

Immune Modulatory Oligonucleotides in the Prevention and Treatment of Allergen-Induced Eustachian Tube Dysfunction in the Animal Model

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This article reviews the current literature investigating the applications and success of immune modulatory oligonucleotides as immunotherapy to treat and prevent allergen-induced eustachian tube dysfunction in animal models. Synthetic DNA-based immunotherapy agents composed of unmethylated cytosine–guanine dinucleotides (CpG ODNs) that bind to Toll-like receptors have been found to have tremendous potential as therapeutic agents and adjuvants. CpG ODNs can induce a shift in the cytokine profile and immune response that favors the T-helper type 1 pathway and suppresses the T-helper type 2 pathway. This makes CpG ODNs promising candidates for treating allergic diseases. Current CpG ODN studies have demonstrated prevention and treatment of acute allergen inflammation of the eustachian tube in an animal model of otitis media. Immune modulatory oligonucleotides in immunotherapy, administered systemically or topically, have been shown to be safe and effective in the animal model.

Introduction

Otitis media with effusion (OME) is defined as chronic inflammation of the middle ear mucosa with presence of fluid in the middle ear. Its etiology is multifactorial. Factors such as eustachian tube dysfunction (ETD), bacterial and/or viral infection of the middle ear, and inflammation within the nose resulting from allergic rhinitis or upper respiratory infection play important and interrelated roles

in the development of OME [1–3]. Although no direct causal evidence links allergy as an etiologic agent in OME, population studies have shown a distinct association between allergy and OME [4,5]. For example, atopic infants in Sweden were five times more likely to develop OME than nonatopic infants [4]. Tomonaga et al. [5] found that more than 87% of those with OME had allergic symptoms and/or were atopic. Jero et al. [6] noted that those with allergy were 4.4 times less likely to clear acute episodes of OME. Other studies report that as many as one half of all patients with OME have allergic rhinitis, and 21% of patients with nasal allergy have OME [7,8]. Nguyen et al. [3] showed that the allergic inflammation observed in atopic patients with OME occurred in the middle ear and the nasopharynx [3].

ETD is thought to be one of the foremost factors contributing to OME. Although the precise manner in which nasal allergic disease contributes to ETD is not fully understood, it is believed that allergic inflammation within or around the nasopharyngeal portion of the eustachian tube (ET) may lead to the loss of its protective functions, resulting in increased risk for development of otitis media [9].

From an immunologic standpoint, it is more important to think of the physiologic components of the middle ear as an intricate interrelated structure composed of the middle ear space, ET, nasopharynx (adenoids), nasal mucosa, and mastoid air cells [10]. The ET plays a vital role in middle ear function by providing a means of interaction between the nasopharynx and middle ear. Swallowing causes the tensor veli palatini to actively open the ET and thus ventilates the middle ear with atmospheric pressure [8,11]. The ET is also a pathway for drainage of secretions into the nasopharynx and protects the middle ear from nasopharyngeal secretions and sound pressure [11,12•,13,14].

Like mucosa elsewhere in the respiratory tract, the ET's mucosal lining contains mucus-producing cells, ciliated cells, plasma cells, and mast cells. Hurst et al. [15,16] and Wright et al. [17] demonstrated eosinophils,

myeloperoxidase, eosinophil cationic protein, and mast cells to be present in middle ear effusions of those with chronic OME, suggesting that the middle ear mucosa in atopic patients responds differently than that in nonatopic patients. Ebmeyer et al. [18] demonstrated that mast cells were a major contributor to the early inflammatory response of the middle ear to bacteria. Sobol et al. [19] found T lymphocytes, eosinophils, and other T-helper type 2 (Th2) cytokines in the effusions of atopic children.

Several means have been proposed to explain ET obstruction due to inflammatory processes. First, poor ET function could result from a retrograde spread of edema due to nasal mucosa congestion. Also, poor mucociliary function, whether intrinsic or due to the inflammatory response, may lead to poor clearance of secretions and obstruction of the lumen of the ET [9,12•]. Furthermore, direct allergic inflammation within the ET may cause obstruction due to intrinsic venous engorgement and hypersecretion of mucus, isolating the middle ear space. Thus, gas exchange occurs and results in the diffusion of nitrogen out of the middle ear space and into the mucosa [20]. The development of a net negative pressure in the middle ear perturbs cellular tight junctions and allows for a transudation of fluids into the middle ear space. The persistent obstruction of the ET with mucus results in chronic middle ear inflammation, mucosal metaplasia, increased glandular activities of goblet cells, and an inability to clear middle ear fluid [10,20].

Until recently, the therapeutic options for decreasing allergic inflammation were limited. However, immune modulatory oligodeoxynucleotides may offer new treatments by altering the allergic pathway. Tokunaga et al. [21] first discovered the immunostimulatory properties of bacterial DNA in the 1980s. They demonstrated that nucleic acid extracted from the *Mycobacterium bovis* strain of bacille Calmette-Guérin induced strong antitumor antibodies. The immunostimulatory properties of bacterial DNA are found in the six-base pair DNA sequence that follows the formula 5'-purine-purine-CpG-pyrimidine-pyrimidine-3'. This bacterial sequence can be mimicked by synthetic oligodeoxynucleotides known as cytosine-guanine oligodeoxynucleotides (CpG ODNs) [22].

Since this discovery, CpG ODNs have been found to have tremendous potential as therapeutic agents and adjuvants. The immunostimulatory effects of CpG ODNs have been found to confer protection against infectious diseases, allergies, and cancer in animal models. This paper highlights the recent progress in the treatment and prevention of allergy-induced ETD in the animal model.

Immunologic Principles

Allergic diseases are immune system disorders associated with an imbalance of the Th1 and Th2 lymphocyte subsets. The Th1 pathway is characterized by Th1 antibody production by B cells, activation of natural killer cells and

phagocytic cells, and the development of T-cell-mediated immunity against viral and bacterial infections or malignant cells [23]. In contrast, the Th2 pathway leads to immunoglobulin class switching from IgG to IgE, therefore mediating the eosinophil/mast cell-dependent immunity. This type of response is important for eliminating parasite infections but is implicated in hypersensitivity responses [24]. Interleukin (IL)-12 and interferon- γ (IFN- γ) are important in the modulation of the Th1/Th2 balance. IFN- γ has also been shown to moderate eosinophil recruitment and inhibit secondary allergic response development.

One proposed model of CpG ODN immunologic function maintains that CpG ODNs are recognized by Toll-like receptors (TLRs) and induce a cascade that favors activation of Th1-dominated immune response. TLRs are expressed on the surface (TLR1, TLR2, TLR4, TLR5, TLR6) and the endosomal compartment (TLR3, TLR7, TLR8, TLR9) of antigen-presenting cells [25••]. The endosomal-expressed TLR9 recognizes the dinucleotide sequence CG, which is more abundant in prokaryotic DNA or synthetic CpG ODNs [26,27]. CpG ODN stimulation of TLR9 induces the promotion of IFN- γ , IL-6, and IL-12 and reduces the production of IL-4, which leads to the development of antigen-specific Th1 cells and interferes with the development of allergen-specific Th2 cells [28].

However, other studies have shown a B-cell-intrinsic, T-cell-independent mechanism for which CpG ODNs directly act on B cells and prevent allergic symptoms. CpG ODNs suppress IgE and IgG production of B cells by inhibiting normal isotype switching (IL-4 and CD40) from IgM to IgE and IgG. These studies have also reported that although CpG ODNs stimulate all B cells, they preferentially stimulate memory B cells as opposed to naïve B cells [29].

However, a different model showed that CpG ODNs induced high levels of indoleamine 2,3-dioxygenase, which acts as an immunosuppressant, via the production of IFNs. This model indicates that CpG ODNs mediate immunomodulatory effects by inducing the death of effector Th2 cells and not by inducing a Th1-cell response [30,31]. These studies suggest there may be more than one possible mechanism involved in mediating the antiallergic effects of CpG ODNs.

Structural Characteristics

Changes in the structural requirements for cellular uptake and activation of CpG ODN can enhance its therapeutic use. It has been observed that distinct immunostimulatory profiles of CpG ODN such as sequence and length, as well as the number and positions of CpG motifs, affect species recognition. The expression of TLR functions as a major pathogen-associated molecular pattern receptor, serving to bind pathogen-derived ligands and induce cell activation via the Toll/IL signal pathway. As a result, the expression of TLR9 correlates with the

responsiveness of CpG ODN [25••]. Current evidence suggests that the receptor shows considerable selectivity for CpG ODN sequences, with different preferences observed in different species [32].

Three structurally distinct classes of synthetic CpG ODNs that differentially activate human TLR9 have been described. “K”-type ODNs (also known as CpG-B) are potent B-cell activators that interact with TLR9 and induce monocytes to proliferate and secrete IgM, IL-10, and IL-6. “K” ODNs have a simple structure that encodes multiple CpG motifs on a phosphorothioate backbone. The “K” ODNs with immunostimulatory activity are 12 bases long, whereas shorter ODNs are less active. “D”-type ODNs (also known as CpG-A) are potent at inducing IFN- α production by plasmacytoid dendritic cells, which indirectly supports the subsequent maturation of antigen-presenting cells. “D” ODNs are structurally more complex than the “K” type. “D” ODNs are constructed using a phosphodiester-phosphorothioate backbone and contain a single hexameric purine-pyrimidine-CG-purine-pyrimidine motif that can result in the formation of tetrads and ODN clusters. The minimum length of an active “D” ODN is 18 bases. “C” type (also known as CpG-C) combines B-cell activation and induction of IFN- α . “C” ODNs contain a central phosphorothioate backbone (as in “K” ODNs), as well as the single hexameric purine-pyrimidine-CG-purine-pyrimidine motif (as in “D” ODNs) [33,34••].

Other clinical modifications on CpG motifs also have been reported. In 2003, Kandimalla et al. [35] joined two CpG ODNs through a 5'-5' linkage and showed enhanced immunostimulative activity. They also designed a synthetic nucleoside with a bicyclic heterobase to replace the C in CpG, resulting in an RpG dinucleotide. Using the RpG dinucleotide, they stimulated the proliferation of peripheral blood mononuclear cells in different species. These chemical modifications of the structural characteristics of the CpG ODNs may overcome the species-specific recognition receptor system [35].

Delivery Methods

Selecting the appropriate route of delivery of CpG ODNs to targeted immune compartments is a critical issue in the optimization of immunostimulatory responses of CpG ODNs. To date, many routes have been tested, including intravenous, subcutaneous, sublingual, interdermal, intranasal, intraperitoneal, transtympanic, and intratracheal. The effectiveness, dosing, and toxicities for each method are important for CpG ODN therapeutic application.

Human studies have revealed that subcutaneous (but not intravenous) administration of a CpG ODN induced a systemic innate immune response [36]. Intravenous application of CpG ODN triggered suppression of T-cell expansion and cytotoxic T-cell lymphocyte activity in the spleen, but subcutaneous injection of CpG ODN resulted

in antigen-specific response in the lymph nodes. However, whereas systemic immunization induces adaptive responses systemically, the induction of mucosal immunity generally requires local exposure of antigen. Recent animal studies have shown that intranasal immunotherapy is more effective than systemic therapy in inducing a systemic and mucosal immune response [37]. Other studies have demonstrated that mucosal vaccination routes are effective for inducing antigen-specific IgA responses. IgA has an inhibitory effect on bacterial adherence and is essential for mucosal immunity [38].

A reliable model of allergy has been created in the Brown Norway rat. Previous studies have demonstrated increased IgE response in the Brown Norway rat in asthma, allergic rhinitis, and food allergies [39–41]. These studies demonstrated that CpG ODNs significantly reduced the systemic and pulmonary manifestations of allergy. The model commonly includes a “sensitization” phase, during which animals are exposed to the allergen, followed by a “challenge” phase, in which the animals are re-exposed to the same allergen [42]. The unified airway concept has shown that allergic inflammation occurs in the ET, middle ear, and nasopharynx.

Therefore, our laboratory has evaluated the effect of CpG ODNs in ovalbumin-induced ETD. Assessment of ETD was measured using a water manometer and the application of positive or negative pressure into the middle ear [43]. Our laboratory has used multiple challenge and treatment approaches. We have investigated the use of CpG ODNs when delivered via subcutaneous injection before transtympanic allergen challenge and found no clinical evidence of middle ear effusion in treated animals compared with untreated animals [39]. Ebert et al. [44] demonstrated that subcutaneous injection of CpG ODNs can prevent allergic-like inflammation of ET after intranasal allergen exposure. Blanks et al. [45] also demonstrated effective mucosal application of CpG ODNs before and after transtympanic allergen exposure. Unlike previous studies, CpG ODNs were placed directly on the middle ear mucosal epithelium via transtympanic injection. This study showed a 50% reduction in the pressure required to open and close the ET in the CpG ODN-treated animals compared with the untreated animals [45]. Kodama et al. [38] has also demonstrated that CpG ODN acts as an immunostimulate in the middle ear of mice nasally immunized with nontypeable *Haemophilus influenzae*.

Side Effects

CpG ODNs' high immunostimulatory ability raises concerns in relation to their clinical tolerability. It is possible that CpG ODNs may enhance the immunogenicity of self-proteins, triggering the development of autoimmune disease, or increase susceptibility of the host to pathologic agents that cause toxic shock. CpG ODNs may trigger the production of reactive oxygen species and the secretion of

proinflammatory cytokines and chemokines [46]. With local intranasal therapy of CpG ODNs, Matheu et al. [37] found that mice developed a mild synovitis [37]. Previous animal studies have shown (in high doses) severe toxicity, including multifocal hepatic necrosis, destruction of lymphoid follicles, extramedullary hematopoiesis, and lethal systemic inflammatory response syndrome [47].

However, no toxicity has ever been reported in normal animals treated with therapeutic doses of CpG ODNs. CpG ODNs have also been administered to humans in clinical trials, with no adverse reactions attributable to CpG ODNs observed.

Conclusions

Several theories have been proposed to explain ETD secondary to an inflammatory process. ETD may result from a retrograde spread of edema attributable to nasal mucosa congestion. Poor ciliary function resulting from an inflammatory response may also contribute to impaired clearance of secretions and obstruction of the ET lumen [9,12]. Additionally, direct allergic inflammation within the ET may cause obstruction from intrinsic venous engorgement and hypersecretion of mucus, thus isolating the middle ear space and thereby affecting gas exchange [20]. As a result, the persistent obstruction of the ET results in chronic inflammation, mucosal metaplasia, increased glandular activities of goblet cells, decrease of ciliated cells, and an inability to clear middle ear fluid [10,20]. Repeated allergic exposures and development of ETD may lead to repeated episodes or persistence of otitis media.

Recent studies have demonstrated that immunotherapy is an effective treatment before or after the development of allergen-induced ETD. CpG ODNs represent a new class of adjuvant for immunotherapy in the treatment of allergic disease.

Disclosures

No potential conflicts of interest relevant to this article were reported.

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