

Review

From Bugs to Drugs: Therapeutic Immunomodulation with Oligodeoxynucleotides Containing CpG Sequences from Bacterial DNA

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ABSTRACT

Several types of immune cells possess pattern recognition receptors (PRR) that can distinguish prokaryotic DNA from vertebrate DNA by detecting unmethylated CpG dinucleotides in particular base contexts (CpG motifs). Bacterial DNA or synthetic oligodeoxynucleotides containing these CpG motifs activate both innate and acquired immune responses that have evolved to protect against intracellular infections. These T helper 1 (Th1)-like immune responses include activation of B cells, dendritic cells, macrophages, and natural killer (NK) cells. CpG DNA-induced immune activation can protect against infection either alone or in combination with a vaccine and is effective in the immunotherapy of allergic diseases and cancer. Human clinical trials using such CpG DNA are currently underway.

INTRODUCTION

WHEN THEY THINK OF DNA, most biologists are interested in its function as the genetic code or perhaps as a tool to use in various antisense, triplex, or aptamer approaches. However, some types of DNA can be immune stimulatory. Bacterial DNA, but not vertebrate DNA, can activate B cells to proliferate and secrete antibody and natural killer (NK) cells to lyse tumor cells and secrete interferon- γ (IFN- γ) (Fig. 1) (Messina et al., 1991; Tokunaga et al., 1984). The immune stimulatory effects of bacterial DNA lie in its content of unmethylated CpG dinucleotides in particular base contexts (Krieg et al., 1995). In contrast to bacterial DNA, vertebrate DNA contains a lower frequency of CpG dinucleotides (CpG suppression), and these are highly methylated, which prevents their immune stimulatory effects (Krieg et al., 1995).

The immune system may be thought of as consisting of two basic types of defenses. The phylogenetically older innate immune system, which is typified by such cells as macrophages, monocytes, neutrophils, and NK cells, detects pathogens by their interactions with pattern recognition receptors (PRR).

PRR accomplish this feat by interacting with certain molecular structures that are highly conserved among many classes of microbes but are not present in vertebrate cells. For example, vertebrates have evolved PRR to detect pathogen structures, such as lipopolysaccharides (LPS), peptidoglycans, viral double-stranded RNA (dsRNA) structures, and high-mannose proteins (Medzhitov and Janeway, 2000). Ligand binding to PRR rapidly triggers innate immune defenses, leading to release of cytokine and chemokine alarm signals within minutes to hours. Recent advances suggest that CpG motifs are yet another type of molecular structure that functions to alert innate immune defenses to the possible presence of pathogens.

In contrast to the limited specificity of innate immune PRR, the acquired immune system, exemplified by B cells and T cells, possesses highly specific antigen receptors that can distinguish extremely subtle structural differences between microbes. However, adaptive immunity suffers from the disadvantage that this extraordinary specificity requires gene rearrangement and about 10–14 days to develop after infection. Moreover, activation of the innate immune defenses is essential for the efficient initiation of acquired immunity. This review discusses the

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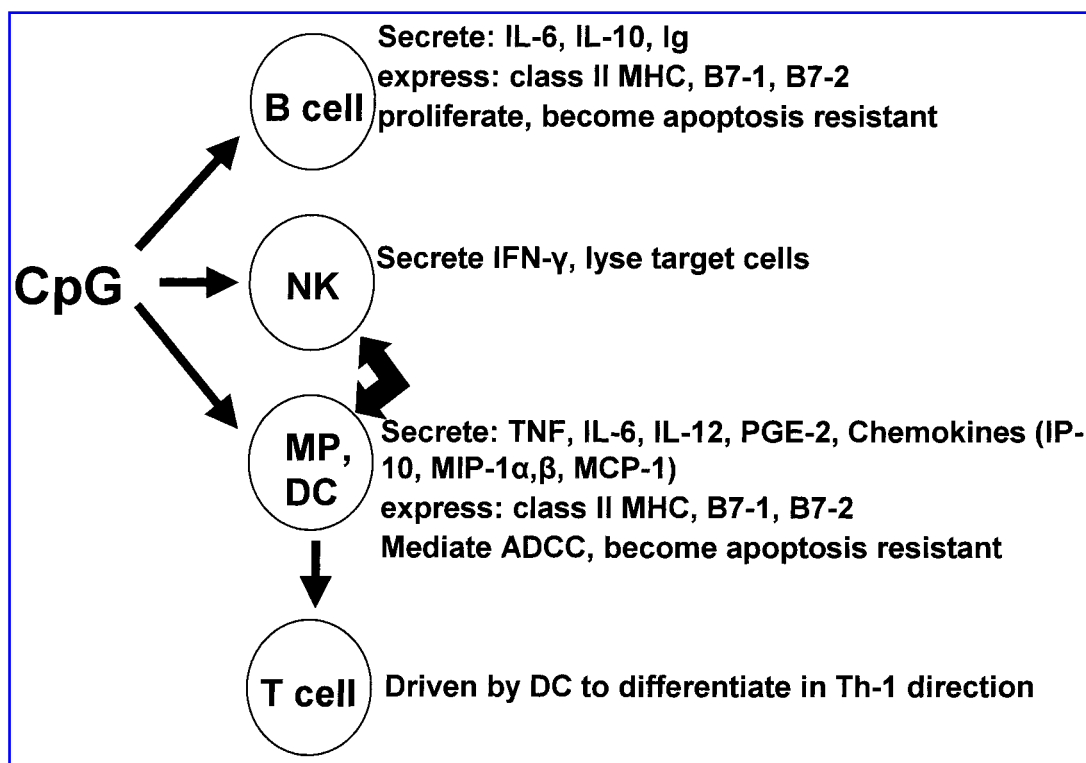


FIG. 1. Cellular effects of immune stimulatory CpG oligonucleotides. CpG oligodeoxynucleotides (ODN) stimulate B cells, NK cells, macrophages (MP), and dendritic cells (DC). Within a few hours, this activation causes the cells to secrete a variety of immune modulatory cytokines and other factors, as noted in the figure. The expression of costimulatory molecules that promote the generation of antigen-specific immune responses is increased within 24–48 hours. In addition, B cells, but not other cells, are induced to proliferate. CpG DNA protects several cell types against spontaneous or induced cell death through several mechanisms. CpG activates several cell types to mediate antibody-dependent cellular cytotoxicity (ADCC), whereby destruction of target cells that have antibodies bound to their surface is enhanced. Among the many secondary effects induced by CpG are the mutually stimulatory interactions whereby cytokines derived from NK or MP or DC stimulate each other. T cells are not directly activated by CpG DNA but are indirectly activated as a result of the enhanced activity of DC and other antigen-presenting cells (APC). T cells can differentiate in either a Th1 or Th2 direction. Differentiation toward Th1 is driven by cytokines, such as IFN- γ and IL-12, whose expression is induced by CpG DNA. Therefore, T cells that differentiate as a result of CpG stimulation become Th1-like T cells, which are associated with the development of cellular immune responses, such as cytotoxic T lymphocytes. Th2 differentiation is typically driven by different cytokines, such as IL-4 and IL-5, and promotes the development of humoral immunity as well as allergic responses that are mediated by IgE antibodies.

mechanisms through which exposure of CpG DNA leads to the activation of innate and acquired immune defenses and summarizes the potential therapeutic applications for this.

MOLECULAR MECHANISMS OF CpG DNA

Although there are cell surface proteins that can bind DNA, the immune stimulatory effects of CpG DNA require cell uptake (Fig. 2) (Krieg et al., 1995; Manzel and Macfarlane, 1999). This DNA uptake is inducible and varies with cell type but is sequence independent (Kimura et al., 1994; Krieg et al., 1991). Uptake probably involves both pinocytosis and endocytosis (Beltinger et al., 1995; Krieg 1995b). Endosomal acidification or maturation or both appear to be essential for CpG-induced immune stimulation, as this is completely blocked by drugs, such as chloroquine, that interfere with endosomal function (Macfarlane and Manzel, 1998; Yi et al., 1998b). The structure of the DNA molecule is also important because it must have an accessible 5'-end (Yu et al., 2000) and preferably has a TpC at the 5'-end (Hartmann and Krieg, 2000).

The nature of the intracellular receptor for CpG DNA is not yet clear. However, recent studies have defined a requirement for a member of the toll-like receptor family, TLR-9, in mediating the immune stimulatory effects of unmethylated CpG motifs (Fig. 2) (Hemmi et al., 2000). It is not yet clear whether TLR-9 directly binds to CpG DNA or whether there may also be TLR-9-independent mechanisms of action for some CpG motifs. The intracellular location of the CpG receptor seems logical if this detection pathway evolved as a defense against intracellular as opposed to extracellular pathogens.

Several intracellular signaling pathways appear to be activated by CpG DNA (Fig. 2). Within 7–10 minutes, the mitogen-activated protein (MAP) kinases p38 and the c-Jun NH₂-terminal kinases are activated in human and murine B cells and macrophages (Hacker et al., 1998; Hartmann et al., 2000; Yi and Krieg, 1998b). In addition, there is a rapid generation of reactive oxygen species (ROS), which appears to be important in the activation of NF- κ B (Sparwasser et al., 1997; Stacey et al., 1996; Yi et al., 1996b; Yi and Krieg, 1998a).

These signaling pathways converge on the nucleus, causing activation of multiple transcription factors and increased ex-

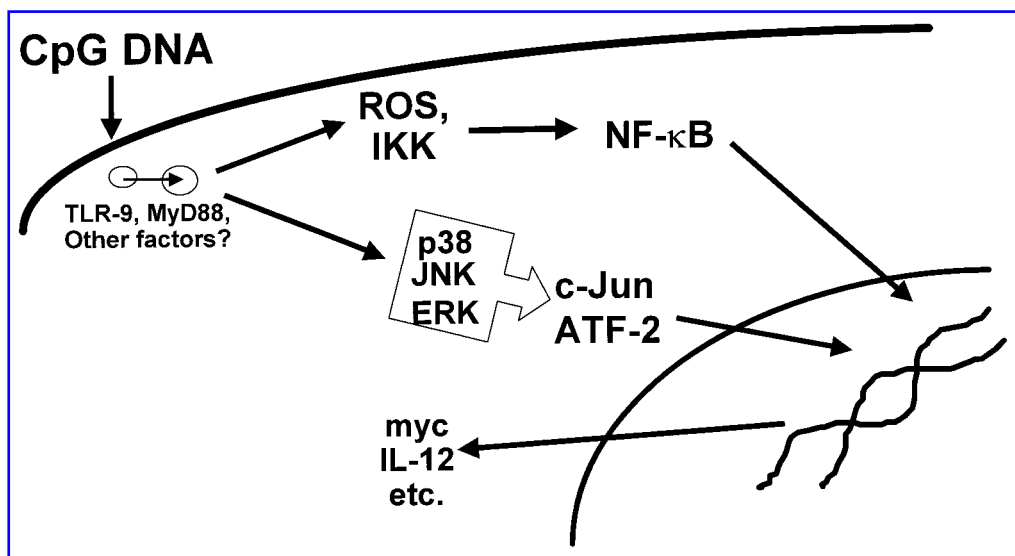


FIG. 2. Molecular mechanisms of CpG-mediated immune activation. CpG oligodeoxynucleotide(ODN) or bacterial DNA is internalized in a sequence-independent fashion into an endosomal compartment. It is possible that the recognition of CpG motifs, which requires TLR-9 and MyD-88, occurs in this endosomal compartment, but that has not yet been fully clarified. In any case, a signaling complex appears to be formed leading to the rapid generation of reactive oxygen species (ROS) and the activation of the inhibitor of NF- κ B kinase (IKK). A second set of signaling factors that are activated by CpG DNA is the mitogen-activated protein kinases, p38, JNK, and ERK. These separate signaling pathways lead to the activation of transcription factors, including NF- κ B, c-Jun, and ATF-2, which induce new gene transcription, such as the cell cycle regulatory gene *c-myc* and Th1-like cytokine genes, such as IL-12.

pression of multiple proto-oncogenes, antiapoptotic proteins, costimulatory molecules, and proinflammatory cytokines and chemokines (Bohle et al., 1999; Chace et al., 1997; Jakob et al., 1998; Redford et al., 1998; Schwartz et al., 1997; Sun et al., 1998b; Sweet et al., 1998; Vallin et al., 1999; Yi et al., 1996a, 1998a,b; Yi and Krieg, 1998a; Zhao et al., 1997). Overall, the most highly produced cytokines in the response to CpG are predominantly the Th1-like cytokines, such as interleukin-12 (IL-12), IL-18, IFN- α , IFN- γ , and tumor necrosis factor- α (TNF- α). However, CpG DNA also induces B cells to produce IL-10, which appears to act in a counterregulatory fashion to limit the inflammatory response to CpG (Redford et al., 1998).

INDUCTION OF Th1-LIKE IMMUNE RESPONSES BY CpG DNA

When the innate immune system is activated by a stimulus, it produces cytokines that determine the type of acquired immune response that will follow. If the innate immune activation is coupled to the production of Th1-like cytokines, such as IFN- γ , IFN- α , and IL-12, the B and T cell responses to any associated antigens will be dominated by a Th1-like response. In contrast, if the innate immune response occurs in a cytokine milieu that is dominated by Th2-like cytokines, such as IL-4, IL-5, and IL-10, associated antigens will more likely induce Th2-like B and T cell responses. Although the way in which the innate immune system decides whether to induce a Th1-like or a Th2-like immune response to an antigen is poorly understood, it appears that the dendritic cell (DC) is the key regulatory cell. CpG DNA is an extremely potent inducer of murine and human DC, triggering them to make high levels of Th1-like cytokines (Bauer et

al., 2001; Bohle et al., 1999; Brunner et al., 2000; Hartmann et al., 1999; Jakob et al., 1998; Schattenberg et al., 2000; Sparwasser et al., 1998; Stacey et al., 1996). Of note, neither IFN- γ nor IL-12 is essential for the ability of CpG DNA to induce Th1-like immune responses (Kline et al., 1999).

Strong CpG motifs are extraordinarily effective B cell mitogens, driving up to 95% of B cells into the cell cycle (Hartmann and Krieg, 2000; Hartmann et al., 2000; Krieg et al., 1995; Liang et al., 1996). The mitogen effects appear to be direct, as they are seen with highly purified B cells. All B cell subsets can be stimulated by synthetic oligodeoxynucleotides (ODN) containing CpG motifs. For ODN with a nuclease-resistant phosphorothioate backbone, the optimal CpG motif for driving murine B cell proliferation is GACGTT, whereas the optimal motif for driving human B cell activation is GTCGTT dinucleotide at the 5'-end (Hartmann and Krieg, 2000; Hartmann et al., 2000). CpG-induced B cells secrete IL-6, IL-10, and immunoglobulin, express increased cell surface levels of costimulatory molecules, and become resistant to apoptosis (Davis et al., 1998; Hartmann and Krieg, 2000; Redford et al., 1998; Wang et al., 1997; Yi et al., 1996a,b; Yi and Krieg, 1998a; Yi et al., 1998a). Similar effects are also seen with malignant B cells from lymphoma patients, which enter the cell cycle and become effective antigen-presenting cells (APC) for T cells (Decker et al., 2000).

CpG DNA is a potent activator of murine and human NK cells, driving them to secrete IFN- γ and to have increased lytic activity (Ballas et al., 1996; Cowdery et al., 1996; Iho et al., 1999; Klinman et al., 1996; Yamamoto et al., 1992). In the case of murine NK cells, CpG DNA does not appear to act directly but rather by inducing adherent cells (presumably macrophages or DC or both) to produce costimulatory cytokines, such as

IL-12, TNF- α , and type I IFN (Ballas et al., 1996; Chace et al., 1997; Cowdery et al., 1996). Human NK cells may be directly stimulated by CpG DNA, although this still appears to be enhanced by cytokine costimulation (Iho et al., 1999).

Although an initial early report using partially purified cells suggested that CpG DNA directly activates T cells (Klinman et al., 1996), subsequent studies have demonstrated that CpG DNA does not have direct stimulatory effects on resting T cells (Lipford et al., 1997a; Sun et al., 1998b). On the other hand, CpG DNA may be able to costimulate human T cells induced by extensive cross-linking of the T cell receptor (Iho et al., 1999).

ACTIVATION OF PROTECTIVE INNATE IMMUNITY BY CpG DNA

The intracellular location of the CpG receptor and the fact that CpG DNA triggers strong Th1-like immune responses suggest the possibility that immune recognition of CpG DNA may have evolved as a defense against intracellular bacteria, viruses, and retroviruses, as the nucleic acids of these pathogens would be potentially accessible to the CpG PRR and the Th1-like immune responses are most protective against infection with these agents. Remarkably, a single dose of CpG DNA protects for at least 2–4 weeks against lethal challenge with a broad range of pathogens, including *Listeria monocytogenes*, *Francisella tularensis*, anthrax, Ebola virus, malaria, leishmaniasis, and schistosomiasis (Elkins et al., 1999; Gramzinski et al., 2001; Krieg et al., 1998; Lipford et al., 1997b; Oxenius et al., 1999; Walker et al., 1999; Weighardt et al., 2000; Zimmermann et al., 1998). Although the exact mechanisms of protection are not yet clear, IFN- γ and IL-12 are required, presumably reflecting activation of nonspecific innate immunity rather than accelerated development of specific immunity. Nevertheless, mice can develop protection against repeat challenge, consistent with the concomitant development of adaptive immune responses. Of note, the protective effect of CpG DNA against *Leishmania* infection is sustained for more than 5 weeks if the CpG is administered in a formulation that may function as a depot for slow release, such as alum (Stacey and Blackwell, 1999).

CpG DNA AS AN INDUCER OF ACQUIRED IMMUNITY

As noted, activation of innate immunity is required for the efficient generation of acquired immune responses, such as antibody production and generation of cytolytic T cells (CTL). Several characteristics of CpG DNA suggest its ability to boost acquired immune responses and, therefore, function as a vaccine adjuvant. First, by activating DC, CpG DNA should enhance their ability to present antigens to T cells. Because it creates a Th1-like cytokine environment, this should result in the generation of Th1 T cells and CTL activity. Second, the B cell-stimulatory effects of CpG DNA suggest that it should turn them into effector APC, which may also be able to stimulate naive T cells or at least restimulate memory T cells. In addition, CpG DNA-induced B cell activation shows synergy with signaling through the B cell antigen receptor (Krieg et al., 1995).

Thus, B cells specific for an antigen administered along with the CpG DNA would be synergistically activated, thereby promoting the development of antigen-specific antibody production. CpG DNA also drives isotype switching of B cells, leading to maturation of the humoral immune response (Davis et al., 1998). Finally, by blocking the apoptosis of B cells, CpG DNA should further enhance sustained antibody responses. Numerous investigators have now demonstrated that CpG DNA is an extraordinarily effective adjuvant for inducing antigen-specific humoral and cellular immune responses against model protein and peptide antigens (Chu et al., 1997; Lipford et al., 1997a; Roman et al., 1997; Sun et al., 1998a), viral and bacterial proteins (Davis et al., 1998; Moldoveanu et al., 1998), tumor antigens (Sun et al., 1996; Weiner et al., 1997), and even some polysaccharides (Chelvarajan et al., 1999). In addition to these studies in mice, CpG DNA is an effective adjuvant in primates (Davis et al., 2000; Jones et al., 1999). In a recent human clinical trial as an adjuvant for the hepatitis B vaccine, the addition of CpG DNA boosted antibody responses, especially to the priming dose of the vaccine (H.L. Davis et al., manuscript in preparation).

The adjuvant activity of CpG DNA does not appear to be dependent on any particular route, as it has been seen with intramuscular, subcutaneous, intranasal, and even oral routes of delivery (McCluskie and Davis, 1998; McCluskie et al., 2000; Moldoveanu et al., 1998). Although immunization during the neonatal period is usually inefficient and biased toward Th2-like immune responses, the addition of CpG DNA allows effective induction of Th1 immune responses (Brazolot Millan et al., 1998; Kovarik et al., 1999). CpG motifs also appear to be essential for the efficacy of DNA vaccines (Gramzinski et al., 1998; Krieg, 1996; Sato et al., 1996).

APPLICATIONS OF CpG DNA IN CANCER IMMUNOTHERAPY

As noted, CpG DNA is an effective adjuvant for tumor vaccines. However, it is also noteworthy that the innate immune activation induced by CpG DNA can also protect against tumor challenge in certain model systems (Dow et al., 1999). Daily injection of CpG DNA into established tumors can induce tumor regression (Carpentier et al., 1999; Smith and Wickstrom, 1998). Indeed, the original discovery of the immune stimulatory properties of bacterial DNA was based on its ability to cause regression of established tumors after intralesional injection (Tokunaga et al., 1984).

Several conclusions emerge from different studies using CpG ODN as adjuvants in various experimental systems. First, the ODN are extremely effective Th1 adjuvants, inducing stronger responses than the gold standard Th1 adjuvant, complete Freund's adjuvant. CpG DNA boosts antibody titers 10–100-fold, especially boosting the isotypes of antibody that are associated with Th1-like responses. CpG DNA also induces the generation of CTL responses, even when it is combined with other Th2-inducing adjuvants, such as alum or cholera toxin. CpG DNA allows a 10–100-fold reduction in the dose of antigen, presumably because of the increased efficiency of APC antigen presentation. CpG DNA appears to be effective in all types of formulations, including saline, emulsion, and lipo-

some, and for all forms of vaccines and antigens, including peptides and live vaccines, with the possible exception of some polysaccharide antigens. Given the very high stability of CpG ODN and the simplicity of low cost production, these qualities appear to make CpG DNA an extremely favorable choice for incorporation into any vaccine where a Th1-like response is desired.

Another method by which CpG DNA can be used for tumor immunotherapy is in combination with passive immune therapy protocols, such as monoclonal antibodies (mAb). Antitumor antibodies bind to the tumor surface and are thought to mediate at least some of their activity through the mechanism of antibody-dependent cellular toxicity (ADCC). Innate immune activation by CpG DNA should dramatically enhance ADCC, improving the antitumor effect of antibody immunotherapy. The addition of CpG DNA to an antitumor antibody immunotherapy in a mouse model improved the long-term survival from approximately 10% to approximately 80% (Wooldridge et al., 1997).

UTILITY OF CpG DNA IN IMMUNOTHERAPY OF ASTHMA AND ALLERGIC DISEASES

The frequency of asthma and allergic diseases in industrialized countries has been increasing dramatically in the past few decades and has even been considered to be an epidemic (Erb, 1999; Kline, 2000). Although the cause of this epidemic is unclear, there has been increasing recent interest in the hygiene hypothesis, that is, that a decrease in frequency of Th1-like infections during childhood due to improvements in hygiene, increased vaccination and use of antibiotics, and smaller family sizes, may result in the initiation of Th2-like responses to common environmental antigens as a default pathway. These Th2-like responses are known to cause allergic disease. Recent studies in mice support this hypothesis by demonstrating that CpG DNA not only can prevent the sensitization of mice to allergens but also can reverse established Th2-like airway inflammation (Broide et al., 1998; Kline et al., 1998; Peng et al., 2001; Serebrisky et al., 2000; Shirota et al., 2000; Sur et al., 1999). Surprisingly, this effect of CpG DNA does not require the presence of either IL-12 or IFN- γ (Kline et al., 1999). In addition to reducing allergen-induced airway inflammation, systemic administration of CpG DNA also blunts the pulmonary inflammatory response to inhaled endotoxin (Schwartz et al., 1999). Nevertheless, inhalation of CpG DNA can cause acute pulmonary inflammation (Schwartz et al., 1997).

ABILITY OF CpG DNA TO INDUCE AUTOIMMUNITY

Because of its potent immune stimulatory effects, it may be expected that CpG DNA would cause systemic autoimmune diseases. However, despite early suggestions that CpG DNA may be a trigger for systemic lupus erythematosus (SLE) (Krieg, 1995a), subsequent studies have demonstrated that treatment of normal or lupus-prone mice neither induces nor aggravates autoimmune disease but actually reduces its severity (Gilkesson et al., 1996, 1998; Mor et al., 1997)! On the other

hand, CpG DNA can be used as an adjuvant to induce autoimmune responses against microbial antigens that are molecular mimics of self-antigens (Bachmaier et al., 1999) and against a central nervous system antigen, myelin basic protein, causing experimental autoimmune encephalomyelitis (EAE) (Segal et al., 1997). In other experimental systems, the administration of CpG DNA can either aggravate or ameliorate EAE disease severity (Boccaccio et al., 1999; Tsunoda et al., 1999). These effects may not be surprising, as administration of IL-12 has also shown variable results in autoimmune models depending on the timing and dosage of administration. DNA vaccines containing CpG motifs are protective against EAE (Lobell et al., 1999; Ruiz et al., 1999). In general, these studies provide encouragement that the administration of CpG DNA as an adjuvant for foreign antigens should be safe and effective. If one considers teleology, this may not be surprising because the immune stimulatory response has presumably evolved to activate protective mechanisms without major breakdowns in self-tolerance.

DISCUSSION

Immune recognition of unmethylated CpG motifs shows the ability of the innate immune system to detect molecular patterns that distinguish pathogens from self. The immune activation triggered by CpG DNA is effective at inducing innate and acquired immune responses, which are biased toward Th1 immunity. CpG DNA has shown efficacy in a variety of preclinical disease models as an inducer of innate immunity, an adjuvant for vaccination, and an immunotherapeutic for allergic diseases and cancer. More than ten human clinical trials using CpG DNA are already underway. In the coming years, the therapeutic potential of this remarkable immunomodulator should become clear, along with a better understanding of its molecular mechanisms of action.

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