

CpG Oligodeoxynucleotides as a Future Vaccine for Allergic Diseases

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ABSTRACT

An astounding feature of the DNA sequences termed CpG motifs is the induction of immune and inflammatory responses in a senseless manner. CpG motifs exist abundantly in microbes and evoke innate immunity that constitutes the first line of defense against microbial infections in vertebrates. CpG motifs that essentially work in an antigen-nonspecific fashion, however, turn into novel immunomodulators that can manipulate acquired immunity in an antigen-specific manner if oligodeoxynucleotides containing CpG motifs (CpG ODNs) are directly conjugated to the antigen. CpG ODNs with potent polyclonal Th1-inducing ability show promise for application in immunotherapy whereby neutralization of dominant allergy-prone Th2 cells is achieved by inducing allergen-specific Th1 cells. The underlying mechanisms include an unexpected enhancement of dendritic cell function as a linker between innate and acquired immunity. In the foreseeable future the mainstream therapeutic role of corticosteroids in anti-inflammatory therapy for allergic diseases could possibly be replaced by immunotherapy using CpG ODN-conjugated antigens.

KEY WORDS

antigen-specific, bronchial asthma, dendritic cell, Th1/Th2 cell

INTRODUCTION

In recent years the incidence of various allergic diseases, including allergic rhinitis, bronchial asthma, and atopic dermatitis, has been increasing throughout the world. Although manifestations vary according to disease, the fundamental pathophysiologic abnormalities underlying these diseases are now considered to be related to the overreaction of Th2 cells to allergens. Novel immunotherapies to treat allergic diseases by inhibiting the causal Th2 cells are therefore anticipated.

REGULATION OF IMMUNE RESPONSES BY T CELLS

Apart from pathogenic microbes, the majority of allergens are innocuous in nature. Allergens do not multiply inside the body. They do not directly destroy tissues. The immune system could ignore them with impunity. For some unknown reasons, however, the immune surveillance system evokes disadvantageous responses to the allergens, resulting in the onset and perpetuation of allergic diseases. Among a miriade of

cells involved in allergic inflammation, Th2 cells are considered the prime lymphocyte subset that dictates a series of allergic reactions.

The immune system appears to be tightly and smartly regulated by various regulatory mechanisms. Among others, T cells have been attracting much attention especially with respect to antigen specificity and major histocompatibility complex (MHC)-restriction specificity at the induction and effector phases. T cells with regulatory functions were formerly called suppressor T cells.¹ After a lull in extensive investigations on suppressor T cells there has been a resurgence in research with accompanying new terminology, whereby regulatory T cells have replaced the old term for suppressor T cells.²⁻⁵ Regulatory and suppressor T cells manifest their principle activities by inhibiting other T cell subsets. Recent advances have enabled us to identify the effector regulatory molecules, such as TGF- β , IL-10 or CTLA-4.^{2,3,6,7}

Some effector T cells can also exert inhibitory functions on other T cells. Typical examples are Th1 and Th2 cells. Th1 and Th2 cells were defined on the ba-

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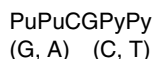


Fig. 1 CpG motifs. CpG motifs consist of a central unmethylated CpG dinucleotide flanked by two 5' purines and two 3' pyrimidines.

sis of the specific cytokines they secrete.⁸ A particularly intriguing phenomenon is that Th1 and Th2 cells with the same antigen specificity mutually inhibit each other's effector functions.^{9,10} These original findings have contributed to furthering the understanding of the basic abnormalities in various diseases and are leading the way toward the development of future therapies. Allergic diseases are a prime candidate for immunotherapy, due to the nature of distal upstream antigen-specific Th2 cells which dictate most allergic manifestations.

CPG MOTIFS AND ACQUIRED IMMUNITY

The function of DNA had long been regarded as limited to the transmission of the genetic information. This dogma was challenged by the astounding findings of Tokunaga and his group.¹¹ They reported that DNA itself directly activates NK cells in a senseless manner.^{12,13} These functions were localized specifically to particular sequences of DNA,¹⁴ now called CpG motifs (Fig. 1).

CpG motifs are frequently found in bacteria, but rarely in vertebrates. Vertebrates recognize CpG motifs as non-self. During the long evolutionary process, vertebrates have somehow acquired the ability to exploit these differences for protection against pathogenic infections. The presence of CpG motifs inside the body is interpreted as an invasion by microorganisms, and as a result defensive immune and inflammatory responses are evoked.

The recognition of microbes is often mediated by receptors that have evolved to bind selectively to structures expressed solely in microbes but not in self-tissues.¹⁵ So far, 10 different kinds of receptors, collectively called toll-like receptors (TLR), have been discovered. One of the TLRs, TLR9, specifically recognizes CpG motifs,¹⁶⁻¹⁸ and the colocalization of CpG motifs with TLR9 is the initial step that ultimately leads to the activation of the acquired immune system, namely T and B cells.^{19,20}

One of the outstanding characteristics of the acquired immune system is the antigen-specificity at the induction and effector phases. The most efficient immune responses are attained when they are evoked in an antigen-specific manner, as is the case with preventive vaccines against microbial infections. However, the effects of CpG motifs on acquired immunity are polyclonal in nature since CpG motifs do not possess inherent antigenic information. Treatment with CpG motifs may only stir the sophisticated

immune regulation.

Since synthetic oligodeoxynucleotides containing CpG motifs (CpG ODNs) mimic the activity of bacterial DNA, CpG ODNs have been used to explore the nature of CpG motifs. In the following discussion, we shall mainly focus on experimental applications of CpG ODNs for the treatment of bronchial asthma in murine models.

CPG ODNs AS ANTIGEN-NONSPECIFIC INHIBITORS OF ALLERGIC INFLAMMATION

Early attempts to reduce allergic airway inflammation were made by administering CpG ODNs without an accompanying antigen. Intraperitoneal or intranasal administration of CpG ODNs was reported to inhibit airway eosinophilia and improve airway hyperresponsiveness. These anti-allergic effects were concomitant with decreases in IL-5 secretion from CD4⁺ spleen cells or decreases in IL-4 levels in bronchoalveolar lavage.^{21,22} Increased levels of Th1 cytokines, such as IFN- γ and IL-12, were also noted.^{21,22}

The mechanisms underlying these observations have remained unknown. CpG ODNs induced the expression of costimulatory molecules^{23,24} and IL-12^{25,26} in dendritic cells (DCs), which are required for the induction of Th1 cells. When these changes were induced independently of the attendant antigen, CpG ODNs would plausibly drive immune responses to skew toward a Th1-dominant phenotype in an antigen-nonspecific fashion, which would in turn inhibit allergen-specific Th2 cells in a bystander manner. In another scenario, CpG ODN-induced Th1 cytokines, including IFN- γ and IL-12, may inhibit Th2-mediated inflammation without direct participation of CpG ODN-induced Th1 cells.

Recently, CpG ODN treatment was found to increase the levels of bronchoalveolar lavage TGF- β in a murine asthma model.²⁷ The authors suggested the possible induction of regulatory T cells by CpG ODNs, although no evidence for the production of TGF- β from T cells was demonstrated.

Regardless of the mechanisms, manipulations by CpG ODNs alone required larger doses of CpG ODNs than that by CpG ODNs with concomitant antigen to attain comparable therapeutic effects. Treatment with high doses of CpG ODNs could eventually highlight the intrinsic adverse effects of CpG motifs, such as an increase in serum TNF- α levels or mortality.²⁸⁻³⁰ In light of the pathogenic roles of Th1 cells in various types of autoimmunity,³¹ the effects of CpG motifs on the activation of autoreactive Th1 cells and autoimmunity are also unpredictable and controversial.³²⁻³⁵

Another report showed that treatment with CpG ODNs plus corticosteroids was synergistically more effective than monotherapy with either agent alone.³⁶ Since neither CpG ODNs nor corticosteroids manipulate the immune response in an antigen-specific man-

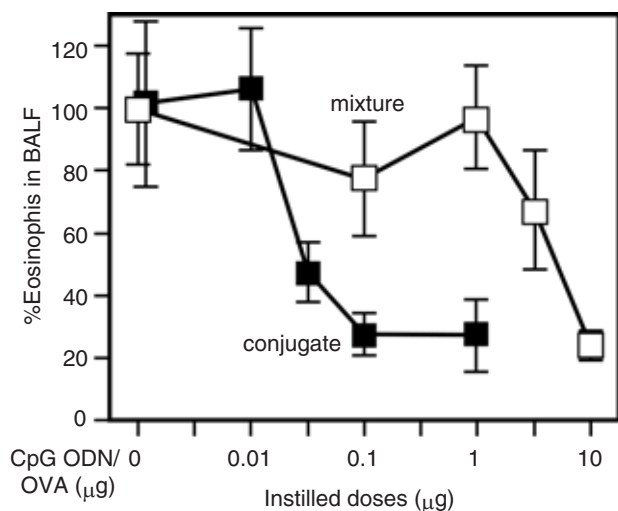


Fig. 2 Comparison of the conjugate with the mixture of CpG ODNs and OVA. OVA-primed BALB/c mice were pre-treated intratracheally with graded doses of the conjugate (■) or a mixture (□) of CpG ODNs and OVA before OVA challenge. The airway eosinophilias were expressed as a percentage relative to the control response. The covalent conjugate was calculated to be 100-fold more potent in the inhibition of airway eosinophilia than the mixture of CpG ODNs and OVA.

ner, treatment with the CpG ODNs would represent nothing more than an anti-inflammatory therapy, the same effect as corticosteroids.

INHIBITION OF ALLERGIC INFLAMMATION BY CPG ODNs IN AN ANTIGEN-SPECIFIC MANNER

It is well known that Th1 and Th2 cells work in a mutually inhibitory manner when both cells recognize the same antigen.¹⁰ CpG motifs have diverse proinflammatory and immuno-potentiating actions, including Th1 induction, although these actions are independent of specific antigens.

In order to augment the potentiation of adjuvant use of CpG motifs in the antigen-specific Th1 responses, CpG ODNs are often coadministered with the antigen. Actually, this idea has demonstrated positive results. Inhibition of airway allergic inflammation was achieved with smaller doses of CpG ODNs when administered together with antigen than when given alone.^{37,38} The anti-inflammatory activity was accompanied by the improvement of airway hyperresponsiveness and the inhibition of Th2 cytokines.^{37,38} The absence of IFN- γ nullified the CpG ODNs' effects, suggesting the possibility for Th1 cell contribution in the inhibition of Th2 cells.³⁷

Established airway eosinophilia was also improved by treatment with CpG ODNs and antigen.^{39,40} In parallel, an improvement of airway hyperresponsiveness

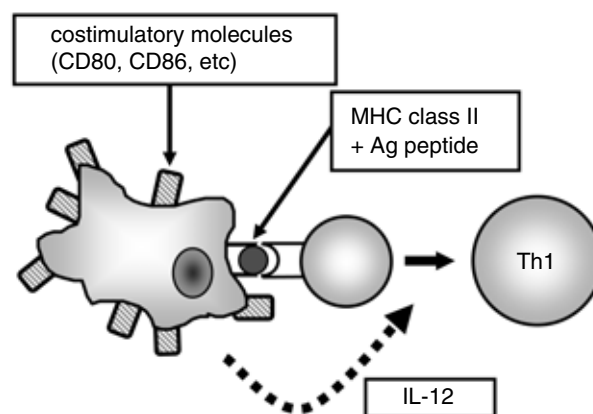


Fig. 3 Three conditions required for the induction of antigen-specific Th1 cells. The conditions are as follows: ① presentation of antigenic peptides in the context of MHC class II molecule by antigen presenting cells, particularly dendritic cells ② maturation and full activation of dendritic cells for the expression of costimulatory molecules, including CD80 and CD86 ③ secretion of IL-12 from dendritic cells.

and a decrease in Th2 cytokines in the bronchoalveolar lavage was also observed. An interesting finding was an increase in IL-10 production and decreases in IL-5 and IFN- γ production from splenocytes treated with antigen and CpG ODNs.⁴⁰ These findings might imply that CpG ODN immunotherapy induces regulatory T cells other than Th1 cells.

DIRECT CONJUGATION OF CPG ODNs TO ANTIGEN

Since coadministration of antigen and CpG ODNs exerted potent anti-allergic effects, direct linkage between antigens and CpG ODNs was introduced to assure intimate contact between the two. As a result, the conjugated form of CpG ODNs plus antigen was 100-fold more efficient in regulating airway eosinophilia than the unconjugated mixture (Fig. 2). The inhibition of airway eosinophilia by the conjugate was antigen-specific, and occurred in parallel with the unresponsiveness of the antigen-specific Th2 cells in the regional lymph nodes.⁴¹ Airway hyperresponsiveness was also improved.^{41,42}

In accord with these *in vivo* observations, the CpG ODN-antigen conjugate was 100-fold more effective than the unconjugated mixture for inducing *in vitro* Th1 differentiation in an IL-12-dependent manner.⁴¹ Thus, the antigen-specific Th1 cells are induced efficiently by the conjugate of CpG ODNs and antigen, and in turn inhibited the Th2 cells dictating allergic inflammation. The CpG motifs were essential as evidenced by a failure of a conjugate between antigen and ODNs containing no CpG motifs to induce Th1 cells. Marshall *et al.* reported that human peripheral

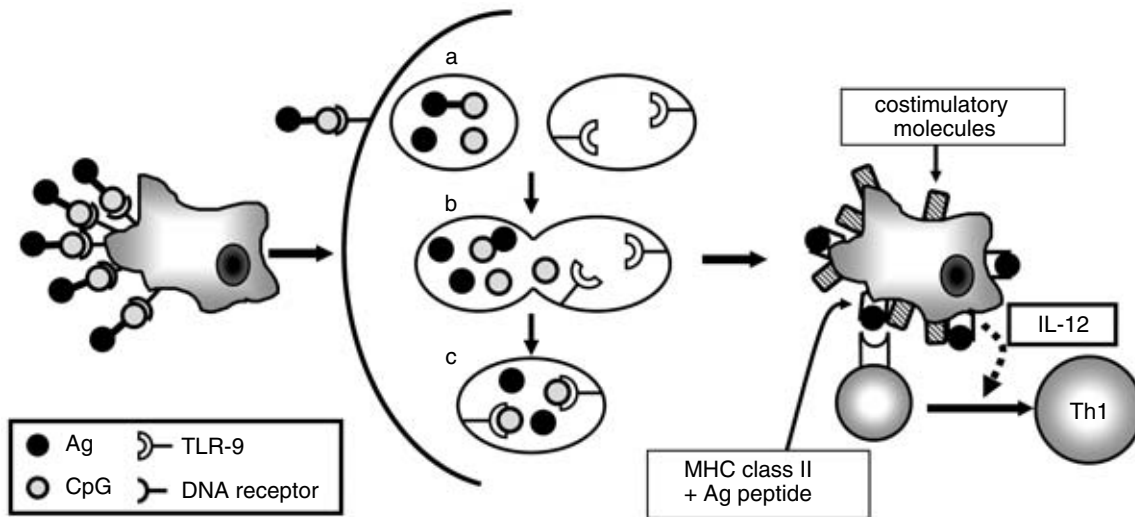


Fig. 4 Advantages of conjugates for Th1 induction. Conjugates between antigens and CpG ODNs first bind to DNA receptors expressed on the surfaces of APCs, and are then incorporated into endosomes by receptor-mediated endocytosis (a). The amounts of antigens incorporated by APCs are greater when the antigens are captured by receptor-mediated endocytosis than compared to ordinary fluid phase endocytosis. The antigens are digested and transferred to the cell surface for the presentation to T cells. On the other hand, endocytosed CpG ODNs colocalize with TLR9 in the late endosomes (c), which trigger activation signals ultimately leading to the expression of costimulatory molecules and the IL-12 secretion. Thus, APCs that fulfill the three conditions shown in Figure 3 induce the differentiation and activation of Th1 cells. ODNs lacking CpG motifs, which can bind to surface DNA receptors and are endocytosed, however, fail to transmit the activation signal due to the inability to colocalize with TLR9 in the endosomes.

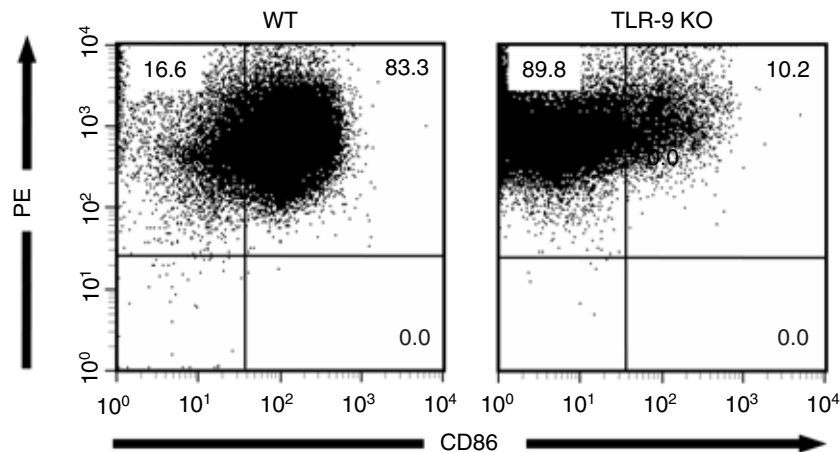


Fig. 5 Absence of need for TLR9 in CpG ODN-mediated Ag uptake by Ia⁺ cells. APCs from TLR9-deficient (TLR9 KO) or wild type (WT) mice were incubated with R-phycoerythrin (PE) conjugated with CpG ODNs overnight, and examined for the expression for PE and CD86. The numbers in figures indicate the percentage of cells in each quadrant. CpG ODN-linked antigens were taken up by APCs regardless of the expression of TLR9, whereas TLR9 was essential for the activation of the APCs as demonstrated by the increased proportion of CD86⁺ APCs.

blood mononuclear cells from patients with ragweed allergy shifted from a Th2 to Th1 cytokine expression pattern when cultured in the presence of the antigen-linked CpG ODNs.⁴³

Another beneficial aspect of the conjugate is the reduction of the allergenicity of antigens. Tighe *et al.* reported that antigen conjugated with CpG ODNs had a diminished ability to react with antigen-specific IgE as evidenced by reduced histamine release from human basophils.⁴⁴ Horner *et al.* demonstrated that increases in the ODN conjugation to antigen ratio resulted in parallel increases in immunogenicity and decreases in allergenicity.⁴⁵ These findings, together with a previous report that CpG ODNs directly inhibited IgE synthesis from B cells through the induction of T-bet,⁴⁶ highlight the effectiveness of CpG ODNs in IgE regulation and offer a possible clinical application for safer immunotherapy.

An extended period of efficacy is another advantageous aspect of conjugates. Horner *et al.* reported that the conjugate-induced Th1-biased immune responses were maintained for at least 1 year,⁴⁷ whereas allergen-independent immunomodulation by CpG ODNs alone was shown to persist for a much shorter period of time.⁴⁸ Differences in the durability might simply reflect differences in the underlying mechanisms involved; conjugates and CpG ODNs work on acquired and innate immunity, respectively.

In addition to applications for allergies, CpG ODN-conjugated antigen was also shown to be beneficial in the treatment of malignant tumors. Melanoma cells were eradicated by the activation of CD8⁺ CTLs induced by the administration of CpG ODN-conjugated tumor antigen.⁴⁹

MECHANISMS FOR CONJUGATE-MEDIATED TH1 INDUCTION

The mechanism for the enhancement of Antigen-specific Th1 responses by the conjugate is of significant interest. Based on the fact that the conjugated forms of antigen and CpG ODNs are in close proximity to each other, initially it seemed conceivable that simultaneous engulfment of both antigen and CpG ODNs was the main reason for the enhanced induction of antigen-specific Th1 cells.^{41,49}

This notion appeared sensible in light of a consideration of the requisites for Th1 induction. Three conditions need to be fulfilled for the efficient induction of antigen-specific Th1 cells (Fig. 3); ① presentation of antigenic peptides in the context of MHC class II molecule by antigen presenting cells, particularly dendritic cells ② maturation and complete activation of dendritic cells in the expression of costimulatory molecules, including CD80 and CD86 ③ secretion of IL-12 from dendritic cells. The first condition derives from the phagocytosed/endocytosed antigen, and the second and third conditions are provided by CpG ODNs. Thus, the incorporation of the conjugate by

antigen-presenting cells (APCs) appears to simultaneously fulfill the three conditions listed above. However, this was not the end of the story.

CpG ODNs in the conjugated form had unexpected advantages in facilitating antigen uptake by APCs. Immature DCs are considered to be well equipped with devices for antigen capture,⁵⁰ although the actual antigen capture is primarily mediated by fluid-phase endocytosis.⁵¹ DCs randomly take up antigens that happen to be in the adjacent neighborhood. In the conjugated form of the CpG ODNs and antigen, however, CpG ODNs work like "glue" to facilitate the adhesion of the conjugate to the cell surfaces of APCs; CpG ODNs bind to cell surface receptors for DNA, which is followed by an increase in antigen uptake by APCs.⁵² A quantitative study revealed that antigen uptake mediated by DNA receptor-mediated endocytosis was 100 times more efficient than that through fluid-phase endocytosis. Thus, CpG ODNs conjugated to antigen exhibit joint properties as promoters of antigen uptake and DC activators, thereby potentiating the ability of DCs to generate Th1 cells (Fig. 4).

The colonization of CpG ODNs with TLR9 in the mature and acidified endosomes is an initial and essential step that triggers intracellular signals.^{16-18,53,54} CpG ODNs, acting as glue, first bind to cell surface receptors for DNA, but not to TLR9, because TLR9 is not expressed on the cell surface. Actually, CpG ODN-conjugated antigens were incorporated into APCs prepared from TLR9 KO mice to the same extent as those from wild mice (Fig. 5). The CpG ODN-conjugated antigen, however, failed to induce Th1 cells due to the lack of the costimulatory molecule expression and the IL-12 secretion in APCs prepared from TLR9 KO mice.⁵⁵

Exogenously administered CpG ODNs first bind to the cell surface receptors specific for DNA, and enter cells through receptor-mediated endocytosis⁵⁵ before colonization with TLR9 in the endosomal/lysosomal compartment.⁵⁶ When antigen is conjugated to ODNs, regardless of the presence or absence of CpG motifs in the ODN sequences, ODNs bind to DNA receptors on the cell surfaces to a comparable extent.^{55,57} After receptor-mediated endocytosis, ODNs with CpG motifs colocalize with TLR9 and trigger signals, whereas those without CpG motifs fail to do so (Fig. 4). These differences ultimately lead to the preferential induction of Th1 or Th2 cells by ODNs with or without CpG motifs, respectively.⁵⁵

FUTURE PROSPECTS

Human clinical trials for ragweed allergic rhinitis using CpG ODNs conjugated to the principal allergenic moiety of ragweed (Amb a 1) are currently in progress in the United States. Six injections of the conjugates over 6 weeks were reported to improve symptoms without any adverse effects.^{58,59} These and other studies show great promise for future break-

throughs in immunotherapy on the horizon.

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