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CpG Still Rocks! Update on an Accidental Drug

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The discovery of the CpG motif in 1995 led to a change in the perception of the immune stimulatory effects of oligodeoxynucleotides (ODN) from an unwanted nonspecific effect to a highly evolved immune defense that can be selectively triggered for a wide range of therapeutic applications. Over the last decade dozens of human clinical trials have been conducted with different CpG ODN in thousands of humans for applications ranging from vaccine adjuvant to immunotherapies for allergy, cancer, and infectious diseases. Along with many positive results have come some failures showing the limitations of several therapeutic approaches. This review summarizes these results to provide an overview of the clinical development of CpG ODN.

Introduction

Experimental artifacts do not usually morph into drugs, but this review will provide an update to the story of one that did. In the early days of the antisense field it was realized that many antisense and control oligonucleotides were unexpectedly immune stimulatory, but this was seen as a quite undesirable toxicity rather than as a potentially useful property. Personally, I was struck by the magnitude of the B cell stimulatory effects induced by some of the "antisense" and control oligodeoxynucleotides (ODN) that I was using in studies of genes that could play a role in B cell activation. In fact, the magnitude of B cell proliferation induced by some of these ODN exceeded that of any other published B cell mitogen. Believing that there must be some underlying function for this effect, I decided to study it, culminating in my discovery that DNA containing CpG motifs activated an immune defense mechanism (Krieg et al., 1995). This understanding made it possible to prevent much of the unwanted immune stimulation in the development of antisense oligonucleotides, either by avoiding CpG motifs in the selection of antisense sequences, or by incorporating 5-methylcytosine, thereby reducing the immune activation. Furthermore, this discovery showed that the immune stimulatory effects of CpG motifs were not a nonspecific toxicity, but actually a highly evolved immune defense mechanism in which the presence of bacterial DNA alerts the immune system to the presence of infection, triggering a broad range of synergistic pathways aimed at containing and then eradicating infectious agents. These salutary effects of CpG ODN provided the basis for improved vaccines as well as immunotherapeutic for cancer, allergic, and infectious diseases, which are being pursued in a variety of ongoing clinical trials (Table 1).

Immune Effects and Mechanisms of CpG Motifs

Five years after the discovery of the CpG motif, Toll-like receptor 9 (TLR9) was identified as the mediator of the immune stimulatory effects of CpG ODN (Hemmi et al., 2000). Since then, a great many studies have explored the direct and indirect immune effects of TLR9 activation. One of the key characteristics of the TLR9-induced innate immune response is that it promotes the development of strong type 1 T helper cell (Th1) adaptive immune responses, including both antigen-specific antibody and $CD8^+$ T cell responses (Krieg, 2006). The family of Toll-like receptors evolved approximately 400 million years ago to detect diverse molecular structures that are present in pathogens but are either absent or sequestered in vertebrates (Kawai and Akira, 2011). The approximately 10 different TLR in vertebrates appear to function as an early warning system for the immune system, detecting the presence of infection by these molecular signatures. Some of the molecules detected by the TLR system are actually unique to pathogens, such as lipopolysaccharide, which is detected by TLR4. These TLR are expressed at the cell surface, where they are best positioned to detect these molecular structures of extracellular pathogens. Other molecular structures, such as CpG motifs, are not completely unique to pathogens but are present at a much lower level in vertebrates due to CpG methylation and suppression (Krieg, 2002; Kawai and Akira, 2011). Further, endogenous nucleic acids are normally sequestered from the endosomal compartment in which the immune system expresses the TLR that detect nucleic acids: TLR9 for DNA and TLR3, TLR7, and TLR8 for RNA structures. Additional immune receptors for nucleic acids are located in the cytoplasm but are outside the scope of this review.

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Table 1. Immune Stimulatory CpG Oligonucleotides in Clinical Development

Oligonucleotide	*Company or sponsor*	*Formulation type*	*Indication*	*Stage of development*
CPG7909	GSK	Vaccine with MAGE-A3 recombinant protein in AS15	Melanoma	Phase 3
CPG7909	GSK	Vaccine with MAGE-A3 recombinant protein in AS15	Lung cancer	Phase 3
CPG7909/NuThrax	Emergent/BARDA/ NIAID	Absorbed in anthrax vaccine	Prophylaxis of anthrax, biodefense	Completed phase 1
CPG7909	NIAID	Malaria vaccines	Prevention or treatment of malaria	Phase 2
CPG7909	Wakayama Medical University and University of Tokyo	Tumor peptide antigens in Montanide ISA 51	Esophageal cancer	Phase 1/2
CPG7909	Stanford University	Whole cell irradiated vaccine	Mantle cell lymphoma	Phase 1/2
CPG7909	Stanford University	Intratumoral injection with local irradiation	B cell lymphoma	Phase 2
CPG7909	MD Anderson Cancer Center, GSK	Vaccine with MAGE-A3 protein formulated in AS15 combined with systemic IL-2	Melanoma	Phase 2
CPG7909	NIAID	Mixed with J5-OMP vaccine	Prevention of meningococcal sepsis	Phase 1
CPG7909	Ludwig Institute for Cancer Research	Various tumor antigen peptides and Montanide	Advanced melanoma	Phase 1
CPG7909	University of Pittsburgh, NCI	Various peptides from NY-ESO-1 in Montanide ISA 720	Immunotherapy of tumors expressing NY-ESO-1	Phase 1
CPG7909	City of Hope Medical Center, NCI	CMV fusion peptide vaccine	Prophylactic vaccine against CMV	Phase 1
ISS1018/Heplisav	Dynavax	Vaccine with hepatitis B surface antigen	Prophylactic vaccination of populations with poor vaccine responsiveness	Completed phase 3, pending BLA
DV-601	Dynavax	Vaccine with surface and core hepatitis B virus antigens	Therapy of subjects chronically infected with HBV	Completed phase 1b
SD-101	Dynavax	Saline	HCV	Completed phase 1b
N8295	Dynavax	Conjugate of CpG ODN to influenza antigens NP and M2e, sometimes combined with H5N1 vaccine	Universal flu vaccine	Completed phase 1b
AZD1419	Dynavax/AstraZeneca		Asthma	Preclinical
IMO-2055	Idera	Saline	Various cancers alone or in combinations with chemotherapy	Phase 2
MGN1703	Mologen	Saline	Colorectal cancer	In phase 2
MGN1601	Mologen	Allogeneic renal cancer cell line	Renal cancer	Phase 1
DIMS0150/ Kappaproct	InDex Pharmaceuticals AB	Given by rectal administration	Refractory ulcerative colitis	Phase 2/3
GNKG168	SBI Biotech	Saline	CLL	Phase 1

Information collected from company websites and publications and www.clinicaltrials.gov; does not include programs known or believed to have been terminated.

BARDA, Biomedical Advanced Research and Development Authority; BLA, Biologics License Application; CLL, chronic lymphocytic leukemia; CMV, cytomegalovirus; GSK, GlaxoSmithKline; HBV, hepatitis B virus; HCV, hepatitis C virus; IL-2, interleukin-2; NCI, National Cancer Institute; NIAID, National Institute of Allergy and Infectious Diseases; ODN, oligodeoxynucleotides.

Structure-activity relationship studies of CpG ODN have defined 3 families with distinct structural and biological characteristics (Hartmann et al., 2003; Marshall et al., 2003; Vollmer et al., 2004). A-class CpG ODN are potent activators of natural killer cells and interferon alpha (IFN-α) secretion from plasmacytoid dendritic cells (pDC), but only weakly stimulate B cells. Canonical A-class ODN contain polyG motifs at the 5′ and/or 3′ ends, which are capable of forming complex higher-ordered structures known as G-tetrads and a central phosphodiester region containing 1 or more CpG motifs within a self-complementary palindrome (reviewed in Krieg, 2006). Typical B-class ODN have a completely phosphorothioate backbone, do not form higher-ordered structures, and are strong B cell stimulators, but induce relatively

little natural killer (NK) cell activity or IFN-α secretion (Krieg, 2002). The structure of C-class ODN is typically based on a phosphorothioate backbone, but is distinct in that the CpG motifs are followed by a 3′ palindrome, which may form a duplex. The C-class CpG ODN have immune properties intermediate between the A and B classes (Hartmann et al., 2003; Marshall et al., 2003; Vollmer et al., 2004; Marshall et al., 2005). None of these CpG ODN classes have any significant immune stimulatory effects in mice deficient in TLR9, and therefore they all require the presence of TLR9 (Vollmer et al., 2004). The mechanism through which the different classes of CpG ODN induce such divergent immune effects through TLR9 remains incompletely understood. It has been proposed that the IFN-α inducing effect of A-class ODN is mediated through interaction with CXCL16, which is expressed on pDC, but not B cells (Gursel et al., 2006). Some studies have also pointed to the possibility that the presence of the different ODN classes in distinct intracellular compartments may contribute to their differential immune effects (Honda et al., 2005).

Infectious Disease Applications of CpG ODN

CpG ODN monotherapy for the prevention or therapy of infectious disease

The Th1-like immune effects of TLR9 activation appear to have evolved not only to detect infection, but also and in particular to stimulate protective immunity against intracellular pathogens. Consistent with this role, studies from many investigators have demonstrated that prophylactic treatment of mice with a synthetic TLR9 ligand can provide transient protection against a wide range of viral, bacterial, and even some parasitic pathogens, including lethal challenge with some Category A bioterror agents or surrogates such as *Bacillus anthracis*, vaccinia virus, *Francisella tularensis*, and Ebola (reviewed in Krieg, 2007). Protection has been demonstrated through multiple routes of administration of B-class CpG ODN, including injection, inhalation, and oral administration. The duration of protection depends upon the specific model, and can range from several weeks to a single day. Although it is appealing to speculate that innate immune activation with CpG ODN could protect against bioterror attacks in humans, and it very well may, the clinical usefulness of the treatment may be complicated by the induction of flu-like symptoms from the CpG, which could create practical difficulties in determining whether people are actually infected or not.

One infectious disease that has been an interesting candidate for CpG ODN therapy is hepatitis C virus (HCV), which infects approximately 170 million people worldwide. Current therapy is based on sustained treatment with a combination of pegylated IFN and ribavirin, to which direct antiviral agents are being added. Infection is cleared spontaneously in about 20% of people who become infected, and this has been associated with the induction of Th1-like innate and adaptive immune activation leading to the development of a strong and diverse Th1 and CD8 cytolytic T cell response against the virus (reviewed in Houghton, 2011). Since TLR9 activation can drive a similar pattern of innate and adaptive immune responses to that seen in spontaneous clearance, we investigated whether A-, B-, or C-class CpG ODN may induce IFN-α secretion in the peripheral blood mononuclear cells (PBMC) of HCV-infected subjects. We found that a C-class CpG ODN, CPG 10101, induced particularly high levels of IFN-α secretion (Vicari et al., 2007), suggesting this agent could be an effective monotherapy against HCV. In a phase 1 dose-escalation study of CPG 10101 in healthy volunteers, CPG 10101 induced serum levels of IFN-inducible genes (Vicari et al., 2007). In a 4-week phase 1b blind, randomized, controlled trial involving 60 HCV-infected subjects, CPG 10101 monotherapy caused a dose-dependent decrease in blood viral RNA levels from baseline of up to 1.69 $\log_{10}$ at the highest dose level of 0.75 mg/kg weekly (McHutchison et al., 2007). Unfortunately, in further clinical trials there was no evidence for induction of an antiviral T cell response, which is thought to be required for clearing the infection, and the antiviral effect of CPG 10101 did not prove to be sustained; clinical development was terminated.

The development of CpG ODN as an adjuvant for infectious disease vaccines

TLR9 activation rapidly enhances innate immunity, which sets the stage for strong antigen-specific humoral and cellular responses. Mechanisms contributing to the strong adjuvant activity of B-class CpG ODN (with which most of the vaccine studies have been performed) for the humoral response may include: (1) synergy between TLR9 and the B cell receptor preferentially stimulating antigen-specific B cells (Krieg et al., 1995); (2) inhibition of B cell apoptosis improving B cell survival (Yi et al., 1998); and (3) enhanced maturation of the antibody response (Davis et al., 1998). TLR9 does not appear to be expressed in resting T cells, and so the enhanced development of antigen-specific $CD4^+$ and $CD8^+$ T cell responses in vaccines containing CpG ODN is attributed to the secretion of Th1-like cytokines and chemokines and to dendritic cell maturation and differentiation (Krieg, 2002).

B-class CpG ODN have been demonstrated to be highly effective vaccine adjuvants for inducing the preferred Th1 type of immune response in many experimental mouse models, using diverse antigens and formulations including peptide or protein antigens, live or killed viruses, dendritic cell vaccines, autologous cellular vaccines, and polysaccharide conjugates (Krieg, 2002). Other groups have focused on the use of A-class CpG ODN in virus-like particles, which also have demonstrated strong adjuvant effects (Jennings and Bachmann, 2008). C-class CpG ODN, too, show strong vaccine adjuvant effects in rodents and primates (Teleshova et al., 2004; Wille-Reece et al., 2005; Wille-Reece et al., 2006).

Many vaccine adjuvants that initially appeared promising in animal studies have failed to strongly boost immunity in humans. Fortunately, this has not been the case with CpG ODN, which have shown efficacy with generally good tolerability in multiple human trials. Several human clinical trials using B-class CpG ODN have demonstrated enhanced vaccine responses to hepatitis B surface antigen (HBsAg), either in combination with alum (Cooper et al., 2004a; Cooper et al., 2005) or alone (Halperin et al., 2003; Halperin et al., 2006). For example, a randomized, double-blind, controlled phase 1/2 dose-escalation study in healthy individuals showed an approximately 10-fold increased antibody response to an alum-absorbed hepatitis B virus (HBV) vaccine with the addition of a B-class ODN, CPG 7909 (Cooper et al., 2004a). Furthermore, the CpG ODN-induced HBsAg-specific antibody responses (anti-HBs) appeared earlier and had a higher avidity (Siegrist

et al., 2004). In studies sponsored by Dynavax Technologies using a different B-class CpG ODN similar enhancement of the immune response to purified HBsAg was seen in the absence of alum, indicating that CpG ODN are effective vaccine adjuvants in humans also in the absence of other adjuvants (Halperin et al., 2003; Halperin et al., 2006). This vaccine, also called Heplisav, has been administered to more than 5,000 people so far with positive results in several phase 3 clinical trials (www.dynavax.com). In a phase 3 clinical trial of more than 2400 adults over age 40, a 2-dose regimen of Heplisav was superior to the FDA-approved 3-dose regimen of the current vaccine, Engerix-B. In another phase 3 trial of Heplisav, more than 500 subjects with chronic kidney disease were randomized to receive Heplisav or Engerix-B, with the result that again, Heplisav provided superior immune responses. Within the next 1 or 2 years Heplisav could become the first CpG-adjuvanted vaccine approved for human use.

Immune-suppressed individuals have an especially high rate of unresponsiveness to the HBV vaccine. Approximately 50% of individuals infected with human immunodeficiency virus (HIV) fail to mount protective levels of antibody. In HIV-infected patients too, a randomized, double-blind, controlled trial demonstrated that addition of CPG 7909 significantly enhanced (1) the mean titers of anti-HBs antibody; (2) the antigen-specific T cell proliferative response to the vaccine, and (3) the proportion of HIV patients who had seroprotective antibody levels at 12 months following vaccination (Cooper et al., 2005).

For vaccines against potential bioterror agents, such as anthrax, the faster seroconversion provided by CpG adjuvants is especially important, because it could enable post-exposure vaccination following an attack. In a phase 1 trial, CPG 7909 dramatically accelerated seroconversion to the approved anthrax vaccine, Biothrax (Rynkiewicz et al., 2011). Control subjects reached their peak titer of toxin-neutralizing antibody at day 46, but this titer was achieved in the subjects receiving CPG 7909 more than 3 weeks earlier, at day 22. Furthermore, the addition of CPG 7909 increased the proportion of subjects who achieved a strong immunoglobin G response to the anthrax protective antigen from 61% to 100%.

In mice, the use of a CpG ODN as vaccine adjuvant enables the antigen doses to be reduced by approximately 2 orders of magnitude, with no reduction in the amplitude of the immune responses (Weeratna et al., 2003). Given the concerns over potential pandemic influenza or other infections where vaccine quantities may be limiting, such an effect could be tremendously beneficial. In a phase 1b randomized, double-blind, controlled clinical trial, the co-administration of CPG 7909 with one-tenth of the normal dose of a commercial influenza vaccine restored the antigen-specific IFN-γ secretion to that induced with full-dose vaccine (Cooper et al., 2004b). Should these results be reproduced in further studies, they could support the use of CpG ODN adjuvants in the development of vaccines for pandemics.

The largest infectious cause of death worldwide is malaria, and current vaccines in development have generally suffered from limited immunogenicity. Major research efforts have been directed at the identification of potentially protective antigens and the development of strong vaccine adjuvants and formulations for these. Following positive results in rodents and primates, a phase 1 dose-escalating study was conducted in 75 healthy volunteers to assess the effects of 3 vaccinations with the recombinant malarial protein vaccine apical membrane antigen 1-combination 1 (AMA1-C1)/Alhydrogel with and without the addition of CPG 7909 (Mullen et al., 2008). Local and systemic adverse events were significantly increased with the addition of CPG 7909, but so was the antibody response (up to 14-fold higher than the controls), and the functional capacity of the antibodies was confirmed by showing the parasite growth was significantly reduced in an *in vitro* growth assay (Mullen et al., 2008). In a follow-up trial, 24 healthy volunteers received the AMA1-C1/Alhydrogel with or without CPG 7909 in different formulations and dosing schedules (Ellis et al., 2009). The peak antibody levels were again increased in the groups receiving CPG 7909, but in contrast to the previous trial of the vaccines, the injections were well tolerated (Ellis et al., 2009). *In vitro* malaria parasite growth inhibition assays followed the antibody levels in showing increased efficacy (Ellis et al., 2009). A further phase 1 study of AMA1-C1/Alhydrogel with or without CPG 7909 was performed in Mali among adults who had been exposed to malaria (Sagara et al., 2009). In this population the vaccines were well tolerated, and there was a more than 2-fold increase in the antibody response with the addition of CPG 7909 (Sagara et al., 2009). Based on the positive results with the AMA1-C1/Alhydrogel plus CpG 7909, 5 healthy malaria-naive volunteers and several unvaccinated controls were immunized with AMA1-C1/Alhydrogel+CPG 7909, and challenged by intravenous inoculation of *Plasmodium falciparum* infected erythrocytes (Duncan et al., 2011). Within the vaccine group there was a significant correlation between the *in vivo* parasite multiplication rate at 48 hours and both the vaccine-induced *in vitro* growth-inhibitory activity and the AMA1 antibody titers (Duncan et al., 2011). However, immunization unfortunately failed to provide any detectable clinical benefit compared to the controls: There was no significant difference in the *in vivo* parasite multiplication rates (Duncan et al., 2011).

Another malaria vaccine candidate, merozoite surface protein 1(42) [MSP1(42)], was formulated with Alhydrogel plus CPG 7909 and evaluated in a phase 1 clinical trial in 60 healthy volunteers (Ellis et al., 2010). Again, the vaccine was generally well tolerated and the addition of CPG significantly enhanced anti-MSP1(42) antibody responses following vaccination by approximately 8-fold at 2 weeks after the third immunization when compared to MSP1(42)-C1/Alhydrogel alone, with a comparable improvement in an *in vitro* parasite growth inhibition assay (Ellis et al., 2010). Both vaccines increased the generation of malaria-specific memory B cells (Crompton et al., 2009). Further investigations of these and other malaria vaccine candidates are underway.

Application of CpG ODN in Allergic Diseases

The development of CpG ODN as an adjuvant for allergy vaccines

The Th1-biased immune response that is induced by CpG ODN opposes and may overcome the Th2-type immune response that is associated with allergies. In allergic mice, CpG ODN are able to redirect the allergic Th2 response and prevent inflammatory disease manifestations, even in mice with established allergic disease (Kline et al., 1998). In one of the relatively few human clinical trials performed with an A-class CpG ODN, scientists at Cytos used virus-like particles to

stabilize the ODN and formulate them with an extract of house dust mite for immunotherapy of 21 allergic patients (Senti et al., 2009). The results of the trial were quite encouraging: The allergic response was virtually eliminated on conjunctival challenge testing and symptoms of allergic asthma and rhinitis significantly reduced for at least 38 weeks after treatment (Senti et al., 2009). Rather than mixing the CpG ODN with the allergen, a conjugate of a B-class-like CpG ODN to a portion of the ragweed allergen was found to be even more effective than a mixture for immunotherapy in mice (Tighe et al., 2000a; Tighe et al., 2000b). This conjugate approach was evaluated in several human clinical trials as an allergy vaccine for allergic rhinitis, with encouraging evidence for a selective and specific redirection of the allergic Th2 response towards a non-allergic and non-inflammatory Th1 response in early phase 1 and 2 clinical trials (Creticos et al., 2006; Simons et al., 2004). Unfortunately, these results were not confirmed in definitive phase 3 clinical trials, and the approach has not been pursued. The reason for the phase 3 failure is unclear, but might have involved enrollment of many subjects who were not ragweed-allergic, or an unexpectedly mild ragweed allergy season at the sites running the trial.

The development of CpG ODN monotherapy for allergy treatment

One of the basic limitations of the concept of an allergy vaccine is that most allergic individuals actually are allergic to multiple different antigens, not just one. It would obviously be more complex and expensive to develop a separate vaccine for every allergen than it would to develop than a single, universal allergy immunotherapeutic. Preclinical studies in mice and primates provided strong support for this universal therapeutic concept, and suggested several potential mechanisms of action (reviewed in Kline and Krieg, 2008). Several companies have conducted clinical development work for such an allergy CpG monotherapy. In 2002 Coley Pharmaceutical Group (Coley was later acquired by Pfizer in 2008) initiated a collaboration with Sanofi-Aventis for an inhaled CpG ODN monotherapy. Phase 1 trials showed evidence of immune activity and good safety, but following several regulatory delays and corporate reorganizations, the program was terminated. In 2006 Dynavax Technologies initiated a collaboration with AstraZeneca in the field of CpG ODN for asthma, and this collaboration has recently led to the selection of a lead compound, AZD1419 (Table 1) for initiation of Investigational New Drug Application (IND)-enabling preclinical studies (www.dynavax.com).

Cytos Biotechnology was initially focused on a vaccine approach for allergy immunotherapy with CpG ODN (see previous discussion). However, in a double-blind, randomized, placebo-controlled phase 2 clinical trial it was found that allergen was not required for clinical benefit in patients with allergic rhinoconjunctivitis treated with an A-class CpG ODN packaged into virus-like particles with no allergen (CYT003-QbG10) (Klimek et al., 2011). In this clinical trial CYT003-QbG10 was injected subcutaneously (SC) weekly for 6 weeks at 1 of 2 different doses into patients with house dust-mite allergy (Klimek et al., 2011). The treatment was generally well tolerated and allergic symptoms were significantly lower in patients treated with the high dose of CYT003-QbG10 as compared with placebo. There was also a 10-fold increase in allergen tolerance upon conjunctival provocation testing in the high dose group, while in the placebo group it remained unchanged (Klimek et al., 2011). Although injection therapy may not be desirable for patients with mild disease, this approach could prove useful in patients with moderate or severe asthma or other allergic symptoms. One would not normally expect an unmodified CpG ODN such as that used in CYT003-QbG10 to be stable *in vivo*, but perhaps the packaging in the virus-like particle protects the ODN from degradation.

Applications for CpG ODN in Oncology

Anti-tumor effects of CpG ODN monotherapy in humans

Several clinical studies of single-agent CpG ODN TLR9 agonists have been completed and shown evidence of antitumor activity. Immunotherapies are often tested in the setting of melanoma and other skin cancers because they tend to be highly immunogenic. One approach to melanoma monotherapy that has been investigated in humans with PF-3512676 is local therapy with intra- or perilesional injection. In a phase 1 trial of low dose intra- or perilesional injection of PF-3512676 there was 1 local regression among 5 patients with metastatic melanoma and 1 complete response (CR) and 4 partial responses (PRs) among 5 patients with basal cell carcinoma (Hofmann et al., 2008). In a different trial involving 24 patients with clinical stage I to stage III melanoma, surgical resection of the primary tumor was followed by randomization to receive either saline or 8 mg PF-3512676 intradermally at the excision site, followed 1 week later by a sentinel lymph node procedure (Molenkamp et al., 2007). PF-3512676 injection induced the release of inflammatory cytokines, decreased the number of regulatory T cells, and induced tumor-specific $CD8^+$ T cells in sentinel lymph nodes of these patients (Molenkamp et al., 2007; Molenkamp et al., 2008; Sluijter et al., 2010). Furthermore, both myeloid and pDCs were activated (Molenkamp et al., 2007), and the degree of pDC activation correlated with the magnitude of the $CD8^+$ T cell response (Molenkamp et al., 2008).

Intratumoral monotherapy with TLR9 agonists has also been evaluated in patients with recurrent glioblastoma (GBM). A minor response was observed in 2 of 24 patients with recurrent GBM receiving intratumoral CpG-28 in a phase 1 dose-escalation trial (Carpentier et al., 2006). A phase 2 trial of this oligo CpG-28 in 34 patients with recurrent GBM at the highest dose reached in the phase 1, 20 mg, had a 6-month progression-free survival rate of 19%, which did not meet the goal of the study (Carpentier et al., 2010). The overall survival rate was 24% at 1 year and 15% at 2 years, which are longer than typical for GBM but could reflect patient selection in this small non-randomized trial.

A second approach to immunotherapy of melanoma has been systemic therapy with SC injection. In a phase 2 study in 20 patients with metastatic melanoma treated with SC PF-3512676, 2 patients (10%) had a PR, and 3 patients (15%) had stable disease (Pashenkov et al., 2006). These patients with possible clinical benefit from PF-3512676 therapy tended to have increased levels of NK cell activity compared to the patients with progressive disease. In these studies the PF-3512676 monotherapy was generally well tolerated and was associated with some antitumor activity. In a second phase 2

clinical trial of SC PF-3512676 in 184 patients with advanced melanoma with or without dacarbazine chemotherapy (described further below) (Weber et al., 2009), there was no statistically significant benefit from CpG therapy overall. Nevertheless, a single patient in one of the monotherapy arms had a prolonged course during therapy with apparently slow progression of the original tumor masses. On surgical excision this was found to be necrotic tissue with inflammatory cells and no remaining detectable tumor (Stoeter et al., 2008).

In a phase 1/2 dose-escalation trial, 39 patients with stage IV renal cell carcinoma (RCC) received weekly PF-3512676 by SC injection at doses ranging from 0.08 mg/kg up to a high dose of 0.81 mg/kg for up to 24 weeks (Thompson et al., 2009). The maximal tolerated dose was not reached, but the side effects of treatment were tolerable, and the efficacy of CpG therapy in this setting was modest with 2 PR, one of which occurred at a weekly dose of 0.16 mg/kg and the other at a dose of 0.54 mg/kg (Thompson et al., 2009).

In a phase 1 study of SC PF-3512676 monotherapy in patients ($N=28$) with refractory relapsed cutaneous T cell lymphoma, 7 patients (25%) achieved an objective response (2 CRs, 5 PRs) (Kim et al., 2010). Patients typically experienced mild to moderate injection-site reactions (erythema, induration, edema, inflammation, and pain) and flu-like symptoms (fatigue, rigors, fever, and arthralgia) (Kim et al., 2010). PF-3512676 has also been studied in a phase 1 trial in patients with refractory non-Hodgkin's lymphoma (NHL). In patients ($N=23$) receiving PF-3512676 (0.01 to 0.64 mg/kg) intravenously (IV) up to 3 times per week, NK cell numbers and activation were enhanced in most subjects and most side effects were again mild to moderate and transient (Link et al., 2006).

The development of CpG ODN as an adjuvant for cancer vaccines

Of the large number of papers published showing beneficial effects of CpG ODN in diverse anti-tumor applications in mouse models, some of the most encouraging results have been as adjuvants for cancer vaccines (reviewed in Krieg, 2008). Since mouse models are notoriously unreliable for predicting outcomes in humans, this review will only touch on the results from human clinical trials. In 8 advanced melanoma patients, CPG 7909 at a dose of 0.5 mg mixed with a melan-A tumor peptide antigen in a water-in-oil emulsion (Montanide) stimulated strong and rapid CD8 T cell responses reaching a mean of >1% of melan-A specific $CD8^+$ T cells (Speiser et al., 2005). The addition of CpG 7909 to the melan-A peptide vaccine not only induced a high frequency of tumor-specific $CD8^+$ T cells (Appay et al., 2006b), but also promoted effector cell differentiation (Appay et al., 2006a; Baumgaertner et al., 2011). In 1 patient, a very strong T cell response was associated with disease stabilization, but the patient's cancer progressed when the tumor apparently mutated to stop expressing the tumor antigen (Speiser et al., 2006). A-class CpG ODN in nanoparticles have also demonstrated strong adjuvant activity for melan-A peptide vaccination in melanoma patients (Speiser et al., 2010).

Positive results have also been seen in vaccine trials with other tumor antigens. In 1 trial in stage III/IV melanoma expressing the tumor antigen NY-ESO-1, patients were immunized with an analog NY-ESO-1 peptide in 1 of 3 ways: (1) peptide in Montanide; (2) peptide in combination with CPG 7909; or (3) with the triple combination of Montanide, CpG, and peptide. Only the patients immunized with the triple combination of Montanide, CpG, and peptide developed a rapid increase of effector-memory NY-ESO-1-specific CD8+ T cells (Fourcade et al., 2008). NY-ESO-1 is expressed in many different tumor types, including for example, 15% to 25% of metastatic prostate cancers, so a vaccine using it could be widely applicable. In a phase 1 clinical study, patients with advanced prostate cancer were vaccinated intradermally with NY-ESO-1 protein mixed with CPG 7909 every 3 weeks for 4 doses (Karbach et al., 2011). Many patients made high-titer antibodies to NY-ESO-1, and 9 of the patients (69%) developed NY-ESO-1-specific CD4+ and/or CD8+ T-cell responses (Karbach et al., 2011). Five of these 9 patients did not express NY-ESO-1 in the autologous tumor (Karbach et al., 2011).

Extensive immunologic studies of patients vaccinated with CpG ODN and other adjuvants together with tumor antigens have revealed that although strong T cell responses can be seen among circulating cells in the blood, the tumor-specific T cells that are isolated from tumor metastases nevertheless show signs of immune "exhaustion" or greatly reduced functional activity (Baitsch et al., 2011). Some of the candidate immune receptors that may suppress immune responses in cancer patients are B and T lymphocyte attenuator (BTLA) (Derre et al., 2010; Fourcade et al., 2011), programmed death ligand 1 (Fourcade et al., 2009), and Tim-3 (Fourcade et al., 2010). A better understanding of the inhibitory mechanisms controlling anti-tumor immune responses should provide further improvements in the efficacy of cancer vaccines.

Based on encouraging results from several early clinical trials, GlaxoSmithKline (GSK) has made a strong commitment to the development of CpG ODN as a key component of a cancer vaccine. GSK is currently conducting, in parallel, two phase 3 clinical trials of patients with tumors expressing the tumor antigen MAGE-3: one trial is in melanoma, and the other is in non-small-cell lung cancer (Brichard and Lejeune, 2007). A major challenge in the cancer vaccine field has been the identification of subjects who are the most likely to respond, since the great majority of patients have not shown benefit in past clinical development programs. In this regard GSK may have made an important advance by identifying a genetic signature in the cells of patients who turn out to respond to the vaccine (Gajewski et al., 2010). By requiring the presence of this signature for enrollment into the phase 3 trials, it is hoped that the chance for a positive outcome is enhanced. The results of these two phase 3 trials may be available in late 2012 or in 2013.

Anti-tumor applications for CpG ODN in combination with other therapies

The classical approach for inducing antigen-specific anti-tumor immune responses has been using vaccination, as described in the preceding section. However, the level of success using this type of approach has been disappointing (Rosenberg et al., 2004), and many groups have been exploring combinations of immunotherapies with other types of therapies, especially using approaches that may weaken the tumor to make it more susceptible to immune-mediated attack. In mice with relatively small tumors, CpG monotherapy can be

sufficient to induce a T cell mediated tumor regression, but to induce rejection of larger tumors the CpG ODN generally needs to be combined with other effective anti-tumor strategies, such as monoclonal antibodies (mAb), radiation therapy, surgery, and chemotherapy (reviewed in Krieg, 2008).

CpG ODN for non-Hodgkin's lymphoma. In murine lymphoma models, the addition of a CpG ODN to anti-tumor mAb therapy provides a synergistic anti-tumor effect (Wooldridge et al., 1997) which depends not only on TLR9 expression in host immune cells, but also on TLR9 expression in the B cell tumor (Li et al., 2007). It appears that activation of TLR9 in tumor cells makes the tumor itself more immunogenic, inducing a stronger anti-tumor response than if the tumor does not express TLR9 (Li et al., 2007). Based on these and other preclinical results, several phase 1 clinical trials have investigated the combination of a CpG ODN with the anti-tumor antibody rituximab in patients with relapsed or refractory NHL. In a dose-escalation study, 12 out of 50 NHL patients (24%) receiving PF-3512676 weekly for 4 weeks IV or SC in combination with rituximab had an objective response (including 5 CRs and 7 PRs), (Link et al., 2006; Leonard et al., 2007). In a separate dose-escalation phase 1 study, 20 patients with relapsed NHL were treated with the SC administration of weekly CpG ODN 1018 ISS following rituximab therapy (Friedberg et al., 2005). Dose-dependent induction of type 1 IFN-inducible genes was seen. In a phase 2 clinical trial performed by the same group, 1018 ISS was administered at a dose of 0.2 mg/kg weekly SC for 4 weeks in combination with rituximab to 23 patients with relapsed/refractory follicular lymphoma (Friedberg et al., 2009). Almost half of the patients showed clinical responses, and many patients also showed evidence of immune response, including infiltration of the tumors with $CD8^+$ T cells and macrophages (Friedberg et al., 2009). Because the trials had no control arm it is impossible to determine to what if any degree the CpG ODN may have contributed to these responses, or if the rituximab therapy was responsible.

More definitive evidence of clinical benefit from CpG ODN therapy was seen in a phase 1/2 clinical trial in NHL patients in which intratumoral injection of the CpG ODN PF-3512676 was accompanied by local radiation to the injected tumor (Brody et al., 2010). In this clinical setting radiation therapy alone can cause local responses, but not systemic regression. Of 15 treated patients, there was 1 CR, 3 PRs, and several other patients with stable regressing disease (Brody et al., 2010). The CpG therapy also resulted in induction of anti-tumor specific $CD8^+$ T cells (Brody et al., 2010). Further studies are underway in an effort to increase the response rate to this approach.

CpG ODN in combination with chemotherapy. It may seem counterintuitive to combine immune suppressive chemotherapy with TLR9 stimulation, but such combinations have shown improved survival in mouse tumor models using several different chemotherapy regimens (Weigel et al., 2003; Balsari et al., 2004; Wang et al., 2004; Pratesi et al., 2005; Bourquin et al., 2006; Gekeler et al., 2006; Mason et al., 2006; Rayburn et al., 2007; Reinis et al., 2007; Petrangolini et al., 2008; Ampollini et al., 2009; Buhtoiarov et al., 2011; Johnson et al., 2011; Sommariva et al., 2011). The increased anti-tumor efficacy of a CpG chemotherapy combination approach required T cells but not NK cells and was associated with the induction of a stronger anti-tumor T cell response in mouse models (Vicari et al., 2009). Based on these and other results, we investigated the effect of adding the B-class CpG ODN PF-3512676 (also known as CPG 7909 when used as a vaccine adjuvant) to standard dacarbazine chemotherapy for the treatment of metastatic stage III/IV melanoma in a phase 2 clinical trial (184 patients), but unfortunately there was no evidence for clinical benefit (Weber et al., 2009). Perhaps in the setting of advanced melanoma the immune system was too suppressed to mount an effective tumor response with this therapeutic combination.

More encouraging initial results were seen in a phase 2 clinical trial evaluating the addition of PF-3512676 to standard taxane/platinum chemotherapy for first-line treatment of stage IIIb/IV non-small-cell lung cancer: There was a significant improvement in response rate from 19% in the patients randomized to standard chemotherapy to 38% in the patients who also received PF-3512676 (Manegold et al., 2008). The secondary endpoint of this trial, survival, showed a trend to improvement from a median survival of 6.8 months in the chemotherapy arm versus 12.3 months in the combination arm, and an improvement in the 1-year survival from 33% to 50% (Manegold et al., 2008). The safety profile of the combination therapy was generally good, and as in the other clinical trials with TLR9 agonists, the most common side effects were mild to moderate injection site reactions and transient flu-like symptoms. In an attempt to confirm and extend these positive results, 2 controlled phase 3 human clinical trials of PF-3512676 combined with doublet chemotherapy in first line treatment of unresectable non-small-cell lung cancer were initiated, but these trials unfortunately failed to replicate the phase 2 result—no difference in survival or other clinical benefit was found from the addition of PF-3512676 in either trial (Hirsh et al., 2011; Manegold et al., 2012). Phase 3 clinical failures are all too common in oncology, and often the reasons are unclear, as is the situation in the case of PF-3512676. Unfortunately no immune studies were performed and no clinical samples collected in these trials, so they did not contribute to our knowledge of the immune effects of TLR9 activation in patients with advanced cancer, nor is it possible to identify any subsets of patients who may have benefited from therapy. Although Pfizer acquired Coley in 2008 for the PF-3512676 and other CpG programs, these oncology applications have not been aggressively pursued.

Safety of TLR9 Activation in Rodents and Humans

It cannot be overemphasized that the safety profiles of CpG ODN differ significantly between rodents and primates. Since TLR9 is expressed in a broader range of immune cells in rodents compared to primates, the rodent tends to over-predict toxicities that will occur in primates. One of the major mechanisms of toxicity in rodents appears to be inducing the secretion of tumor necrosis factor (TNF)-a and other cytokines and chemokines from the direct stimulation of TLR9 in monocytic cells (Cowdery et al., 1996; Sparwasser et al., 1997; Yi et al., 2001; Campbell et al., 2009). In humans, monocytic cells do not express TLR9 or secrete TNF-a in response to CpG ODN *in vitro* or *in vivo* and so these toxicities have not been observed (Krieg, 2003; Krieg et al., 2004; Campbell et al., 2009). Chronic dosing of phosphorothioate (PS)-ODN in rodents results in a dose-dependent mononuclear cell infiltration in

the liver and kidneys as the major organs of ODN deposition, but these cell infiltrates do not occur in monkeys or humans, possibly because of the broader distribution of TLR9 expression within the immune cells of rodents, as previously noted (Henry et al., 1997; Vollmer and Krieg, 2009).

In human clinical trials, the safety profile of several TLR9 agonists has been studied over a dose range from 0.0025 mg/kg to 0.81 mg/kg. To the best of my knowledge, the maximum tolerated dose of a CpG ODN in humans has not been identified. The primary adverse events are dose-dependent local injection reactions (e.g., erythema, pain, swelling, induration, pruritus, or warmth at the site of injection) or systemic flu-like reactions (e.g., headache, rigors, myalgia, pyrexia, nausea, and vomiting) and are consistent with the known mechanism of action of TLR9 agonists and the cytokines and chemokines whose expression they induce. Depending on the dose, systemic symptoms typically appear within 12 to 24 hours of dosing and persist for 1 to 2 days and in rare cases, longer. Even without the addition of a TLR9 agonist, vaccines commonly induce injection site reactions. With some vaccines containing a CpG ODN adjuvant there appears to be a slight increase in the frequency of injection site reactions compared to the frequency observed with the vaccine alone, but these still are generally mild. More significant injection site reactions can be seen with high dose SC injection of CpG ODN for oncology applications, and seizures have been described upon intracranial administration, although it is not clear whether these resulted from the CpG ODN or from the procedure (Carpentier et al., 2010).

CpG ODN treatment can exacerbate autoimmunity in several mouse models but can prevent or reduce inflammatory disease in others (reviewed in Krieg and Vollmer, 2007). Among the thousands of humans who have been treated with different CpG ODN in the clinical trials reviewed here, I am only aware of a single report of autoimmune disease developing: A normal subject in one of the Heplisav trials was diagnosed with Wegener's granulomatosus following the second injection of the vaccine. The Heplisav trial was on a prolonged clinical hold during an FDA investigation of this case, but in the end, no association with the treatment could be identified, and the case presumably represents the kind of rare spontaneous event that tends to occur during clinical development of any new drug as large numbers of patients are exposed to it. The clinical experience to date indicates that CpG ODN treatment of normal humans, cancer patients, or individuals infected with HIV or HCV does not readily induce autoimmune disease. However, the duration of therapy has usually been less than 6 months; only a few patients have received chronic therapy with CpG ODN for longer than 3 years. It is worth bearing in mind that TLR9 activation induces the secretion of IFN-α, and since treatment with recombinant IFN-α induces an autoimmune disease in 4%–19% of chronically treated patients (Ioannou and Isenberg, 2000), continued vigilance would be prudent until larger numbers of patients have been treated with TLR9 agonists for longer periods of time.

Conclusion

The CpG motif was described in 1995, and the first human clinical trial began in 1999. In a little more than a decade since then, more than a dozen TLR9 agonists have progressed into human clinical trials, including several investigational products in phase 3 trials and multiple drugs in phase 2 development. Many of the early clinical results have been quite encouraging, with strong indications of substantial clinical benefit that indicate that the targeted activation of TLR9 will enhance the treatment of cancer and infectious diseases and decrease the harmful inflammatory responses of asthma and other allergic diseases. Although longer-term follow-up of larger numbers of patients is needed, the safety of these TLR9 agonists appears acceptable, and their potential clinical contributions are enormous. As with any new drug platform, not all of the trials succeed, and there have been painful and costly failures along the way. Many drugs that are blockbusters today experienced similar or greater challenges during their early development.

Important lessons have been learned along the way. Immunotherapy with CpG ODN has demonstrated considerable potential for therapeutic applications, especially in the area of enhancing vaccine efficacy. This is the indication in which the first drug approvals are likely to come. It seems likely that as vaccines containing CpG ODN are approved and used more widely, they will be incorporated into other vaccines or used in settings where their ability to provide rapid seroconversion or reduced antigen doses provides a particular advantage.

Great potential also exists for the applications of CpG ODN in asthma and allergic diseases, assuming that the safety profile is acceptable for this patient population. Oncology is an extremely challenging field for drug development, with arguably the highest rate of phase 3 clinical failures of any field. CpG ODN clearly have activity as a monotherapy in multiple different tumor types, but the efficacy is relatively modest, and the focus has been on identifying more potent combination approaches. Vaccines adjuvanted with CpG ODN can induce immune responses in patients with advanced metastatic disease, but it seems logical to assume that the chances for clinical success would be greater at earlier stages of disease. In patients with advanced malignancies, more work needs to be done to find therapeutic combinations in which CpG ODN may realize the full potential of therapeutic benefit. New applications for these TLR9 agonists seem certain to emerge as the field advances.

Author Disclosure Statement

AMK is an inventor on patents relating to CpG ODN and may receive royalties from these.

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