

Adjuvant Properties of CpG Oligonucleotides in Primates

Daniela Verthelyi

Summary

Unlike mammalian DNA, bacterial, plasmid, and synthetic DNA containing unmethylated CpG dinucleotides in specific sequence contexts are recognized by the Toll-like receptor 9 expressed by B cells and plasmacytoid dendritic cells, and trigger the activation of the innate and adaptive immune system. Upon signaling, CpG DNA induces B cells, natural killer cells, macrophages, and dendritic cells to proliferate, differentiate, take up, and present antigen and secrete a variety of immunoglobulins, chemokines, and predominantly Th1-type cytokines. Preclinical studies in mice and primates show that DNA sequences containing CpG motifs can selectively promote cellular and/or humoral immune responses in vivo. Early results from ongoing clinical studies indicate that CpG oligonucleotides (ODN) are well tolerated and improve the immune response to microbial vaccines. This work examines the progress in utilizing CpG ODN as adjuvants in conventional and DNA vaccines.

Key Words: CpG ODN; TLR-9; innate immunity; vaccine adjuvant; DNA vaccines.

1. Introduction

The concept of immunological adjuvants was first described by Ramon in 1924 as “substances used in combination with a specific antigen that produce more immunity than the antigen alone” (1). Indeed, adjuvants can improve the response to vaccines by (1) increasing the immunogenicity of a weak antigen, (2) accelerating and prolonging the immune response to the antigen, and (3) modulating the cytokine milieu to favor Th1- or Th2-type immune responses to the antigen. Despite intensive research, in the intervening 80 yr, the Food and Drug Administration (FDA) has only approved aluminum-based mineral salts (ALUM) as vaccine adjuvants. Although Alum enhances antibody responses, it is not effective as promoter of cell-mediated immunity or mucosal protection.

In recent years, well-defined synthetic molecules that mimic pathogen associated molecular patterns (PAMPs) have been developed. These PAMPs activate the innate immune system acting on germ-line encoded pattern recognition receptors (PRR), including Toll-like receptors (TLR), expressed on granulocytes, monocytes, B, natural killer (NK), and dendritic cells (DCs). Activation of the innate immune system by this route results in the production of reactive oxygen and nitrogen intermediates (ROI and RNI), cytokines, chemokines, and polyreactive antibodies as well as enhanced antigen presentation to the cells of the adaptive immune system. Whereas some TLR ligands can be very toxic, others can be harnessed to act as effective vaccine adjuvants. Among the candidates for vaccine adjuvants, are the CpG oligonucleotide (ODN), synthetic strands of single-stranded DNA encoding one or more unmethylated CpG motifs that mimic bacterial DNA. Combined with protein antigens or DNA vaccines CpG ODN support the induction of strong Th1-type responses, characterized by the generation of CTL and the secretion of IFN γ when administered by parenteral, oral, or mucosal routes (reviewed in [ref. 2](#)). This chapter focuses on the studies to assess the safety and effectiveness of CpG ODN as adjuvants to conventional and DNA vaccines.

1.1. CpG ODN

Immunostimulatory bacterial products were first used as vaccines adjuvants over 60 yr ago. Pisetsky ([3](#)) and Tokunaga ([4](#)), respectively, realized that bacterial DNA stimulated B-cell and NK-cell activity, however it was not until 1995 that Arthur Krieg and Dennis Klinman identified the contribution of the unmethylated CpG dimers (CpG motifs) to the immunogenicity of bacterial preparations ([5](#)). The activity of bacterial DNA, or the synthetic oligodeoxynucleotides (CpG ODN) that mimic it, was first characterized in mice. They stimulate a polyclonal B-cell activation characterized by cell proliferation, reduced apoptosis, secretion of interleukin (IL)-6, IL-10, and polyclonal IgM, and the upregulation of costimulatory molecules such as class II major histocompatibility complex (MHC), CD80, CD86, and CD40 ([6](#)) (*see Fig. 1*) ([7](#)). CpG ODN also act on professional antigen-presenting cells (APC) monocytes, macrophages, and DCs, increasing their antigen uptake and presentation as well as augmenting the expression of costimulatory molecules, and the secretion of pro-inflammatory cytokines such as IP-10, type 1 IFNs, and IL-12 ([8–13](#)). These cytokines, in turn, activate NK cells, CD4⁺, CD8⁺, and $\gamma\delta$ T cells enhancing their lytic activity and the secretion of high levels of IFN γ ([14–17](#)). The increased antigen presentation in a Th1 cytokine milieu supports antigen-specific humoral and cellular responses. The ability of CpG ODN to promote antigen crosspresentation by DCs to prime CD8⁺ T in a CD4⁺ T-

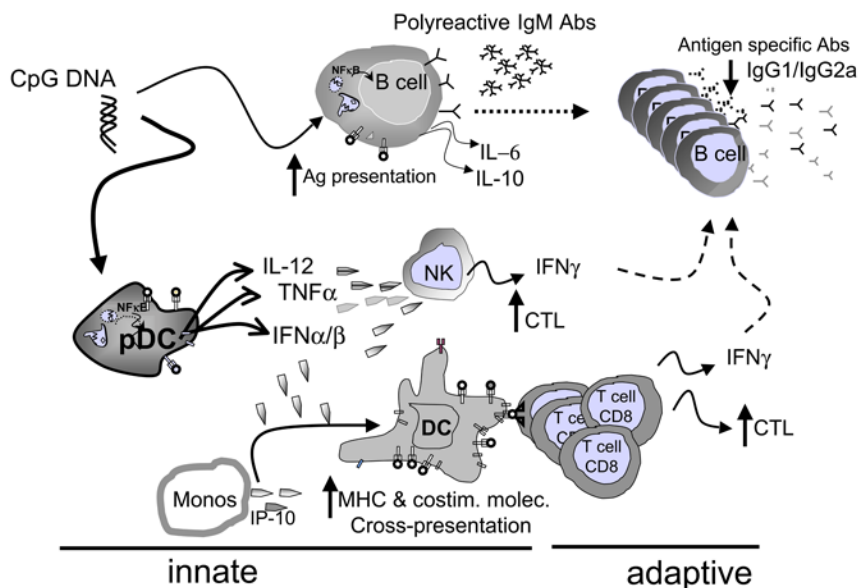


Fig. 1. Immunomodulatory effects of DNA containing unmethylated CpG motifs on the immune system. CpG DNA triggers polyclonal activation of B cells and stimulates plasmacytoid dendritic cells (pDC). The pDC mature and produce high levels of IFN α/β , which in turn activate natural killer cells to produce IFN γ and foster the maturation of monocytes into mDC. These active and mature mDC present antigen to CD4 $^{+}$, CD8 $^{+}$ T cells increasing the cellular and humoral immune response.

cell-independent manner make these adjuvants particularly attractive for use in immunocompromised HIV-infected subjects (18,19).

1.2. Types of CpG ODN

Although the recognition of bacterial DNA is conserved from fish to men, CpG ODN motif recognition by TLR9 has diverged throughout evolution, so that the specific motifs that activate rodent immune systems are only weakly stimulatory in primates and vice versa. Two distinct types of CpG ODN termed K and D ODN that are immunostimulatory for primates have been identified (14,20–22). In addition, a third type of CpG ODN, class “C,” has been recently developed that combines features of both D and K type ODN (21,22).

Type “K” (also known as B [7]) ODN encode multiple TCG motifs on a phosphorothioate backbone, and primarily stimulate B-cell proliferation and IgM, IL-10, and IL-6 secretion (see Fig. 2). On pDC, the K ODN promote maturation (defined by upregulation of CD80, CD86 and MHCII), increased survival and TNF α and IL-8 secretion, but induce low and transient secretion

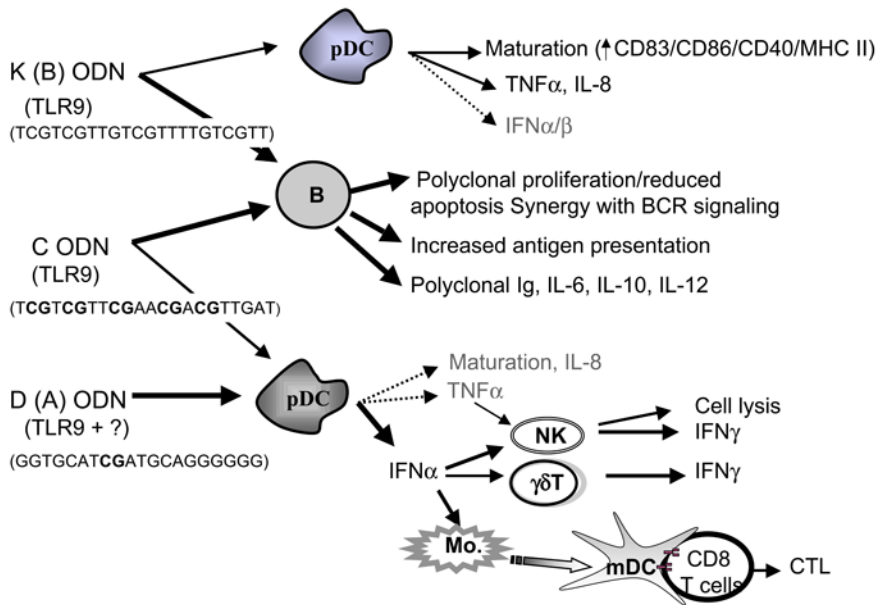


Fig. 2. CpG DNA activate the innate immune system by acting on TLR9-bearing B cells and plasmacytoid dendritic cells (pDC). The activation of T cells, natural killer (NK) cells, and monocytes is indirect. B cells proliferate and secrete IgM, IL-6, and IL-10 and upregulate antigen presentation along with cross-presentation on major histocompatibility complex I. pDC mature and secrete high levels of tumor necrosis factor (TNF) α and type I IFNs leading to NK cell activation and the maturation of monocytes into functional active mDC. The net result of CpG DNA mediated activation of the immune system is increased antigen presentation in a strong Th1 cytokine milieu.

of type I IFN that is only evident at very low ODN concentrations (23). ODN type D (also known as A [24]) have more complex structures marked by flexible mixed phosphodiester/phosphorothioate backbones and a self-complementary core sequence that is thought to fold into a stem-loop structure exposing the single hexameric purine/pyrimidine/CG/purine/pyrimidine motif on the apex. At the 3'-end, the molecules are capped by poly G tails that combine to form G tetrads (14,25). Although D ODN act through the TLR9/MyD88 pathway, they are unable to stimulate B cells and induce only weak maturation on pDC (see Fig. 2) (14,23). Instead, D ODN trigger high and sustained levels of IFN α/β secretion by pDC that mediates monocyte activation and maturation into functionally active DC, as well as NK and $\gamma\delta$ T cells activation with increased cell lysis and IFN γ secretion fostering a strong cellular immune response (11,12,14,20,23,26). The third class of ODN (Type C), are hybrid molecules, in that they have a phosphorothioate backbone, and several K-like

TCG motifs on the 5'-side, as well as a hairpin forming region on the 3'-end that allows it to stimulate the production of type I IFNs from pDC. C type ODN induce IL-6 and Ig levels comparable with those induced by K ODN, but the levels of IFN α secreted are lower than those achieved with D ODN. Given their potential for induction of strong cellular and humoral adaptive immune responses, these ODN are being actively studied for clinical application.

1.3. CpG ODN Uptake and Cellular Activation

The role of TLR9 as a CpG ODN receptor was first suggested by studies in mice lacking MyD88 and TLR9 and then confirmed by showing that unresponsive human HEK293 cells were activated by K type ODN when transfected with human TLR9 (27–30). The interaction between CpG ODN and TLR9 is highly specific because a single base change in the ODN sequence can abrogate the response. Indeed, this receptor confers the species specificity to ODN recognition as HEK293 cells transfected with human TLR9 responded to K-ODN but not to murine CpG ODN, and vice-versa (29,30). Unlike other TLR receptors, which are present on the cell membrane, TLR9 is located on the inner surface of the endoplasmic reticulum of resting B cells and pDC and migrates to the endosomes upon cell activation (29,31).

Synthetic ODN are internalized rapidly by monocytes, DCs, and B cells regardless of the presence of a CpG motif (32). Although direct interaction has not been confirmed, after uptake, K ODN travel to the late endosomes where they colocalize with TLR9 and adapter molecule MyD88 (33). Localization of the ODN to the endosomes is blocked by wortmanin suggesting that phosphatidylinositol-3 kinase plays a role in ODN trafficking (34). Once in the endosomes, maturation and acidification of the endosomes are necessary as blockers of endosome acidification (chloroquine or bafilomycin) prevent TLR9 mediated activation (7). Recent studies suggest that optimal signaling requires recruitment of two or more receptors as ODN that have multiple CpG motifs (K and C ODN) or polymers of a single CpG motif (D ODN) have stronger stimulatory effects (25,35). TLR activation is followed by the sequential recruitment and phosphorylation of MyD88, IL-1 associated kinase (IRAK)-4, and tumor necrosis factor (TNF) receptor associated factor-6 (TRAF6) leading to I κ B degradation and nuclear translocation of NF κ B.

The stimulatory pathway for D ODN is less well defined, as they are unable to stimulate primary B cells, B-cell lines bearing TLR9, or HEK293 cells transfected with human TLR9 (14,32,36). A recent study in mice using D-type ODN shows that the IFN α secretion induced by D ODN was absent in cells from TLR9 or MyD88 KO mice (26), leading to the speculation that in addition to TLR9, one or more co-receptors, chaperones, or adaptor molecules are involved in the homing/signaling of D ODN (36).

2. Results and Discussion: CpG ODN as Vaccine Adjuvants

2.1. Studies in Mice

Early studies assessing the adjuvant effects of CpG ODN showed that CpG ODN induced high levels of IL-12 and IFN γ secretion by mouse macrophages and NK cells. Further, when co-administered with hen egg lysozyme (HEL), CpG ODN induced high levels of HEL-specific IFN γ secreting cells (37). These studies were followed by the demonstration that CpG ODN enhanced the specific immunity to recombinant hepatitis B-surface antigen (38). Effectiveness as a vaccine adjuvant for infections that require a Th1-type immune response was established when Walker et al. demonstrated that co-administration of CpG ODN with *Leishmania* major antigens to susceptible Balb/c mice resulted in protection of 40% of the animals from subsequent challenge (39). These studies were successfully replicated in several murine models of vaccines where protection against challenge required humoral and/or cellular adaptive immune responses (40–42) (Table 1).

To optimize the availability and half-life of CpG motifs several modifications have been devised (Table 2) (43): the most widely used is the substitution of one or more oxygen moieties with sulfur groups on the ODN backbone creating phosphorothioate ODN that have a prolonged half-life (30–60 min compared with 5–10 min for phosphodiester) in vivo (43). Other changes include the point substitutions for synthetic bases such as nucleosides (44,45), or the co-administration of immunostimulatory sequences with cytokines or chemokines to increase uptake by specific cell types (Table 2). In addition, several studies showed that the adjuvant properties of CpG DNA are improved 10- to 100-fold when the ODN and the antigen are in close proximity and likely to be taken up by the same cell. A number of mechanisms have been designed to achieve this, ranging from the simple induction of a depot effect using mineral oils, to lipid emulsions, microparticles, nanospheres, and direct physical conjugation (Table 2) (46–48).

Although vaccines have been traditionally administered by intramuscular (IM), subcutaneous (SC), or intradermal (ID) route, the vast majority of infectious diseases are transmitted via the gastrointestinal, respiratory, or genitourinary mucosa. Use of CpG ODN as adjuvants in intranasal, oral, or intrarectal vaccines resulted in increased systemic and mucosal immune responses in murine models of hepatitis B (HBV), tetanus toxoid, and influenza (49–51). The responses were characterized by high levels of antigen-specific IgA in local and distant mucosal secretions (e.g., saliva, feces, lung washes) as well as increased systemic IgG2a, CTL and antigen-specific IFN γ (reviewed in ref. 51) suggesting that CpG-containing vaccines administered by these routes may provide both systemic and mucosal protection.

Table 1
CpG ODN as Vaccine Adjuvants

Species	Infectious agent	Reference
Conventional vaccines		
Mice/primates	Papilloma virus	84
Mice/primates	Hepatitis B	38,62,65
Mice/primates	Malaria	64,85,86
Mice/primates	Leishmaniasis	20,39,87
Mice	Herpes	88
Mice	Brucellosis	85
Mice	Chlamydia	89
Mice	HIV	90
Mice	Aspergillosis	91
Mice	Mycobacteria	92
Mice	Tripanosomiasis	93,94
Guinea pigs	Foot and Mouth Disease	95
Pigs	Actinobacillus pleuropneumoniae	96
DNA vaccines		
Primates	HBV	97
Chicken	Bursal disease virus	98
Mice	Hepatitis B	99
Mice	Hepatitis C	100
Mice ^a	HIV	60,101
Mice	CMV	102
Mice	Dengue	103
Mice	Listeria	104

^aAdditional CpG cloned into a second co-administered plasmid.

It is important to note that CpG ODN do not improve the efficacy of all vaccines in mice, and that increased immune responses to a pathogen do not necessarily correlate with better clinical outcome. Two recent studies illustrate this: Prince et al. showed that cotton rats immunized transmucosally with respiratory syncytial virus fusion protein and CpG ODN developed higher titers of neutralizing antibodies and reduced viral load upon subsequent live challenge. However, lung inflammation in these animals was more severe than in control animals (52). Similarly, mice vaccinated subcutaneously with *Helicobacter pylori* lysate plus CpG ODN had a 10-fold reduction in the number of *H. pylori* in their gastric mucosa but aggravated gastritis (53).

Table 2
Mechanisms for Increasing the Effectiveness of CpG ODN as Adjuvants

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1. Delivery of the CpG ODN and the antigen to the same cell
 - a. Depot effect (e.g., mineral oils) ([105,106](#))
 - b. Liposomes ([48,107](#))
 - c. Micro- and nanospheres ([46,108](#))
 - d. Direct conjugation to antigen ([47](#))
 - e. Plasmid manipulation to encode increased number of CpG motifs ([60](#))
 2. Increasing the ODN half-life
 - a. Backbone modifications (e.g., phosphorothioate)
 - b. Synthetic bases ([109](#))
 - c. Termolabile groups (Daniela Verthelyi, submitted)
 - d. Virus particles ([110](#))
 3. Homing of the ODN to specific tissues or cells
 - a. Co-administration with cytokines or chemokines ([111](#))
 - b. Cationic microparticles ([112](#))
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2.2. CpG Motifs and DNA Vaccines

CpG motifs contribute to the immunogenicity of DNA vaccines. Sato et al. ([54](#)) first provided evidence that these motifs were important to vaccine immunogenicity by substituting a CpG motif containing an ampr gene for a kanr gene in a β -galactosidase expression plasmid. This re-engineered plasmid elicited a higher (>200-fold) IgG antibody response, more CTL activity, and greater IFN γ production than the original vector. Klinman et al. ([55](#)) confirmed the importance of CpG motifs in DNA vaccine immunogenicity, in a study showing that elimination of CpG motifs by selective methylation reduced the ability of a DNA vaccine to induce cytokine production in vitro and antibody responses in vivo. In addition to stimulatory motifs, utilizing DNA from adenovirus, Krieg et al. ([56](#)) identified neutralizing or suppressive DNA sequences and demonstrated that the removal of these neutralizing sequences also resulted in improved immunogenicity. A number of vectors have been designed since to optimize the ratio of stimulatory to neutralizing (or suppressive) motifs in neonate and adult mice ([55,57–59](#)).

In addition, to engineering CpG motifs into the DNA vaccines, additional CpG motifs have been administered as synthetic ODN and in co-administered plasmids. For example, Kojima et al. ([60](#)) co-administered plasmids encoding 20 CpG motifs with a DNA vaccine encoding the envelope glycoprotein of HIV to BALB/c mice to increase the HIV-specific cell mediated and humoral immunity. However, some studies have raised the concern that administration of synthetic ODN together with a DNA vaccine may result in reduced antigen expression ([61](#)).

2.3. Studies in Primates

Nonhuman primate peripheral blood mononuclear cells (PBMCs) (including chimpanzees, aotus monkeys, rhesus macaques, and cynomolgus macaques) respond to the same D-, K-, and C-type ODN sequences with the same cytokine profile as human PBMC (20,62). This makes primates the preferred species for preclinical studies (63). Early studies in *Aotus* monkeys showed that co-administration of *Escherichia coli* DNA with a DNA vaccine encoding hepatitis-B surface antigen (HBs) augmented the HBs-specific antibody response sixfold. Jones et al. (64) reproduced the effect of *E. coli* DNA using K-type ODN as adjuvant for PADRE-45, a malaria peptide, in a Montanide 720 emulsion. Similarly, co-administration of CpG ODN type K and Engerix B—a licensed HBV vaccine—in cynomolgus macaques, induced a 60-fold increase in IgG anti-HBs Ab levels after priming and a 15-fold increase in boosted animals when compared with vaccine alone (62). In the same study, the Ab response of chimpanzees ($n = 2$) to Engerix B was boosted two- to threefold by the addition of K-type ODN (62). The adjuvant activity was not limited to K ODN because a later study showed similar results utilizing ODN type D (65).

The choice for optimal CpG ODN adjuvant appears to depend on the pathogen as well as on the type of response required for protection, as K-, but not D-type ODN improved the antibody response to anthrax recombinant protective antigen (see Fig. 3). The opposite was true in a model of cutaneous leishmaniasis, where protection requires a strong cellular immune response characterized by high levels of IFN γ and low levels of IL-10. In this model, animals immunized with heat killed *Leishmania* vaccine (HKL V) and D ODN were protected from a live parasite challenge 14 wk after immunization, whereas those that received HKLV alone, or HKLV plus K or control ODN were not (see Fig. 3) (20). Similarly, D ODN improved the response to leishmanization, the controlled induction of leishmaniasis employed in several countries as a means of vaccination (65a). Indeed, D ODN were more effective at controlling *Leishmania* lesion size than other TLR7, 8 or 9 ligands (*manuscript in preparation*). These results suggest that D type ODN are more effective in inducing a protective cellular immune response, whereas the K-type of ODN appear to foster high antibody titers, and the selective application of one or the other will allow some control over the effectiveness of the immune response elicited.

2.4. CpG ODN as Vaccine Adjuvants in Immunocompromised Hosts

Immature or defective immune systems render neonates and immunocompromised patients particularly vulnerable to infection. For the same reason, vaccination of these subjects is often unsuccessful. Therefore, development of an adjuvant that improves the immune response in these popu-

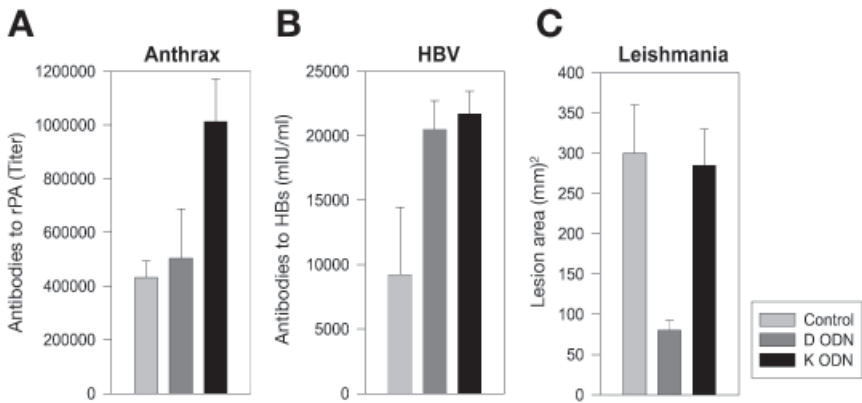


Fig. 3. Differential adjuvant activity of D and K oligonucleotides (ODN) in primate models. **(A)** Illustrates the mean maximal antibody titer in macaques ($n = 5\text{--}6/\text{group}$) immunized with anthrax recombinant protective antigen (rPA) in ALUM alone, or together with 500 μg of D or K ODN. Note that the addition of K ODN improved the antibody titer. **(B)** Antibodies to surface hepatitis antigen in macaques immunized with a pediatric dose of Engerix B, a licensed vaccine, alone or together with K or D ODN ($n = 5/\text{group}$). Note that both D and K ODN improve the titers of antibodies achieved. **(C)** Rhesus macaques were primed and boosted with heat killed *Leishmania* parasites in alum alone or with 500 μg of D or K ODN ($n = 6/\text{group}$). The figure illustrates the peak lesion size following a challenge with live *Leishmania amazonensis* 14 wk after priming. Note that macaques that received D ODN as vaccine adjuvant had a significant reduction in lesion severity.

lations is a priority. Studies conducted in young mice show that the in vitro response to CpG ODN is incomplete, with reduced activation of pDC, and lower cytokine levels (66–68). However, several studies have shown that CpG ODN can foster the priming of antibodies response to peptide and polysaccharide antigens in neonatal mice, suggesting that CpG ODN would be an effective adjuvant for vaccination in this population (66,68,69).

A study showing that CpG ODN improved the response to a hepatitis B vaccine in naturally unresponsive orangutans suggested that CpG ODN might be effective in immunocompromised adults (70). In vitro, the response of PBMC to K ODN is unaltered in HIV infected patients or simian immunodeficiency virus (SIV) infected macaques, whereas the IFN α and IFN γ responses to stimulation with D ODN are weak. The reduced responsiveness correlates with viral load and CD4T cell counts and it is possibly a result of the fewer and altered pDC and NK cells in these subjects (71). As observed in humans, SIV infected macaques are unable to mount a protective response to a hepatitis B vaccine. Using this model, we demonstrated that co-administration with K or

D CpG ODN, significantly improved the antibody titer in animals with viral loads $>10^7$ copies/mL. Clinical trials are underway to assess the safety and effectiveness of CpG ODN in these patients.

2.5. Human Trials and Safety of CpG ODN

Safety is an overriding concern in vaccine development. Concerns regarding the use of CpG ODN have been discussed in detail elsewhere (7,63,72). They center on the uncontrolled activation of the innate immune system leading to toxic shock, and the development of autoimmunity. The available studies show that systemic administration of ODN at doses used for vaccine adjuvants (5–100 μ g/dose) do not lead to adverse effects (72,73). At higher doses (>10 mg/kg) or when administered daily there is evidence of splenomegaly, draining lymph node hyperplasia, extramedullary hematopoiesis, complement and thromboplastin activation, and even death (74,75). These effects are largely sequence independent and can be attributed to the toxicity of the phosphorothioate backbones. Toxic shock was observed when therapeutic doses of CpG ODN were administered in conjunction with sublethal doses of LPS or in mice presensitized with d-galactosamine (76,77). Administration of CpG ODN at restricted sites, such as the intraarticular space leads to arthritis. The arthritis is mediated by increased local TNF α production, is dependent on the presence of the CpG motifs, and can be inhibited by local or systemic administration of suppressive ODN (78,79).

The results from studies in macaques at doses that are effective as vaccine adjuvant (<1 mg/kg or <5 mg/macaque) showed that there were no changes in body weight and the serology and hematology did not differ from that of untreated controls (20). At higher doses, thrombocytopenia, delayed coagulation, complement activation, and glomerulonephritis have been observed.

Hundreds of subjects have received CpG ODN in the context of more than a dozen ongoing phase I clinical trials testing the safety of D and K CpG ODN as vaccine adjuvants, or immunotherapeutics for allergy or cancer. Reports from two studies using K type CpG ODN in concert with a hepatitis B antigen show that addition of CpG ODN resulted in earlier seroconversion and higher anti-HBs Ab titers than vaccine alone (80). Adverse events observed to date mainly include injection site reactions (e.g., pain, redness, swelling, and induration). The most frequent systemic effects consisted of headaches and flu-like symptoms. None were severe (81–83). Although transient increases in antibodies to dsDNA have been observed, no autoimmune disorders have been reported.

Vaccines are still the most cost-effective manner of treating disease. With the advent of well-defined synthetic small molecules designed to mimic pathogen associated molecular patterns, it is now possible to envision the development of highly defined and reproducible synthetic vaccines that promote very

specific immune responses. The studies in primates have established that the different types of CpG ODN are effective as vaccine adjuvants in primates and have low toxicity. Importantly, because these adjuvants activate multiple cells of the innate immune system, they appear to be effective in even in immunocompromised subjects. Available data from the phase I clinical trials, including CpG containing DNA vaccines suggest that these adjuvants can be used safely in people. As clinical studies progress we will have a better understanding of the immunological effects of CpG ODN. Increased understanding of the intracellular activation pathways that allows for more selective triggering of immune responses is bound to minimize the adverse effects of these immunomodulators.

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