

The immunobiology and clinical potential of immunostimulatory CpG oligodeoxynucleotides

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Abstract: Over 100 years ago, Coley first explored the use of bacterial products as immunostimulatory therapy for nonbacterial disease. It is now clear that bacterial DNA, and synthetic oligodeoxynucleotides containing specific motifs centered on a CpG dinucleotide (CpG ODN), are potent immunostimulatory agents. The molecular mechanisms responsible for the immunostimulatory effects of CpG ODN have yet to be elucidated fully, although it is clear that CpG ODN act rapidly on a variety of cell types. This includes activation of B cells, natural killer cells, and antigen-presenting cells including monocytes, macrophages, and dendritic cells. These effects have led to evaluation of CpG ODN as immune adjuvants in immunization where they have been shown in animal models to enhance the development of a TH1-type immune response. Preliminary results from clinical trials using CpG ODN as an immune adjuvant are promising. Preclinical studies suggest CpG ODN can also enhance innate immunity against a variety of infections, synergize with monoclonal antibody to enhance antibody-dependent cellular cytotoxicity, and alter the Th1/Th2 balance as a possible treatment for allergic diseases and asthma. Clinical evaluation has recently begun to determine whether promising preclinical results with CpG ODN can be translated into effective and tolerable clinical treatment approaches. *J. Leukoc. Biol.* 68: 455–463; 2000.

Key Words: *B cells · natural killer cells · antigen-presenting cells · allergic diseases · asthma*

INTRODUCTION

The first reported systematic attempt to use an immunostimulatory therapy for a nonbacterial disease took place in the 1890s when Dr. William Coley, a New York surgeon, performed a series of studies evaluating the anti-tumor activity of bacteria. In his initial studies, Dr. Coley injected live streptococci directly into the tumor masses of his patients. This resulted in tumor regression in his first patient that lasted for 7 years. However, dangers of infection were high, with the first patient almost dying of erysipelas. For the next two decades, Coley explored use of heat-killed gram-positive and gram-negative bacteria as immunotherapeutic agents for cancer [1,

2]. This preparation, known as “Coley’s Toxin,” resulted in tumor regression in some patients, although the response rate was less than that seen with live organisms. In subsequent decades, much of the antitumor activity of Coley’s toxin was attributed to endotoxin [3]. However, it is worth noting that Coley’s original success was with streptococcus; a gram-positive organism that does not produce endotoxin. Additional bacterial components, such as bacterial DNA, may well have played a role in the observed responses.

It was almost 100 years between Coley’s studies and the recognition that bacterial DNA itself can stimulate the immune system. Shimada, Yamamoto, and colleagues demonstrated that bacterial DNA could enhance natural killer (NK) cell activity [4, 5], and Messina, Pisetsky, and colleagues found that such DNA was also capable of inducing B cell activation [6]. In the mid-1990s, Krieg and colleagues gained additional insight into the moieties in bacterial DNA that are responsible for the immunostimulatory effects of bacterial DNA. As with many pivotal discoveries, this one was serendipitous. While studying the effect of anti-sense DNA on B cell activation, these investigators found that a number of “control” oligodeoxynucleotides (ODN) also mediated B cell activation. After making and testing hundreds of ODN, it was determined that the immunostimulatory effects of these “control” ODN were dependent on an unmethylated CpG dinucleotide in a particular sequence context [7]. Specifically, the optimal stimulatory motif was determined to be as follows: $R_1R_2CGY_1Y_2$ where R_1 is a purine (preference for G), R_2 is a purine or T, and Y_1 and Y_2 are pyrimidines. Klinman et al. found these ODN not only induced B cell activation, but also induced production of a wide variety of cytokines, indicating a more complex pattern of immune activation [8].

Although teleological explanations are risky, it is fascinating to consider whether an immune response to unmethylated CpG motifs enables the mammalian immune system to distinguish microbial DNA from self DNA. Indeed, it is easy to conceive how having bacterial DNA serve as a “danger signal” would be advantageous. Sequence differences between bacterial DNA and vertebrate DNA appear to make this possible. CpG dinucleotides are present at the expected frequency in bacterial DNA (1 in every 16 dinucleotides) while mammals have

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CG suppression, with CG dinucleotides being found at approximately one-fourth the expected frequency [9]. Furthermore, the majority of cytosines present in CG dinucleotides are methylated in mammals. Although bacteria can methylate select bases, there is no methylation specificity for CG dinucleotides. Thus, unmethylated CG dinucleotides are much more common in bacteria than in mammals and other vertebrates.

We now know that the immunostimulatory effects of specific motifs within bacterial DNA, including CG dinucleotides, likely play an important role in the immunostimulatory effects of various bacterial preparations that have been observed over the years. Attenuated mycobacteria are currently used for treatment of superficial carcinoma of the bladder [10]. Yamamoto et al. found that mycobacterial DNA could result in tumor regression, and that this was accompanied by induction of interferon secretion, and NK lytic activity [11]. In further studies, these investigators found that DNA from a variety of bacteria could cause interferon (IFN) secretion and tumor regression, whereas vertebrate DNA did not [5].

Over the past 5 years there has been a dramatic increase in our understanding of the molecular and cellular effects of CpG DNA, and its effects *in vivo* in animal models. Studies to date suggest CpG DNA could have significant therapeutic promise in the treatment of a variety of disorders, including infectious disease, allergy, and cancer. Despite these rapid advances, there is still much we have to learn about these potent immunostimulatory agents. Clinical trials in these areas have only recently begun. In this review, we will outline what is known (and what is not known) about CpG DNA at the molecular, cellular, and intact animal level, and will discuss the potential for clinical utilization of this novel class of compounds (see **Table 1** for summary).

MOLECULAR EFFECTS OF CPG ODN

Ongoing investigations are exploring the molecular mechanisms responsible for the immunostimulatory effects of CpG ODN, as our knowledge in this area is far from complete. As a starting point, it is important to recognize that ODN, which are polyanions, can have both sequence-specific and nonsequence-specific immunological effects (see below). Therefore,

careful controls need to be included when studying the sequence-specific effects of any ODN, including both anti-sense and CpG ODN [12]. An additional basic question is whether the sequence-specific immunostimulatory effects of CpG ODN are mediated by binding of ODN to a CpG-specific surface receptor. This issue remains controversial. Liang et al. found that Sepharose beads coated with stimulatory, but not nonstimulatory ODN, induce B cell proliferation similar to that seen with soluble ODN, suggesting specific surface receptors are present [13]. In contrast, Krieg et al. found that CpG ODN covalently linked to a solid support are nonstimulatory. In addition, fluorescein isothiocyanate (FITC)-labeled CpG ODN and non-CpG ODN have similar surface binding, uptake, and intracellular trafficking [7]. Unlike antigens that trigger many surface receptors, CpG ODN do not appear to induce detectable Ca^{2+} flux, changes in tyrosine phosphorylation, or phosphatidylinositol-3-kinase activation. In addition, non-CpG ODN can compete with CpG ODN and inhibit both uptake and immune stimulation [14].

Whether or not a specific surface receptor is involved, uptake of CpG ODN is saturable and both energy and temperature dependent [14]. As is the case for other ODN [15], CpG ODN appear to be taken up in a nonspecific manner through acidic vesicles in the endosomal compartment. Drugs that interfere with endosomal acidification, such as quinacrine, inhibit the immunostimulatory activity of CpG ODN [14, 16].

Transcriptional activation induced by CpG ODN is very rapid, and is detectable within 15 min [17, 18]. Although there are hints that CpG ODN binds specifically to select proteins in both the cytoplasm and nucleus, the identity of CpG binding proteins has yet to be determined. Description of such proteins will be of obvious importance as we work to understand the molecular mechanisms responsible for the immunostimulatory effects of CpG ODN. Given the importance of CG methylation in transcriptional control, an area of intense interest is how CpG ODN might interact with transcription factor complexes. Indeed, the complexity of these structures may account for the difficulty encountered to date in identifying the CpG receptor.

What we do know is that generation of intracellular reactive oxygen species (ROS) occurs within minutes of exposure of cells to CpG ODN, and is associated specifically with CpG ODN and not with non-CpG ODN [17, 18]. Multiple down-

TABLE 1. Immunostimulatory CpG ODN—Major Effects

Molecular effects	Cellular effects	<i>In vivo</i> effects	Therapeutic potential
Uptake of ODN is active and dependent on backbone, but not sequence specific	Directly activates B cells, and is synergistic with signaling via B cell receptor	Induces B cell proliferation and Ig production	Enhance innate immunity
Receptor/binding protein not yet identified	Enhances expression of MHC and costimulatory molecules by antigen presenting cells	Enhances innate immunity as indicated by resistance to select organisms	As a treatment for allergy and asthma
Induction of Reactive Oxygen Species occurs early	Induces production of Th1-type cytokines by Antigen Presenting Cells	Functions well as a non-toxic immune adjuvant	To enhance the efficacy of monoclonal antibody therapy
Activates multiple downstream signaling pathways	Activates NK cells by indirect and direct pathways	Enhances development of a cellular immune response when used as a vaccine adjuvant	As an immune adjuvant for vaccines for infectious diseases and cancer

stream changes then occur, including nuclear factor- κ B (NF- κ B) activation [19]. Indeed, activation of NF- κ B by ODN was noted before the immunostimulatory effects of CpG ODN were described [20]. Changes that follow NF- κ B activation include production of tumor necrosis factor (TNF) and interleukin (IL)-1 γ [19]. CpG ODN can also impact on additional signaling pathways, and can induce changes such as activation of mitogen-activated protein kinases (MAPKs) and stress kinases such as JNK1 [14, 18].

Investigation into the interaction of CpG ODN with cytokine gene promoters is supplying further evidence for the molecular complexity of the mechanisms responsible for the immunological effects of CpG ODN. Takeshita et al. have used site-directed mutagenesis of the IL-6 promoter to demonstrate CpG ODN regulation of IL-6 gene expression involves both enhancer and derepression mechanisms [21]. More detailed descriptions of signaling and CpG ODN have recently been published [22, 23]. Our knowledge with respect to the specifics of CpG ODN-mediated signaling is sure to expand rapidly in the near future given the number of excellent investigators currently working in this area.

CELLULAR EFFECTS OF CPG ODN

The impact of CpG ODN has been evaluated extensively at the cellular level. Initial studies demonstrated that B cells proliferate and secrete Ig in response to CpG ODN. In fact, higher concentrations of CpG ODN induce activation of over 90% of primary murine B cells. At lower concentrations, B cell activation by CpG ODN synergizes with signals through antigen-specific activation pathways [7]. The effect of CpG ODN has also been studied on cells, such as WEHI-231, that undergo apoptosis in response to cross-linking of the B cell receptor. CpG ODN rescues these cells from anti-IgM-induced cell cycle arrest and apoptosis [24]. This activation results in phenotypic changes such as up-regulation of Class I MHC, Class II MHC, and costimulatory molecules [7]. Human B cells also respond to CpG ODN treatment, although the proliferative effect of CpG ODN on human B cells is not as profound as that seen in the murine system. It is interesting that similar changes are seen with malignant cells, including chronic lymphocytic leukemia cells [25, 26] and other malignant B cells [unpublished results].

CpG ODN can also activate antigen-presenting cells (APCs) including monocytes, macrophages, and dendritic cells [27]. This plays a central role in the overall immune response to CpG ODN. Effects of CpG ODN include induction of cytokine production and change in phenotype. Production of a growing number of cytokines is increased by CpG ODN including IL-6, IL-12, IFN- α , IFN- β , TNF- α , IL-1 β , and IL-18 [8, 28, 29]. Phenotypic changes on professional APCs are similar to those seen with B cells, and include induction of MHC and costimulatory molecule expression. Actual changes vary with different CpG ODN and from cell type to cell type, however, a common theme is that CpG ODN induce maturation of immature APCs. For example, dendritic cells derived from both bone marrow and skin up-regulate class I, class II, CD80, CD86, and CD40, and produce IL-12 [30–32].

NK activity is also increased by CpG ODN [33]. Much of this effect is indirect because highly purified NK cells are not strongly activated by CpG. IL-12 and IL-18 likely play a key role in this response. There appears to be a positive feedback loop between APCs and NK cells [34]. NK cells secrete IFN- γ after being stimulated by IL-12 and IL-18 that is produced by CpG ODN-stimulated APCs. This IFN- γ further activates the APCs.

The effects of CpG ODN on T cells remains controversial. Clearly, the activation of APCs by CpG ODN has potent, if indirect, effects on T cells [35]. Some investigators have reported that CpG ODN also has direct effects on T cells, and can supply a costimulatory signal directly to the T cell [36, 37]. We have been unable to detect such an effect. Because different sets of ODN were used by these two groups, it is possible the costimulatory effect on T cells was due to non-CpG motifs in the ODN, and not to the CpG motif itself. Further work is needed in this area. Nevertheless, this points out the complexity of the responses to immunostimulatory DNA.

IN VIVO EFFECTS OF CPG ODN

Given the diverse and potent effects CpG ODN has on a variety of immune cell subsets, it is not surprising that these agents are potent when administered *in vivo*. Studies in humans are only now beginning. However, extensive studies have been done in rodents, and some studies have been done in non-human primates. The observed *in vivo* data fits well with the *in vitro* data outlined above. Mice treated with CpG ODN develop splenomegally due to expansion of the B cell compartment, proliferation of NK cells, and extramedullary hematopoiesis [38]. They also have an increase in serum immunoglobulins, and increased production of a broad range of cytokines as outlined above. These changes confirm the shift toward a Th1 response [39], with increased levels of IgG2a, and IFN- γ [8, 40]. IL-6 and IL-12 secretion is increased within 4 h after *in vivo* treatment with CpG ODN. CpG ODN is, in general, well tolerated whether CpG ODN is given intravenously, subcutaneously, or intraperitoneally with no formation of sterile abscesses as is seen with complete Freund's adjuvant.

Toxicity can be seen in select conditions. Repeated large doses of CpG ODN can cause a wasting syndrome, and ultimately can be fatal to mice, apparently due to massive B cell proliferation and cytokine production. Toxicity has also been observed with the combination of CpG ODN and lipopolysaccharide (LPS), with CpG effectively priming mice for a Schwartzman-like reaction when a small dose of LPS is given a few hours after CpG ODN [40]. As clinical trials progress, particular caution will need to be taken when CpG ODN are combined with other agents that induce activation of monocytes and macrophages. It will also be important to monitor whether CpG ODN therapy increases the toxic systemic effects of bacterial infection.

HETEROGENEITY OF CPG ODN EFFECTS

ODN backbone itself can impact on *in vitro* and *in vivo* immunostimulatory activity [41]. Phosphodiester DNA with CpG

motifs, including synthetic ODN and bacterial DNA, can stimulate large amounts of cytokines *in vitro* and *in vivo* [40]. However, phosphodiester CpG ODN do not induce massive splenomegally seen with phosphorothioate CpG ODN, and phosphodiester CpG ODN are not as effective as phosphorothioate CpG ODN when used as immune adjuvants [42]. This is most likely because ODN with a phosphodiester backbone are very sensitive to nucleases and are degraded rapidly.

In addition, the anti-sense literature teaches us that the phosphorothioate backbone of ODN has non-sequence-specific immunostimulatory effects. For example, non-CpG phosphorothioate ODN have been shown to activate the transcription factor Sp1, which plays a key role in translation of multiple genes [43]. It is important to note that stimulatory effects of non-CpG phosphorothioate ODN are relatively limited when compared to the effects of phosphorothioate CpG ODN.

A variety of cell populations are activated by CpG ODN, and CpG ODN with different sequences vary in their ability to activate various cell populations. There is also variability from species to species [44]. This makes definition of the immunological effects of a specific CpG ODN complex. Despite this heterogeneity, a number of patterns of cellular activation appear to be emerging, which is allowing us to designate various classes of CpG ODN. Some CpG ODN induce significant activation of APCs (monocytes, dendritic cells), NK cells, and production of IFN- α , but have little impact on B cells. This class of ODN has recently been designated as "CpG- α ." Other CpG ODN activate APCs and NK cells, but induce little IFN- α . These CpG ODN are potent activators of B cells. These are designated "CpG- β ." The rules related to which CpG ODN will be CpG- α and which will be CpG- β are only now being worked out. What is clear is that the CpG motifs are not the only sequences that are relevant and that the nucleotides preceding and following the CpG motifs can have a significant impact on the immune effects of the ODN [33, 41, 45, 46].

CPG ODN TO ENHANCE INNATE IMMUNITY AND AS AN IMMUNE ADJUVANT FOR IMMUNIZATION

Given the potent immunostimulatory effects of CpG ODN, there are a number of conditions for which such agents might prove useful clinically. Solid animal model data suggest the shift of the immune response to a Th1 response by CpG ODN could be of benefit for the treatment of infectious diseases by enhancing innate immunity or by serving as an immune adjuvant during vaccination. CpG ODN therapy also holds promise for "Th2" diseases such as allergy and asthma. Finally, there are a number of scenarios where CpG ODN could be used as a component of cancer immunotherapy. Each of these areas is under intensive investigation.

The ability of CpG ODN to enhance the innate immune system, as indicated by activation of NK cells and monocytes, could be of use in itself. CpG ODN is a highly effective therapeutic agent for the treatment of murine models of leishmaniasis [43] and is also protective against a challenge with *Listeria monocytogenes* [47]. CpG ODN is currently being in-

vestigated for its ability to enhance innate immune defenses against other infectious organisms.

In addition to enhancing innate immunity, CpG ODN may be useful in preventing and treating infectious diseases based on its ability to enhance an antigen-specific response. CpG ODN is also attractive as a component of cancer vaccine strategies. Immune adjuvants used most extensively today enhance the Th2 response, and do not markedly enhance cellular immunity. Aluminum hydroxide is used in most commercial vaccine preparations and has been shown to actually block activation of CD8⁺ cytotoxic T lymphocytes (CTLs) in mice [48]. Other adjuvants that induce an enhanced Th1 response are currently being evaluated in both pre-clinical and clinical studies [49]. These include threonyl-muryl dipeptide [50], a variety of attenuated or killed bacteria [51] and bacterial derivatives [52], BCG [53] and *Quillaja saponaria* 21(QS21) [48], which has shown promise in preliminary clinical trials. However, none of these adjuvants are ideal due to toxicity (including both systemic toxicity and local inflammation after repeated immunization), limited efficacy at stimulating a cellular response, or difficulties associated with production.

A variety of characteristics of CpG ODN make them attractive as immune adjuvants. CpG ODN is inexpensive to produce. As reviewed above, there is significant synergy between signals delivered through the B cell receptor and CpG ODN that results in enhanced production of antibody. CpG ODN also has potent effects on APCs and enhances their ability to present antigen. Studies in a range of animal models have demonstrated that CpG ODN can serve well in this capacity. Using hen-egg lysozyme (HEL) as the antigen, Chu et al. found that immunization in incomplete Freund's adjuvant (IFA) resulted in Th2-dominated immune response characterized by HEL-specific secretion of Th2 cytokines (i.e., IL-5 but not IFN- γ). In contrast, immunization with HEL and CpG ODN switched the immune response to a Th1-dominated cytokine pattern (high levels of IFN- γ and decreased IL-5) [39, 54]. CpG ODN also enhanced production of anti-HEL IgG2a when compared with IFA-HEL. This Th1 response was more marked than that seen with complete Freund's adjuvant (CFA) despite a lack of local inflammation with CpG ODN. Davis and colleagues also found that CpG ODN markedly increased antigen-specific IgG and particularly IgG2a, using hepatitis virus B surface antigen as the immunogen [55]. It is important to note that they also found that CpG ODN enhanced development of a hepatitis virus B surface antigen-specific cytotoxic T cell response. Lipford et al. found a similar humoral and cellular response using ovalbumin as the target antigen [56]. Results with models of influenza are also promising, and have demonstrated that CpG ODN can enhance immunization delivered via the mucosa [57].

Our studies in a tumor model utilized the Id from the 38C13 murine lymphoma model as the target antigen. CpG ODN was as effective as CFA at inducing an antigen-specific antibody response, and was associated with less toxicity [42]. Again, CpG ODN induced a higher titer of antigen-specific IgG2a than did CFA. Therapeutically, mice immunized with CpG ODN as an adjuvant and Id-KLH as the immunogen were protected from tumor challenge to a degree similar to that seen in mice immunized with CFA and Id-KLH but with less toxicity [42]. In

addition, there was synergy between CpG ODN and granulocyte-monocyte colony-stimulating factor (GM-CSF) [31].

Studies in primates are also promising. Davis et al. have found that CpG ODN can markedly enhance the response of orangutans to hepatitis B immunization. A significant fraction of orangutans are hyporesponsive to current commercial hepatitis B vaccines. The addition of CpG ODN to the vaccine markedly increased the seroconversion rates and greatly enhanced the antibody response [58]. After two doses, 100% of animals immunized with CpG ODN plus the vaccine had protective levels of antibodies compared to only 8% of animals immunized with the commercial vaccine.

Most importantly, similar results have recently been reported in the first human trial with CpG ODN. Two weeks after the first immunization of normal volunteers, 92% of subjects receiving CpG ODN combined with vaccine had antibodies compared to 0% of the subjects receiving vaccine alone. Two and four weeks after the second dose, antibody titers were more than 30 times higher in subjects receiving CpG ODN plus vaccine when compared to vaccine alone (Arthur Krieg, personal communication).

Data in both the murine and human systems demonstrate that CpG ODN can enhance T cell activity by activating APCs and improving their ability to present antigen and activate T cells. This concept fits well with the recent focus of adoptive immunotherapy studies on therapy with *ex vivo* activation of APCs. Studies evaluating the immune effects of dendritic cells (DCs), which are extremely potent APCs, are particularly promising [59, 60], particularly given their ability to induce a cellular immune response [61, 62–64]. Levy and colleagues have demonstrated induction of an antigen-specific cellular response after treatment with antigen-pulsed DCs in a small clinical trial [65]. This was in stark contrast to the studies, reported by the same group, which demonstrated that immunization of patients with Id-KLH leads to an intense humoral response [66]. Enhanced immune activation in patients immunized with DCs pulsed with melanoma-associated antigens has also been reported recently [67].

Studies exploring the effect of CpG ODN on DCs have only recently begun. These preliminary investigations indicate CpG ODN can enhance the ability of some subpopulations of DCs to present antigen and induce an antigen-specific cellular response. Sparwasser et al. have shown that CpG ODN induces maturation of immature DC obtained from murine bone marrow and activates mature DC to produce cytokines, including IL-12, IL-6, and TNF- α [32]. Jakob et al. found that treatment of DCs derived from murine fetal skin decreased E-cadherin-mediated adhesion, up-regulated MHC class-II and co-stimulatory molecules, and enhanced accessory cell activity. Injection of CpG ODN into murine dermis led to enhanced expression of MHC class II and CD86 by the Langerhans cells [30]. We also found that CpG ODN markedly enhance the production of cytokines, including IL-12, from DCs derived from murine bone marrow through the use of GM-CSF and IL-4 [31], and that CpG ODN can enhance the survival, maturation, and differentiation of primary human DCs isolated from the peripheral blood [45]. Clearly, DCs do not represent a single population of cells. The ideal source of DCs or approach to *in vitro* expansion, activation, exposure to antigen, and re-infusion has

yet to be determined. Nevertheless, there is reason to believe CpG ODN could be useful in enhancing the immunological response to DCs.

Another area of intense interest in the field of immunotherapy is the use of immunization using DNA constructs containing sequences that code for the antigen of interest [68–70]. The intent of such therapy is to have host cells take up the DNA, produce protein coded by the DNA, and express peptides derived from that protein in host class I MHC, thereby inducing a cellular immune response directed toward that antigen. It remains unclear whether these functions can be performed by any cells (such as myocytes) that take up the DNA, or whether professional APCs are required [71, 72]. Nevertheless, the inclusion of sequences containing the CpG motif as part of the construct seems to enhance the resulting immune response in animal models. Sato et al. found that human monocytes transfected with plasmid DNA or double-stranded oligonucleotides containing CpG sequences transcribed larger amounts of IFN- α , IFN- β , and IL-12 when compared with cells transfected with DNA that did not contain such sequences [73]. Thus, modifying the CpG content of vectors intended for DNA immunization can have a significant impact on their ability to induce development of a cellular response [74]. The ability to construct vectors that encode for a specific protein and enhance a Th1 response to peptides derived from that protein would have clear implications in the area of vaccination for intracellular parasites and cancer and is currently undergoing intense investigation.

CpG ODN AS A TREATMENT FOR ALLERGY AND ASTHMA

Allergic diseases including asthma are an increasing problem in developed countries [75]. It has been suggested that the asthma and allergy “epidemic” may actually be a side-effect of progress in the area of infection control. As public health has improved, there has been a decreased rate of bacterial infection in the population as a whole. If bacterial products, including bacterial DNA, induce a shift toward a Th1 response, the lack of exposure to such products throughout life could conceivably result in a shift in the immune system, at the population level, toward Th2 type responses. This is highly speculative, and the Th1/Th2 dichotomy is far from straightforward. Nevertheless, it does raise the question of whether CpG ODN can be used on an individual basis to shift the Th1/Th2 balance in patients with diseases, such as allergy and asthma, that are associated with a strong Th2 response. Animal studies indicate this may be possible.

Kline et al. have used an inhalation mouse model to demonstrate that systemic CpG ODN can prevent the development of airway eosinophilia and bronchial hyperreactivity in animals sensitized to an allergen (schistosome eggs) [76]. This therapeutic response is associated with a decrease in IL-4, and an increase in IFN- γ and IL-12 in the airway fluid. An area of some controversy relates to whether conjugation of the allergen to the ODN is required to obtain an optimal therapeutic response. Raz and colleagues have performed a series of studies suggesting this is the case. The most convincing of these

studies involved conjugation of a ragweed allergen to CpG-containing immunostimulatory DNA sequences (termed "ISS"). This conjugate suppressed the IgE response in a number of species [77].

As outlined above, one effect of CpG ODN is to drive immature APCs toward induction of a Th1-type response. Thus, the activation of APCs and B cells raises the possibility that treatment with CpG ODN could lead to the induction of autoantibody production and other forms of autoimmunity [78]. Animal models are mixed in this regard, with some failing to demonstrate induction or exaggeration of autoimmunity [79, 80], whereas others suggest ODN could enhance the severity of disease in model systems of autoimmunity [81]. Further work is clearly needed in this area.

CPG ODN AS A CANCER THERAPY

A number of different approaches could be used to apply CpG ODN to the treatment of cancer. As outlined above, CpG ODN have potent effects on innate immunity and as an immune adjuvant. With respect to innate immunity, the therapeutic effect of Coley's toxin in cancer was likely due to production of a number of cytokines, and bacterial DNA in this preparation may have played an important role in this activity. We now know that CpG ODN induce enhanced production of a number of the cytokines that, individually, have anti-tumor activity, including TNF- α , IL-12, and IFN- γ [29, 33, 40]. These cytokines are now available in recombinant form, and are known to have anti-tumor effects in both animal models and clinical trials [82–84]. Unfortunately, clinical responses to these cytokines have, in general, been limited, and significant toxicity has been noted. The immune response normally involves the integrated production of a variety of cytokines that work in concert both locally and systemically. It is rational to hypothesize that CpG ODN, which is able to orchestrate the production of cytokines by the host both temporally and spatially, could be more effective and less toxic than recombinant cytokines at inducing an anti-tumor response. In addition to stimulating cytokine production, CpG ODN have direct effects on immune cell subpopulations that play an important role in anti-tumor immunity, including NK cells [33], B cells [7], monocytes and macrophages [32, 85], and dendritic cells [30, 31, 45].

In vivo, systemic administration of CpG ODN as a single agent can have anti-tumor effects that appear to be related to enhanced NK activity. Smith et al. used a murine model of lymphoma to evaluate the anti-tumor effects of an antisense phosphorothioate DNA designed to block the *c-myc* oncogene. Both antisense DNA and control sequences inhibited tumor growth. Further investigation demonstrated that the immunostimulatory effect of the DNA, and not anti-sense activity, was responsible for the observed anti-tumor effects [86]. Carpentier et al. have demonstrated that CpG ODN can induce *in vivo* rejection of neuroblastoma xenografts, most likely due to activation of NK cells [87]. We have found that CpG ODN inhibits the *in vivo* growth of B16 melanoma cells and EL4 lymphoma in both immunocompetent and severe combined immunodeficiency mice that lack T or B cells but retain NK function.

Removing NK cells eliminated the anti-tumor response [unpublished results]. Although toxicity from CpG ODN in these and other animal models has been limited, toxicity from non-specific anti-cancer therapy often limits clinical efficacy. Clinical trials currently underway will help us determine whether CpG ODN used to stimulate the innate immune system have promise in the treatment of cancer.

Enhancement of the innate immune system can be used to increase anti-tumor activity in a more specific manner if used in combination with passive administration of agents that allow for tumor targeting, such as monoclonal antibodies. Recent clinical studies demonstrate unlabeled monoclonal antibodies as single agents have significant anti-tumor activity in a number of tumor types, including lymphoma and breast carcinoma [88, 89]. Despite this success, there continues to be significant room for improvement, with most patients responding only transiently. Antibody-dependent cellular cytotoxicity (ADCC) mediated by NK cells and monocytes/macrophages likely plays a large role in the observed clinical responses. The addition of CpG ODN to activate NK cells and monocytes/macrophages, could therefore enhance the efficacy of antibody therapy.

Indeed, we found that CpG ODN-activated murine splenocytes or human peripheral blood lymphocytes mediate ADCC more effectively than do unactivated lymphocytes. *In vivo*, CpG ODN alone had no effect on survival of mice inoculated with the 38C13 murine B cell lymphoma. However, a single injection of CpG ODN enhanced the anti-tumor response to anti-tumor antibody therapy [90]. The combination of antibody plus a single dose of CpG ODN was more effective than antibody with multiple doses of IL-2 at inhibiting tumor growth. More recently, we have found that repeated doses of antibody plus CpG ODN can eliminate tumor load estimated to be 30-fold greater than can antibody alone [unpublished results]. Thus, use of CpG ODN to enhance the efficacy of antibody therapy remains promising. A clinical trial designed to assess this possibility will begin shortly.

Although vaccination for infectious diseases has had a major impact on worldwide public health, development of cancer vaccines has been more difficult. Most challenging in the development of cancer vaccines is the need to break immune tolerance against an antigen, and induce a tumor-specific immune response, including a cellular response strong enough to induce tumor cell destruction [64, 91, 92]. According to classic immunological teaching, intracellular proteins are processed and presented in class I molecules, and this leads to a cellular immune response. In contrast, extracellular antigens are taken into the cell and presented in class II molecules, which leads to a humoral response. It is now accepted that there is cross-talk between the class I and class II pathways, with some extracellular antigens taken up by APCs and processed in a manner that leads to presentation in class I molecules and development of a CTL response [93]. There is now evidence in a number of systems that CpG ODN enhances "cross-priming." In these systems, APCs process exogenous antigen and present peptides derived from such antigens in class I MHC. CpG ODN enhances this effect so that an effective cellular response can be induced by immunization with an intact model antigen [56]. Clinical trials will be needed to determine whether such a response can be elicited in humans.

There is also growing evidence that CpG ODN can impact on cells that are not strictly considered part of the immune system. CpG ODN induces mobilization of hematopoietic cells and extramedullary hematopoiesis, particularly in the spleen [38]. Ongoing studies are exploring the effect of CpG ODN on marrow reconstitution after a variety of myelosuppressive treatments. Whether similar effects on hematopoiesis will occur in humans remains to be seen.

CONCLUSIONS

Recent advances in our understanding of the relationship between the immune system and infection, autoimmunity, and cancer have reawakened interest in the field of immunotherapy. Recognition of the potent immunostimulatory effects of CpG ODN suggest that such agents may well be important agents in the basic immunology laboratory, and in the treatment and prevention of a broad range of diseases. Preliminary studies suggest CpG ODN can be effective in a variety of scenarios when used alone or in combination with other agents. Despite this promise we still do not understand the molecular mechanisms responsible for the immunostimulatory effects of CpG ODN. All CpG ODN are not alike, and more needs to be learned about the heterogeneous responses that occur based on host organism, cell subset, or CpG ODN sequence. Most importantly, we have not yet explored their clinical effects. Further work with CpG ODN in both the laboratory and the clinic is needed before we can know their true promise as investigational immunological and therapeutic agents.

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