune mechanisms. Bacterial DNA is one such immunostimulatory microbe-associated ligand, whose properties can be mimicked by oligodeoxynucleotides (ODN) containing unmethylated cytosine-guanine dinucleotides in specific base sequences (CpG motifs), motifs characteristic of prokaryotic DNA that have been suppressed in eukaryotic DNA. Based initially on observations that CpG ODN induced Th1-type patterns of immune responses, we proposed that CpG ODN might represent a novel therapeutic strategy for the prevention and treatment of atopic disorders. Current understanding suggests multiple mechanisms of action of CpG ODN, but our initial hypothesis has been supported by extensive studies demonstrating, in animal models, efficacy in both incipient and established atopic asthma. These preclinical studies are now being translated into clinical trials exploring this new approach to immunotherapy for atopic disease.

Keywords Asthma · Hygiene Hypothesis · Immunotherapy · CpG oligodeoxynucleotides · T-regulatory cells

Asthma, the Hygiene Hypothesis, Th1/Th2 balance, and CpG DNA

Asthma is a clinical syndrome characterized by variable airflow obstruction, bronchial hyperresponsiveness, and airway inflammation [1]. Its prevalence and severity have markedly increased in the past three decades, especially in modern, industrialized societies.

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Underdeveloped or third-world nations have been relatively spared this epidemic, as are certain populations. Among other groups with reduced risk are included: individuals raised in rural, agricultural settings with an early-life exposure to barns [2, 3]; late birth-order children from larger families [4], and those who enter day-care at an early age [5]; and survivors of certain non-respiratory infections [6]. Children are at highrisk (e.g., with atopic parents) for developing asthma are somewhat protected if they grow up with a dog in the house [7], and children who are raised in parasite-endemic areas demonstrate increased rates of asthma if they are de-wormed [8, 9]. These observations and others suggested a link between childhood infections (e.g. illnesses brought home from school by an older sibling) and microbes (e.g., those found in high concentrations in agricultural settings) and resistance to atopic disorders.

The linking of reduced susceptibility to asthma with early life exposures to pathogens or microbial products was initially puzzling, as infections and the resulting inflammation have long been associated with asthma exacerbations rather than prevention. Termed the ''Hygiene Hypothesis [5],'' such epidemiologic observations are now understood as representing the result of engagement, or lack of engagement, of ancient defense mechanisms that comprise an important arm of the innate immune system. Atopic disorders are characterized by a skewing of immune responses toward a Th2 pattern; Th2 cytokines promote eosinophilia, class switching of B-cells to the production of IgE antibodies, goblet cell metaplasia and airway mucus hypersecretion, and airway hyperreactivity among other effects. Although the fetal immune system is typically Th2-oriented [10], newborns ordinarily exhibit a rapid decline in this tendency; one hypothesis is that early-life exposure to microbial products induces a Th1 milieu, and since Th1 and Th2 responses are counterregulatory, suppression of Th2 activity may result. It would follow, therefore, that this suppression does not occur (perhaps resulting in a life-long tendency towards Th2 responses to otherwise innocuous antigens) in the absence of early life infections or related exposures, providing an immunologic basis for the Hygiene Hypothesis.

How exposure to microbes or their products (PAMPs, Pathogen-Associated Molecular Patterns) might promote such a profound immunologic shift has been the subject of intensive study over the past 15 years. Pattern recognition receptors have evolved to aid in the crucial distinction between ''self'' and ''non-self,'' or ''danger;'' examples of this class include mannose receptors (that bind to terminal mannose groups on microbial glycoproteins, facilitating their endocytosis), nucleotide-binding oligomerization domain proteins (NODs, that promote intracellular recognition of microbial peptidoglycans), and the Toll Like Receptors (TLRs). TLRs are highly conserved receptors originally identified in drosophila that share both structural and functional characteristics. TLRs are found both on the cell surface and within the cell, where they facilitate recognition of and response to microbes and their components, such as endotoxin (TLR-4), bacterial flagellin (TLR-5), viral RNA, (TLR-3, -7, -8) and bacterial DNA (TLR-9). Downstream of ligand/TLR engagement are cascades that induce the transcription of cytokines, maturation of inflammatory cells, and ultimately engagement of both additional innate and adaptive immune mechanisms.

Well before identification of the TLR system, it was recognized that bacterial DNA is immunostimulatory. Initial explanations for this property centered on a need for palindromic sequences, perhaps allowing auto-dimerization. More than a decade ago, Dr. Arthur Krieg first reported that the cytosine-guanine dinucleotide (known, because of the phosphate bond, as CpG), in specific base sequences (CpG motifs) in bacterial DNA, were immunostimulatory and capable of strong B-cell activation [11]. Prokaryotic DNA contains the expected frequency of CpG dinucleotides (1:16 base pairs) which are suppressed

in eukaryotic DNA (1:50–1:100 basepairs); moreover, when present, the cytosine in eukaryotic DNA is often methylated, which silences or reduces the immunostimulatory properties of the motif. Subsequent studies demonstrated that synthetic oligodeoxynucleotides (ODN) centered on CpG motifs (CpG ODN) recapitulated the patterns of activation induced by bacterial DNA, setting the stage for the use of CpG ODN as immunotherapeutic agents.

CpG oligodeoxynucleotides and prevention of asthma

Although current understanding of the immune effects of CpG DNA suggest a complex, redundant, and interactive series of responses, early observations included the induction of a strong Th1-type pattern of inflammation. Among the cytokines induced by CpG DNA are type I Interferons (IFN-α and IFN-β), IFN-γ, and IL-12 [12, 13]. Since, these cytokines can suppress Th2 responses in vitro, we hypothesized that CpG ODN may provide a similar effect in vivo. Soon after Krieg's initial report [11], we began to examine the effects of CpG ODN on manifestations of atopic asthma using a murine model.

Initial studies utilized a model in which animals were sensitized to schistosome eggs by intraperitoneal injection (a model of allergic inflammation that requires no additional adjuvant) and then challenged by transnasal administration of soluble schistosome egg antigen (SEA, 7 and 14 days after sensitization) [14]. This model induces both pulmonary (airway eosinophilia, bronchial hyperreactivity) and systemic (elevated serum IgE levels, Th2 cytokine production) manifestations of atopic asthma. To explore the influence of CpG ODN on this system, we administered the ODN (30 mcg; in later studies, we found benefits with greater than one log reduction in dose) along with the schistosome eggs [14]. This treatment markedly altered the response to airway challenge, with reductions in all parameters studied; airway eosinophilia was reduced by more than 80%, serum IgE by nearly 60%, and bronchial hyperreactivity to inhaled methacholine to about one third of the level without treatment.

In addition to these classic markers of atopic asthma, we also found that in vivo treatment with CpG modulated the cytokine milieu within the lungs. Bronchoalveolar lavage levels of IL-4 were suppressed and those of IFN-γ and IL-12 increased in mice treated with CpG ODN, in comparison with mice challenged with allergen alone. Subsequent studies demonstrated that these responses were mirrored elsewhere in the mice; in vitro allergen rechallenge of splenocytes demonstrated a similar shift towards Th1 and away from Th2 responses [15].

As many of the clinical sequelae of chronic asthma relate to airway remodeling, we developed a model of repeated allergen inhalation challenge to determine the effects of CpG ODN on these parameters [16]. In this model, mice were sensitized to OVA/alum, in the presence or absence of CpG ODN (only during sensitization); all mice were then subjected to 6 weeks of thrice-weekly OVA inhalation exposure. In addition to the parameters modulated in the acute models of atopic disease, CpG-treated mice in this protocol also significantly demonstrated less goblet cell hyperplasia and subepithelial fibrosis, cardinal features of airway remodeling in asthma patients as well as in animal models. These experiments demonstrated that immunomodulatory effects of CpG ODN are longlasting as well as potent.

Our initial studies of the effects of CpG ODN utilized a common route, systemic administration via intraperitoneal injection. Since exposure to allergen significantly occurs by inhalation, we chose to examine whether administration of CpG ODN to the airway

mucosa was capable of preventing manifestations of allergic asthma [17]. Mice were sensitized as usual by intraperitoneal exposure to OVA/alum, and then later challenged by serial inhalations of allergen. CpG ODN was administered by nasal inhalation during sensitization and/or around the time of inhalation challenge. We found that administration within three days of sensitization led to marked reduction in the response to antigen inhalation challenge, but the administration of CpG ODN alone at the time of inhalation had only modest suppressive effects. These results demonstrated that the mucosal immune system in the airway is quite capable of modulating systemic immune responses, and suggested that inhalation may be an effective route of administration of immunomodulators to treat atopic disease.

Besides inhalation of aeroallergens, ingestion is the second most frequent route of exposure to allergens among affected patients. Like the airway, the gastrointestinal tract has an extensive network of immune responders, including GALT (gut-associated lymphoid tissue) such as Peyer's patches, and the sub-/intra-laminal lymphoid aggregations found throughout the intestines. We found that oral administration of CpG-ODNs around the time of sensitization, suppressed antigen-induced airway responses in a dose-dependent manner. The amount of CpG ODN required for effect was more than a log greater than that needed using systemic or transnasal administration, but at a dose of 1,000 μg, atopic responses (airway eosinophilia, bronchial hyperresponsiveness) were nearly completely abrogated [18]. Interestingly, oral CpG ODN appeared much less effective at preventing induction of Th2-type immunoglobulin responses (antigen-specific IgE, IgG1) than other routes of administration, but did induce Th1-associated IgG2a.

CpG ODN and immunotherapy of established asthma

Establishing that exposure to CpG ODN at the time of allergen sensitization prevents the development of manifestations of atopic asthma provides a plausible mechanism to explain the observations that led to the Hygiene Hypothesis. The clinical relevance of models of prevention, however, is far less than that of those, which serve as proof of concept for therapeutic approaches. We were very interested, therefore, in determining whether CpG ODN may serve to reverse the manifestations of established atopic asthma. To answer this question, we first explored the effects of systemic administration of CpG ODN in established atopic asthma. Mice were sensitized to, and then challenged with inhaled OVA; after establishment of the atopic airway inflammation, some mice remained untreated while others were treated (by subcutaneous administration) with OVA alone, CpG ODN alone, or a combination of OVA and CpG ODN [19]. After four biweekly treatments, mice were rechallenged with OVA inhalation, and then sacrificed.

We found that mice treated with CpG ODN alone demonstrated no significant change in airway eosinophilia (from $1.57 \pm 0.29 \times 10^6$ to $1.23 \pm 0.40 \times 10^6$ eosinophils), but those treated with OVA and CpG ODN demonstrated nearly complete reversal of the eosinophilic inflammation ($0.11 \pm 0.04 \times 10^6$ eosinophils, $P < 0.005$). Likewise, bronchial responsiveness to inhaled methacholine was only decreased in the mice subjected to this combination immunotherapy (Penh Index at 50 mg/ml of methacholine—the fold increase over baseline Penh—was 6.24 for the untreated mice and 2.62 for the mice treated with OVA and CpG ODN-treated mice, $P < 0.01$). OVA-specific IgE was reduced by about 2/3 in this group as well. Similar responses were also seen in alternate models of immunotherapy (mouse strains tested included C57 BL/6, BALB/c, and He/J; allergens used included, in addition to OVA, schistosome eggs and the house dust mite antigen *der P*).

The difference in ability to reverse established disease between CpG ODN alone or in concert with allergen were striking regardless of the model system utilized for these studies.

We next studied whether mucosal administration of CpG ODN was also capable of reversing established airways disease [17]. As with the systemic immunotherapy model, OVA-sensitized/challenged mice were treated (by three biweekly transnasal administrations) with CpG ODN, OVA, or a combination of ODN and allergen. Since, our studies of systemic administration of CpG-based immunotherapy suggested the need for antigen along with the CpG ODN, we also evaluated, whether, spontaneously inhaled OVA (administered around the time of treatment) would suffice for the antigen requirement, or whether, a higher dose of antigen (administered directly to the nasal mucosa along with the ODN) was needed. We found that, while administration of the combination immunotherapy was most effective at reversing airway inflammation and bronchial hyperreactivity, the CpG ODN alone, in conjunction with spontaneous allergen exposure, was also quite effective; in contrast, the spontaneous allergen exposure alone actually worsened the same parameters rather than serving to tolerize the mice. Again, CpG ODN administered to mice that had no other exposure to the allergen was only modestly effective at reversing the asthma manifestations. These results suggest that administration of CpG ODN to the airway mucosa, in the setting of environmental allergen exposure, may be an effective route for immunomodulatory treatment of atopic airway disease.

Because most allergic patients are not mono-sensitized but rather demonstrate allergic responses to a wide array of aeroallergens, we next chose to study, whether the protection provided by CpG ODN and allergen was specific to that allergen or rather provided a broader protection. To examine this question, we modified our immunotherapy model so that mice were sensitized to two allergens (OVA and either schistosome eggs or house dust mite). After sensitization, all mice were challenged with inhaled OVA, then treated with OVA and CpG immunotherapy (or remained untreated), and finally challenged with the second allergen. We found that treatment of mice with OVA and CpG ODN also effectively suppressed responses to a second, un-related, allergen. Although in the case of the schistosome-sensitized animals eosinophilia was not significantly decreased (most likely because the eggs remain intraperitoneally, continually re-sensitizing the animals), dust mite-induced eosinophilia was reversed, and airway responses were improved in both cases. Thus, although allergen and CpG ODN together are required for maximal protection against rechallenge in established atopic disease (although the mice may be exposed to the allergen by spontaneous exposure), the protection engendered is effective against other non-related allergen sensitization as well. This broad protection suggests an ‘‘active’’ regulatory or suppressive mechanism of action rather than the induction of allergen-specific tolerance.

Mechanisms of action of CpG ODN in atopic disease

TLR-9, the mandatory receptor for CpG DNA, is one of a family of innate immune receptors. Constitutively expressed by only a limited number of immune cells in humans (plasmacytoid dendritic cells and B-cells), this receptor is more broadly distributed in other mammals. CpG DNA undergoes endoctyosis, binds to TLR-9, and is translocated to the nucleus where it induces activation of NFκ-B, resulting in a series of downstream immune responses. Early effects mostly involve the innate immune system: B-cells and plasmacytoid DCs are activated to release IL-10, type-I IFNs, IL-12, IFN-inducible protein-10

(IP-10) and other cytokines and chemokines, inducing a regulatory/Th1-oriented inflammatory milieu. Downstream responders to these signals include NK-cells, T-cells, and other cells, which amplify and modulate the immune response. Later effects include induction of costimulatory receptors, immunoglobulin isotype switching by B-cells, and activation of a cascade of cellular responses promoting adaptive immune responses.

Based on the literature, our initial hypothesis was that CpG ODN would be an effective immune modulator in atopic disease through induction of Th1-type immune responses that would, in turn, suppress the development or maintenance of Th2-type inflammation. This was in accordance with the reports of induction of IFN-γ from NK-cells [12], IFN-γ (and IL-12 from lymphocytes [13], and IL-12 from antigen presenting cells [20], resulting in marked adjuvant activity promoting Th1 responses [21]. Indeed, our initial studies supported this postulated series of events, with CpG ODN treatment resulting in reduced Th2 cytokines both in pulmonary compartments and systemically, as well as promoting the induction of Th1 cytokines (IFN-γ and IL-12) by allergen rechallenge [14].

We soon recognized, however, that induction of the classic Th1 cytokines (e.g., IFN-γ and IL-12) was not required for the protection offered by CpG ODN against the development of atopic inflammation; using cytokine gene knockout mice and anti-cytokine blocking antibodies, we found that CpG ODN confers protection against both airway eosinophilia and bronchial hyperreactivity in the absence of either or both cytokines [22], although there is a resulting shift in the dose-response curve. Likewise, CpG ODN effects were preserved in the absence of Type I interferon signals (using IFN-α-R KO mice) and IL-18 (using blocking antibodies). Likewise, in vitro studies confirmed that CpG ODN can suppress antigen rechallenge-induced Th2 responses by splenocytes isolated from OVA-sensitized mice in a concentration-dependent manner [15]; induction of Th1 cytokines, however, was maximal at lower concentrations of CpG ODN, suggesting that their induction was coincident to, but not causal for, the anti-Th2 responses. In contrast to the Th1 cytokine responses, CpG-induced IL-10 [23] inversely correlated with IL-5 suppression. In the absence of IL-10, CpG ODN induced a much more vigorous Th1 response, and CpG-induced protection was significantly (although incompletely) blocked in the absence of IFN-γ, IL-12, and IL-10. CpG ODN appear to engage both cytokine-mediated (e.g. IFN-α, IFN-γ, IL-12, IL-10) and cell-mediated (NK activity, regulatory cell responses) mechanisms in their effects.

Conclusions and future directions

CpG ODN are potent immunomodulatory agents that are derived from initial observations of the immune effects of bacterial DNA. As one arm of the innate immune system, responses to CpG ODN provide a framework for understanding the Hygiene Hypothesis, though the true explanation for the observed relationship between reduced exposure to PAMPs and increased susceptibility to atopic disorders likely involves multiple ligands and their receptors. CpG ODN currently are being studied for therapeutic effects in a range of disorders, from infectious diseases to cancer. Preclinical studies (and early clinical experience) suggest that CpG ODN has a high potential for development as novel therapeutic agents to treat allergic asthma and related disease. Characterized by strong Th2 inflammatory responses to otherwise innocuous environmental agents, atopic disorders appear to respond to CpG ODN with marked suppression of inflammation and other characteristics of the clinical disorders. This protective effect can be seen both in the setting of primary

prevention as well as in counteracting established disease. Although the initiating mechanisms (engagement of TLR9) and the final sequelae (suppression of eosinophilic inflammation and related events) are clear, the intervening steps remain uncertain. Ongoing preclinical and clinical studies will help to identify both the relevant mechanisms of action of CpG ODN as well as their appropriate positioning as immunotherapeutic agents.

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