

The CpG Motif:

Implications for Clinical Immunology

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Abstract

The immune system appears to have evolved a mechanism to detect prokaryotic nucleic acids based on their content of unmethylated CpG dinucleotides in particular base contexts. This response promotes the activation of antigen-specific B cells and the secretion of T helper-1-like cytokines. Preliminary animal studies indicate the potential therapeutic utility of CpG DNA as a vaccine adjuvant, and for the immunotherapy of cancer and allergic diseases. On the other hand, CpG DNA can also serve as a trigger for the sepsis syndrome and may have a pathogenic role in certain inflammatory immune-mediated diseases.

1. Background

A wide variety of nucleic acids have been reported to have immune effects. For example, poly(I,C) has been known for decades as an inducer of macrophage and natural killer (NK) cell activation and interferon (IFN) production.^[3-5] Aside from immune activation by this RNA polymer, a DNA polymer consisting of guanosine and cytosine, poly(dG,dC), causes B cell proliferation and immunoglobulin secretion as well as activating NK cells and inducing secretion of interferon- γ (IFN γ).^[6,7] Mycobacterial DNA has also been reported to activate NK cells and induce IFN secretion.^[8] Vertebrate DNA was found to lack these immune stimulatory effects.

In further studies, certain specific synthetic oligonucleotide sequences were found to mimic the immune stimulatory effects of mycobacterial DNA.^[9] The immune stimulatory effects of these oligonucleotides were attributed to certain hexamer palindrome sequences. The differential immune activa-

tion by bacterial DNA, but not vertebrate DNA, was thought to result from a higher frequency of these palindromes rather than from other structural differences between bacterial and vertebrate DNAs such as methylation of CpG dinucleotides.^[10] Bacterial DNA was also reported to induce B cell activation and immunoglobulin secretion, although vertebrate DNA did not.^[11]

Around the same time, certain investigators using antisense oligonucleotides began to observe unexpected sequence-specific immune stimulatory effects.^[12-15] These oligonucleotides all caused B cell proliferation with or without immunoglobulin secretion, but there was no apparent significant homology between the different immune stimulatory oligonucleotide sequences. We independently observed immune stimulatory effects of certain synthetic oligonucleotides, some of which contained palindromes, but most of which did not. In order to identify the feature or features responsible for immune stimulation by these oligonucleotides, we synthesised a large pool of point mutations of the

immune stimulatory oligonucleotides and analysed the varying effects. This eventually led to the identification of a CpG dinucleotide in particular base contexts as being the sole motif responsible for immune stimulation.^[16] Synthetic oligonucleotides ranging in length from 8 to 30 or more bases could cause immune stimulation if there was only a single CpG dinucleotide as long, as this was not preceded by a C or followed by a G. Most importantly, the CpG dinucleotide had to be unmethylated: if the C was replaced by a 5-methyl cytosine, then the oligonucleotide lost its immune stimulatory activity.^[16]

This definition of the CpG motif tied together the previously observed immune stimulatory effects of bacterial DNA and the various 'antisense' oligonucleotides, which contained one or more CpG motifs. CpG dinucleotides are relatively common in bacterial DNA and are unmethylated, but are methylated in vertebrate DNA as well as being 'suppressed'. They only occur about one-quarter as frequently as would be predicted on the basis of random base utilisation.^[17] Furthermore, CpG dinucleotides in vertebrate genomes are most commonly preceded by a C and/or followed by a G, which would make them non-immune stimulatory.

Thus, the presence of these 'CpG motifs' in prokaryotic DNA could enable it to be detected as foreign by the host immune system. The detection of such prokaryotic DNA in host tissues could indicate infection, and thus be used as a signal to trigger an appropriate immune response. Indeed, similar pattern recognition molecules are sensitive to microbial products such as lipopolysaccharides and mannans and are a mainstay of the innate immune defences.

Interestingly, it appears that many viruses may have evolved to avoid triggering the CpG host defence mechanism. Virtually all small DNA viruses and retroviruses have a very high degree of CpG suppression, with only 6 to 20% of the CpGs predicted based on random base utilisation.^[18,19] This degree of CpG suppression was not simply the result of a small genome, since there is no CpG suppression in bacterial phage. Instead, it appears

likely that CpG suppression in viruses has arisen as an adaptation to replication in a eucaryotic host.

2. Mechanisms of Action and Immune Effects of CpG DNA

One possible mechanism of action for CpG DNA could involve the binding to a cell surface receptor which then transduces a stimulatory signal. Indeed, evidence in support of this model has been published recently.^[20] However, our own experiments using a different experimental model system and mouse cells instead of human cells have led to the opposite conclusion.^[16] It has long been recognised that there are DNA-binding proteins on the cell surface and that DNA is internalised by cells spontaneously into an endosomal compartment where it is acidified and degraded.^[21-24] Several cell surface proteins that bind DNA have been described, but their role in mediating DNA uptake remains uncertain.^[25,26] Internalisation of the DNA may be mediated by adsorptive endocytosis.

Nearly all cell types appear to be capable of internalising DNA. Among cells of the immune system, uptake is inducible and generally appears to be highest among monocyte-derived cells and B cells.^[27,28] B cells and monocytic cells that have internalised CpG DNA rapidly show signs of activation, including generation of reactive oxygen species, activation of the mitogen-activated protein kinase (MAPK) signalling pathways, activation of transcriptional regulatory proteins such as nuclear factor κ B (NF κ B), and induction of transcription of multiple proto-oncogene and cytokine mRNAs.^[16,29-35] The earliest activation events such as the reactive oxygen burst, NF κ B activation and MAPK activation (which occur within a few minutes of exposure to CpG DNA) all appear to be mediated through a specific chloroquine-sensitive pathway, as these are completely blocked by low concentrations of chloroquine and related compounds, but B cell or monocyte activation by other activators such as endotoxin or CD40 are not inhibited.^[32,36]

The cytokines induced by CpG DNA are most notable for the predominant secretion of the T

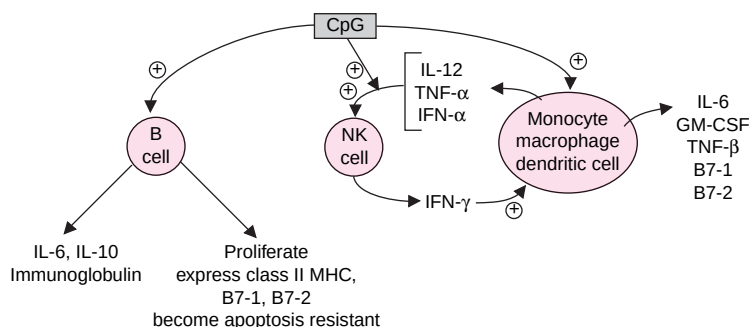


Fig. 1. Overview of the cell types that respond to CpG DNA. CpG DNA, which can be derived either from prokaryotic genomic DNA, plasmids, or synthetic oligodeoxynucleotides, directly activates both B cells and monocytes, including monocyte-derived cells such as macrophages and dendritic cells. It does not appear to directly activate highly purified natural killer (NK) cells; however, it greatly enhances the activation of NK cells in response to low doses of interleukin-12 (IL-12).^[1] T cells do not appear to be directly activated by CpG DNA, although a recent study reported that it could costimulate T cells that were activated through the antigen receptor.^[2] Although many cytokines are produced in the response to CpG DNA, that response is dominated by the T helper-1-like effects of interferon- γ (IFN γ) and IL-12. **B7-1** = CD80; **B7-2** = CD86; **CpG** = CpG DNA; **IL** = interleukin; **IFN** = interferon; **MHC** = major histocompatibility complex; **TNF** = tumour necrosis factor.

helper-1-like (Th1-like) cytokines, IL-12 and IFN γ (fig. 1).^[9,33,37] The IL-12 is predominantly derived from monocytic cells such as macrophages and dendritic cells, which also produce other pro-inflammatory cytokines such as type 1 interferons, IL-6, and tumour necrosis factor- α (TNF α). In addition, these cells are induced to upregulate their surface expression of class II MHC molecules and of costimulatory molecules. Thus, CpG DNA creates a Th1-like cytokine environment and enhances the function of antigen-presenting cells. Together with CpG DNA, the IL-12 activates NK cells to secrete IFN γ and to have enhanced lytic activity.^[38,39] The IFN γ secretion promotes the B cell response to CpG DNA, including both IL-6 secretion and IgM secretion. Of note is the fact that although CpG DNA can be an extremely powerful mitogen that drives more than 95% of B cells into the cell cycle, at lower concentrations the DNA stimulates strongly with signals mediated through the B cell antigen receptor (BCR).^[16] Thus, B cells that have bound specific antigen will be preferentially activated by CpG DNA. Like monocytic cells, B cells also upregulate their expression of costimulatory molecules and class II MHC upon activation with CpG DNA.^[16,40]

3. Therapeutic Applications of CpG Motifs

If immune activation by CpG DNA is indeed a defence mechanism against infection, then it seems logical to hypothesise that exposure to CpG DNA would promote resistance against infection. In order to test this hypothesis, we primed mice with low doses of CpG DNA and then challenged them by injection with a lethal dose of *Listeria monocytogenes*. Mice primed with CpG DNA showed a dramatically enhanced resistance to infection, which persisted for at least 2 weeks after a single dose of DNA.^[41]

In addition to enhancing antigen-nonspecific innate immune defences, several lines of evidence suggested that CpG DNA might promote the generation of an antigen-specific immune response. Firstly, CpG DNA activation of B cells synergises with activation through the antigen receptor. Secondly, CpG DNA makes B cells resistant to activation-induced apoptosis, which may help to promote a more sustained immune response.^[29,31,42] Thirdly, the cells and other antigen-presenting cells are induced to upregulate the expression of

class II MHC and other costimulatory molecules, as mentioned above.

Indeed, several groups have now reported that CpG DNA functions as a superb adjuvant for inducing antibody- and cell-mediated immune responses to a variety of protein antigens whether exposure occurs via intramuscular, subcutaneous, intradermal or mucosal administration.^[2,40,43-46] Recent studies indicate that CpG DNA is also an effective adjuvant for neonatal immunisation (Davis, personal communication) and for immunisation to polysaccharide antigens (Waldschmidt et al., unpublished observation). In addition, CpG motifs have been reported to be required for effective DNA vaccination.^[47,48] As would be expected from the cytokines induced by CpG DNA, these immune responses are strongly Th1-like with a predominance of IgG2A antibodies and strong induction of cytotoxic T lymphocyte (CTL) responses. It is worthy of note that CpG DNA is actually a stronger Th1-like vaccine than complete Freund's adjuvant.^[44]

CpG DNA can be used to induce antitumour immune responses to tumour antigens,^[45] or to activate NK cells to attack tumours that are NK-sensitive (Ballas et al., unpublished observation). CpG DNA is also a very strong inducer of antibody-dependent cellular cytotoxicity, and can be used together with antitumour antibodies to drive strong curative antitumour immune responses.^[49] Recent experiments have demonstrated that the Th1-like effect of CpG DNA can be used to reverse the T helper-2 (Th2) immune response to an allergen, preventing disease in a mouse model of asthma.^[50]

The therapeutic potential of CpG DNA is enhanced by its relative lack of toxicity (at least at low doses) and by its ease of synthesis. For example, nuclease-resistant phosphorothioate oligodeoxynucleotides containing CpG motifs can be synthesised at a cost of approximately \$US200/g (Agrawal, personal communication). Since an estimated effective dose for a human vaccine would be approximately 500 µg, this is quite economical. Human clinical trials using CpG DNA as a vaccine enhancer are scheduled to begin near the end of 1998.

4. Pathogenic Effects of CpG DNA

Excessive or inappropriate immune activation contributes to the pathogenesis of many diseases. As a powerful immune activator, CpG DNA may initiate inappropriate immune responses in certain clinical conditions. An obvious example of this is the sepsis syndrome, where immune activation causes substantial morbidity and mortality. We have demonstrated that CpG DNA-primed immune responses cause mortality in mice upon subsequent challenge with a nonlethal dose of endotoxin.^[39] This increased mortality was dependent upon the induction of IFN γ secretion by CpG DNA, compatible with the role of IFN γ in other models of this sepsis syndrome. CpG DNA can also cause mortality in mice that have been primed with D-galactosamine, which renders them highly susceptible to the toxic effects of TNF α .^[51]

Aside from such systemic exposures to CpG DNA, the lungs would be exposed to this immune stimulator during pulmonary infections. To investigate this possibility, sputum samples were obtained from patients with cystic fibrosis who had chronic pulmonary infections. Bacterial DNA was detected using a specific polymerase chain reaction amplification technique, and was found to comprise approximately 0.1% of the DNA present in pulmonary secretions.^[52] Installation of synthetic oligonucleotides containing CpG motifs or bacterial DNA into the lungs of mice caused acute inflammatory responses. These studies suggest a possible role for CpG DNA in mediating pulmonary inflammation during chronic infections.

CpG DNA is also an attractive candidate as a trigger for induction of systemic lupus erythematosus. In this case, we might hypothesise that B cells specific for DNA would bind CpG DNA through the antigen receptor and also be activated by its mitogenic effects. Several other lines of circumstantial evidence also support the hypothesis that CpG DNA may act as a trigger for lupus (reviewed by Krieg^[53]). Although immunisation of mice with CpG DNA complexed to protein can indeed induce the production of anti-DNA antibodies in normal and lupus-prone mice, it does not induce

or accelerate autoimmune disease.^[54] Studies with naked DNA have also demonstrated a failure to induce or accelerate autoimmune disease.^[55]

Despite these negative results, the multifactorial nature of autoimmune diseases must leave open the possibility that, in certain genetically predisposed individuals, CpG DNA could act as a trigger. Indeed, infections have long been recognised as a trigger for a variety of immune-mediated inflammatory diseases. The significance of this for the clinical development of therapeutic CpG DNA is unclear. Presumably, during an individual's lifetime he or she will be exposed to CpG DNA many times as a result of infections. The added risk of an additional exposure to CpG DNA might be expected to be very small.

Indeed, there may actually be a risk from the failure to be exposed to CpG DNA. The incidence of asthma and other allergic diseases has been increasing dramatically in industrialised countries. This cannot be explained by exposure to pollution, but has recently been linked to a reduced incidence of childhood bacterial infections.^[56,57] Current childhood vaccines are given in alum, which promotes a Th2-type immune response and may even aggravate asthma. These data raise the possibility that childhood exposure to CpG DNA may restore a Th1 immune influence and reduce the incidence of asthma and other allergic diseases. Further studies will be required to investigate this possibility.

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