

Immunostimulatory sequences in immunotherapy

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Purpose of review

To review the current literature regarding immunostimulatory sequences of DNA for immunotherapy with respect to signaling mechanisms, cytokine profiles, structural characteristics and the applicability and success of this strategy to treat allergic disease.

Recent findings

The binding of synthetic DNA-based immunotherapy agents composed of unmethylated cytosine–guanine dinucleotides (CpG ODN) to toll-like receptors have been found to be species-specific. CpG ODNs are capable of inducing a shift in the cytokine profile and immune response that favors the Th1 pathway and suppresses the Th2 pathway. This makes using CpG ODNs a promising candidate for the treatment of allergic diseases, which are known to be mediated by Th2-based response. Current CpG ODN studies have demonstrated prevention and reversal of acute allergen inflammation, airway hyper-reactivity and remodeling. Early animal and human trials of CpG ODNs have shown them to be both well tolerated and effective.

Summary

The use of immunostimulatory sequences in immunotherapy, although still in the early stages of development, has thus far been shown to be both well tolerated and effective, and offers the potential for a better tolerated, more rapid, more efficacious and longer-lasting therapy over current immunotherapy protocols.

Keywords

CpG ODN, immunostimulation, toll-like receptors

Introduction

The basis of the allergist's therapeutic options has been standard allergen immunotherapy since 1911. This approach is limited, however, by the potential for systemic allergic reactions, including anaphylaxis, which is caused by the large doses of allergen often required for efficacy. Furthermore, standard allergen immunotherapy is difficult to administer because it requires regular frequent dosing, over an extended period of time – usually 3–5 years. Therefore, there is a need for new immunotherapeutic agents [1•].

Tokunaga *et al.* [2], in the 1980s, first discovered the immunostimulatory properties of bacterial DNA. They demonstrated that nucleic acid extracted from *Mycobacterium bovis* strain BCG induced strong antitumor antibodies. The ability of the host to recognize bacterial DNA is based on the occurrence of unmethylated cytosine–guanine (CpG) dinucleotides in particular sequences, called CpG motifs [3]. In 1995, the first preliminary characterization of the DNA sequences required for immunostimulation was carried out by Krieg *et al.* [4]. The results showed that the immunostimulatory activity of CpG DNA is contained in the six base pair DNA sequence that follows the formula 5'–purine–purine–CpG–pyrimidine–pyrimidine–3'. The presence of bacterial DNA can be mimicked by synthetic oligodeoxynucleotides (CpG ODNs) [5].

Since this discovery, CpG ODNs have been found to have a tremendous potential to be used 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 article will highlight the recent progress in the treatment of allergy.

Basic immunological principles

The precise mechanisms of the induction of immune response following CpG DNA have not been fully elucidated, although antigen processing, presentation and recognition are well established. The immune response is initiated and expanded by exogenous and endogenous antigen presentation [6]. Exogenous antigens are taken up by antigen-presenting cells (such as macrophages and dendritic cells). These cells function in the immune response by presenting peptide fragments of the antigen on their surface in the context of major histocompatibility complex (MHC) class II molecules. These peptide–class II complexes are transported to the surface and

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Abbreviations

MHC major histocompatibility complex
TLR toll-like receptors

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are recognized by specific CD4⁺ T cells. Endogenous proteins produced within the cells are also presented to the immune system. These proteins are degraded into peptides within the cytoplasm and are bound to the MHC class I molecules for delivery to the cell surface. CD8⁺ T cells recognize this MHC class I/peptide complex.

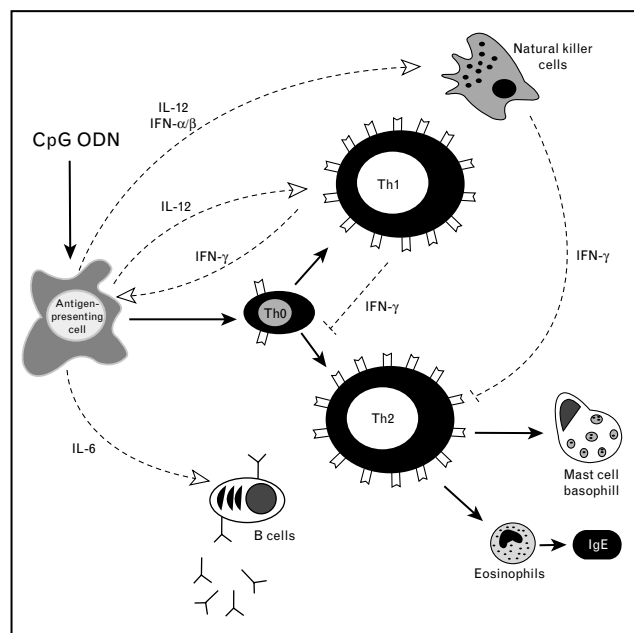
While exogenous and endogenous antigen processing are usually strictly separated, CpG DNA may offer the opportunity to activate both pathways. CpG DNA can be taken up from extracellular spaces; the DNA is then transcribed and translated and packed into MHC class I molecules. The CpG DNA can also be secreted into extracellular spaces and becomes available for dendritic cells and presentation to MHC class II molecules. Therefore, CpG DNA vaccination may improve interaction of antigen-specific CD4⁺ and CD8⁺ T cells, resulting in enhanced mechanisms due to activation of both T-cell subsets [7]. Consequently, modification of CpG DNA may provide the opportunity to favor either the endogenous or exogenous pathway of antigen processing, and, in so doing, eliciting the desired type of immune response or improving immunogenicity.

Antiallergic properties

CpG ODNs can modulate the immune system in a number of ways. These include activation of natural killer cells and antigen-presenting cells, including macrophages and dendritic cells; increasing dendritic cell migration; upregulating the expression of MHC class II, CD40, CD80 and CD86; inducing a Th1 immune response and therefore suppressing IgE synthesis, but promoting IgG and interferon (IFN)- γ production; and initiating the production of IFN- γ , IFN- α/β and interleukins (IL) 6, 12 and 18, which promote Th1 responses and enhance cell-mediated immunity [8^{**}]. CpG ODNs can also directly activate B cells to proliferate or secrete antibodies and can stimulate T cells directly or indirectly, depending on their activation state. As shown in Fig. 1, CpG ODNs initiate a complex network of cell–cell interactions and cytokine cascades that result in enhancement of immune functions in an antigen-independent manner.

Allergic diseases are disorders of the immune system 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. 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 ridding parasite infections but is implicated in hypersensitivity responses [9].

Figure 1 Simplified diagram of the response to CpG ODNs



'Antigen-presenting cell' refers to either macrophages or dendritic cells. Dashed arrows indicate positive and negative feedback loops. CpG ODNs induce a Th1-cell response by inducing IL-12, IL-6, IFN- γ and IFN- α/β .

The mechanism for the downregulation of allergic inflammation in mice treated with CpG ODN conjoined to allergen is unclear. Currently, there are studies that support a variety of mechanisms that are probably responsible and include the interference of antigen presentation to Th2 cells, induction of interferons and even one study inducing the apoptosis of effector Th2 cells. A few of these studies include a study [10] in mice demonstrating that CpG ODN can prevent and even reverse acute allergen-induced eosinophilic inflammation, airway hyper-reactivity and airway remodeling. While CpG ODNs are thought to promote Th1 cytokines and inhibit Th2 cytokines, studies have suggested that IL-12 and IFN- γ (important Th1 cytokines) are not required. Hessel *et al.* [11] demonstrated that CpG ODN blocked allergic inflammation by altering lung antigen-presenting cell function to Th2 cells which prevented production of Th2 cytokines. A different mouse model of asthma showed that CpG ODNs induced high levels of indoleamine 2,3-dioxygenase via the production of interferons, in the lungs, which acts as an immunosuppressive. This model indicates that CpG ODNs mediate immunomodulatory effects by inducing the death of effector Th2 cells and not via induction of a Th1-cell response [12,13]. These studies suggest that there may be more than one possible mechanism involved in mediating the anti-allergic effects of CpG ODN.

Structural characteristics

While developing human vaccines, it has been observed that distinct immunostimulatory profiles of CpG ODN are species-specific, depending on structural characteristics, such as sequence, length and the number and positions of CpG motifs within the ODN. ODNs with CpG motif positioning at the 5'-end have demonstrated the greatest stimulatory activity [8^{••}].

The recognition of CpG motifs requires toll-like receptors (TLR). In 2000, Hemmi *et al.* [14] showed that TLR9-deficient mice did not respond to CpG DNA. In 2001, Takeshita *et al.* [15] demonstrated that human cell lines and peripheral blood mononuclear cells recognized CpG ODN through the expression of TLR9. Thus, the expression in immune cells of TLR9 correlates with the responsiveness to CpG ODN. Other studies [16,17], however, have shown that TLR2 and TLR4 are also major determinates in the susceptibility to the development of asthma and allergy. The engagement of the TLR triggers a cascade of cell-signaling events. These innate immune responses control the spread of infection and direct the development of adaptive immune responses. Overall, the molecular mechanisms of recognition and the interactions of CpG ODN sequences with TLR are not well understood [18,19].

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. 'D'-type (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, which can result in the formation of tetrads and ODN clusters. 'C'-type (also known as CpG-C) combines both 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) [9,20^{••}].

Other clinical modifications on CpG motifs have also been reported. In 2003, Kandimalla *et al.* [21] 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 ODN might overcome the species-specific recognition receptor system. Schmidt *et al.* [22[•]] have also designed a CpG ODN without the phosphodiester backbone (PO-ODN), to possibly avoid any ODN side effects. The use of PO-ODNs in allergic disease has yet to be studied. Currently, different forms of CpG ODNs still continue to be synthesized and investigated.

Vaccine adjuvant

An increasing number of studies have shown that certain CpG motifs in plasmids can be used for genetic immunization to enhance immunogenicity and the Th1 bias. Certain CpG motifs suppress the adjuvant effect of the immunostimulatory CpG DNA; removal of such CpG motifs demonstrates increased IgG2a/IgG1 ratio as well as cytotoxic T-lymphocyte activity. A feature of CpG DNA-based adjuvants is their high potential to induce IL-12 – the central mediator of Th1 reactions. This is of special interest to the treatment of allergy [20^{••},23[•]].

The adjuvant properties of CpG ODNs are markedly improved by ensuring that the ODNs remain in close proximity to the antigen. Therefore, physically binding ODN to the antigen, by cross-linking or coin-corporation into lipid vesicles, generates IgG responses that were 10–1000-fold greater than the antigen alone [24].

Experimental studies [25[•],26] in mice have used allergens such as ragweed and house-dust mite to demonstrate that allergic asthma could be reduced or eliminated by the administration of CpG ODNs during the sensitization phase of the response. In humans, Creticos *et al.* [27^{••}], in a randomized, double-blind, placebo-controlled study, showed long-term efficacy in the treatment of ragweed-allergic rhinitis after a 6-week regimen of vaccine consisting of Amb a 1 (a ragweed-pollen antigen) and CpG ODN. Although Creticos's study did not demonstrate a significant change in the nasal vascular permeability (primary endpoint), patients reported a better quality-of-life score for the first and subsequent ragweed season.

Route of delivery

Different routes of delivery of CpG ODN have been proposed as a way to achieve optimal protection. Studies [28] in mice have demonstrated a superior protection with intratracheal administration of CpG ODN compared with intraperitoneal administration when challenged with intratracheal *Klebsiella pneumoniae*. Similarly, intranasal administration of CpG ODNs has shown a protective effect in the development of Eustachian tube dysfunction in ovalbumin-sensitive rats [29]. Other studies [20^{••}] have observed the opposite effect, demonstrating better CpG ODN protection when it is given via subcutaneous

injection rather than inhaled after a respiratory challenge of parainfluenza virus. Teleshova *et al.* [30[•]] demonstrated that locally administered intranodal CpG ODN has both a local and a systemic effect in macaques. Teleshova considered that this could be due to migration of IFN- γ secreting cells from the periphery to the injected lymph nodes in response to secreted chemokines.

In human studies [31], subcutaneous but not intravenous administration of CpG ODN induced a Th1-like immune response. Subcutaneous injection of the antigen plus CpG ODN resulted in the antigen-specific response in lymph nodes, but intravenous injection triggered suppression of T-cell expansion and cytotoxic T-cell lymphocyte activity in the spleen. Wingender *et al.* [32^{••}] demonstrated that CpG ODN can have adverse effects and suppress T-cell responses. They determined that the anatomical site of application is critical in determining the immunosuppressive compared with stimulatory effects of CpG ODN. Therefore, these studies suggest that it is still worthwhile to continue to compare different routes of delivery.

Side effects

The fact that CpG ODNs are highly immunostimulatory raises concerns regarding their clinical tolerability. A possibility exists that ODNs might 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 pro-inflammatory cytokines and chemokines. These effects can result in inflammatory responses, such as aggravating joint inflammation, draining lymph nodes and increased levels of the acute phase reactant A1-acid glycoprotein [8^{••}].

The ability of CpG ODNs to impact on the development of autoimmune diseases is supported by studies [33] showing that high doses of bacterial DNA elicit the production of autoantibodies against double-stranded DNA in normal mice. Immunization of lupus-susceptible NZB/W mice with *Escherichia coli* DNA but not with calf thymus DNA resulted in elevated levels of anti-DNA-antibodies. Despite the increased anti-DNA antibodies, the treatment improved survival and reduced autoimmune symptoms [34]. Bacterial DNA also stimulates the production of IL-6 and blocks the apoptotic death of activated lymphocytes – functions that predispose to the development of autoimmune disease [9].

The possibility of CpG ODNs to facilitate the development of toxic shock is also a concern [35]. Other agents such as lipopolysaccharide (LPS) and D-galactosamine cause toxic shock by triggering the overproduction of TNF. CpG ODNs can indirectly stimulate macrophages

to produce this cytokine. When CpG ODNs were coadministered to mice with subpathological doses of LPS, severe mortality and morbidity ensued [36].

No toxicity, however, has ever been reported in normal animals treated with therapeutic doses of DNA vaccines or CpG ODNs. CpG ODNs have also been administered to human subjects in more than a dozen clinical trials. No serious adverse reactions attributed to CpG ODNs have been observed.

Conclusion

The possible therapeutic applications of CpG ODNs include the induction of protective innate immunity against certain viral or bacterial infections and upregulating the expression of Th1 while decreasing the Th2 response in cases of asthma or allergic disease. The activation and role of TLRs in the shaping of host immunity and the predisposition towards allergic disease will play a critical part in future prevention and treatment methods. Further studies of the TLR pathway will be required to determine areas for feasible intervention strategies.

Numerous reports have provided evidence of the advantages of CpG ODN immunostimulation, and also of the beneficial effects of these substances in most vaccination applications. Several candidate CpG ODNs are currently being evaluated in clinical applications with cancers, viral and bacterial infections, allergies and asthma.

The molecular basis for the recognition of CpG ODN by the immune system appears to be one of the most important mechanisms for initiating host-innate immune responses against invading organisms. Thus, investigations of the molecular mechanisms of recognition, as well as the interactions of CpG ODN sequences with receptors and the immuno-modulatory functions that result, are not only required for expanding clinical applications, but also to provide new insights in immunobiology. Continuing studies will need to examine the benefit of introducing synthetic bases into the backbone of CpG ODNs, creating multideterminant ODN complexes, and varying the number and sequences of CpG motifs.

We have used CpG ODNs in our laboratory studying the prevention and treatment of ovalbumin-induced allergy on the Eustachian tube in rats. Our results have shown that giving CpG ODNs before and after allergen exposure reduces the allergic response. Our experiments have looked at both subcutaneous and mucosal absorption of CpG ODNs and we have experienced encouraging results with both treatment methods. Therefore, we feel that CpG ODNs offer substantial future promise in the treatment of allergic disease.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

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- of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 295–296).

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