METHODS OF TREATING CANCER USING IMMUNOSTIMULATORY Oligonucleotides

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Appl. No.: 10/719,493
Filed: Nov. 21, 2003

Prior Publication Data
US 2004/0087538 A1 May 6, 2004

Related U.S. Application Data
Continuation of application No. 09/337,619, filed on Jun. 21, 1999, now Pat. No. 6,653,292, which is a division of application No. 08/960,774, filed on Oct. 30, 1997, now Pat. No. 6,239,116, which is a continuation-in-part of application No. 08/738,652, filed on Oct. 30, 1996, now Pat. No. 6,207,646, which is a continuation-in-part of application No. 08/386,063, filed on Feb. 7, 1995, now Pat. No. 6,194,388, which is a continuation-in-part of application No. 08/276,358, filed on Jul. 15, 1994, now abandoned.

Int. Cl. C12N 15/11 (2006.01)
C12N 15/00 (2006.01)
C12N 5/00 (2006.01)
A61K 48/00 (2006.01)
C07H 21/02 (2006.01)
C07H 21/04 (2006.01)
C12P 21/06 (2006.01)

Field of Classification Search : 514/44; 536/23.1; 536/24.1; 536/24.5; 435/69.1; 435/320.1; 435/325; 435/455

See application file for complete search history.

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ABSTRACT
Nucleic acid sequences containing unmethylated CpG dinucleotides that modulate an immune response including stimulating a Th1 pattern of immune activation, cytokine production, NK lytic activity, and B cell proliferation are disclosed. The sequences are also useful as a synthetic adjuvant.

30 Claims, 19 Drawing Sheets
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FIG. 3
**FIG. 12**

- **CELL COUNT \(10^6\)**
- **AMOUNT OF OLIGONUCLEOTIDE (\(\mu g\))**

- **EOSINOPHILS**
- **TOTAL CELL COUNT**
METHODS FOR TREATING CANCER USING IMMUNOSTIMULATORY OLIGONUCLEOTIDES

RELATED APPLICATION


GOVERNMENT

The work resulting in this invention was supported in part by National Institute of Health Grant No. R29-AR42556-01. The U.S. Government has certain rights in the invention.

FIELD OF THE INVENTION

The present invention relates generally to oligonucleotides and more specifically to oligonucleotides which have a sequence including at least one unmethylated CpG dinucleotide which are immunostimulatory.

BACKGROUND OF THE INVENTION


Lymphocyte ODN uptake has been shown to be regulated by cell activation. Spleen cells stimulated with the B cell mitogen LPS had dramatically enhanced ODN uptake in the B cell population, while spleen cells treated with the T cell mitogen ConA showed enhanced ODN uptake by T but not B cells (Krieg, A. M., F. Ginzlig-Meyling, M. F. Gourley, W. J. Kisch, L. A. Chrisley, and A. D. Steinberg. 1991. “Uptake of oligodeoxyribonucleotides by lymphoid cells is heterogeneous and inducible.” Antisense Research and Development 1:161).


Bonneville, and J.-J. Fournie. 1994. “Stimulation of human γδ T cells by nonpeptide mycobacterial ligands.” Science 264:267. This report indicated the possibility that the immune system may have evolved ways to preferentially respond to microbial nucleic acids.

Several observations suggest that certain DNA structures may also have the potential to activate lymphocytes. For example, Bell et al. reported that nucleoskeletal protein-DNA complexes (but not naked DNA) in spleen cell supernatants caused B cell proliferation and immunoglobulin secretion (Bell, D. A., B. Morrison, and P. VandenBygaart. 1990. “Immunogenic DNA-related factors.” J. Clin. Invest. 85:1487). In other cases, naked DNA has been reported to have immune effects. For example, Messina et al. have recently reported that 260 to 800 bp fragments of poly (dC)* (dC) and poly (dG* dC) were mitogenic for B cells (Messina, J. P., G. S. Gilkeson, and D. S. Pisetsky. 1993. “The influence of DNA structure on the in vitro stimulation of murine lymphocytes by natural and synthetic polynucleotide antigens.” Cell. Immunol. 147:148). Tokunaga, et al. have reported that dG* dC induces γIFN and NK activity (Tokunaga, S. Yamamoto, and K. Nama. 1988. “A synthetic single-stranded DNA, poly(dC, dG), induces interferon-α/β and γ, augments natural killer activity, and suppresses tumors growth.” Jpn. J. Cancer Res. 79:682). Aside from such artificial homopolymer sequences, Pisetsky et al. reported that pure mammalian DNA has no detectable immune effects, but that DNA from certain bacteria induces B cell activation and immunoglobulin secretion (Messina, J. P., G. S. Gilkeson, and D. S. Pisetsky. 1991. “Stimulation of in vitro murine lymphocyte proliferation by bacterial DNA.” J. Immunol. 147:1759). Assuming that these data did not result from some unusual contaminant, these studies suggested that a particular structure or other characteristic of bacterial DNA renders it capable of triggering B cell activation. Investigations of mycobacterial DNA sequences have demonstrated that ODN which contain certain palindromic sequences can activate NK cells (Yamamoto, S., T. Yamamoto, T. Kataoka, E. Kurotomo, O. Yano, and T. Tokunaga. 1992. “Unique palindromic sequences in synthetic oligonucleotides are required to induce INF and augment INF-mediated natural killer activity.” J. Immunol. 148:4072; Kuramoto, E., O. Yano, Y. Kimura, M. Baba, T. Makino, S. Yamamoto, T. Yamamoto, T. Kataoka, and T. Tokunaga. 1992. “Oligonucleotide sequences required for natural killer cell activation.” Jpn. J. Cancer Res. 83:1128).


The eAMP response element binding protein (CREB) and activating transcription factor (ATF) or CREB/ATF family of transcription factors is a ubiquitously expressed class of transcription factors of which 11 members have so far been cloned (reviewed on de Groot, R. P., and P. Sassone-Corsi: “Hormonal control of gene expression: Multiplicity and versatility of cyclic adenosine 3',5'-monophosphate-responsive nuclear regulators.” Mol. Endocrin. 7:145, 1993; Lee, K. A. W., and N. Masson: “Transcriptional regulation by CREB and its relatives.” Biochim. Biophys. Acta 1174:221, 1993). They all belong to the basic region/leucine zipper (bZi) class of proteins. All cells appear to express one or more CREB/ATF proteins, but the members expressed and the regulation of mRNA splicing appear to be tissue-specific. Differential splicing of activation domains can determine whether a particular CREB/ATF protein will be a transcriptional inhibitor or activator. Many CREB/ATF proteins activate viral transcription, but some splicing variants which lack the activation domain are inhibitory. CREB/ATF proteins can bind DNA as homo- or hetero-dimers through the AMP response element, the CRE, the consensus form of which is the unmethylated sequence TGAAGGTCA (SEQ. ID. No. 103) (binding is abolished if the CpG is methylated) (Iguchi-Arima, S. M., and W. Schaffner: “CpG methylation of the eAMP-responsive enhancer/promoter sequence TGAAGGTCA (SEQ. ID. No. 104) abolishes specific factor binding as well as transcriptional activation.” Genes Dev. 3:612, 1989).


Aside from their critical role in regulating cellular transcription, it has recently been shown that CREB/ATF proteins are subverted by some infectious viruses and retroviruses, which require them for viral replication. For example, the cytomegalovirus immediate-early promoter, one of the strongest known mammalian promoters, contains eleven copies of the CRE which are essential for promoter function (Chang, Y.-N., S. Crawford, J. Stall, D. R. Rawlins, K.-T. Jeang, and G. S. Hayward: "The palindromic series 1 repeats in the simian cytomegalovirus major immediate-early promoter behave as both strong basal enhancers and cyclic AMP response elements." *J. Virol.* 64:264, 1990). At least some of the transcriptional activating effects of the adenovirus E1A protein, which induces many promoters, are due to its binding to the DNA binding domain of the CREB/ATF protein, ATF-2, which mediates E1A inducible transcription activation (Liu, F., and M. R. Green: "Promoter targeting by adenovirus E1A through interaction with different cellular DNA-binding domains." *Nature* 368:520, 1994). It has also been suggested that E1A binds to the CREB-binding protein, CBP (Arany, Z., W. R. Sellers, D. M. Livingston, and R. Eckner: "E1A-associated p300 and CREB-associated CBP belong to a conserved family of coactivators." *Cell* 77:799, 1994). Human T lymphotropic virus-1 (HTLV-1), the retrovirus which causes human T cell leukemia and tropical spastic paraparesis, also requires CREB/ATF proteins for replication. In this case, the retrovirus produces a protein, Tax, which binds to CREB/ATF proteins and redirects them from their normal cellular binding sites to different DNA sequences (flanked by G- and G-rich sequences) present within the HTLV transcriptional enhancer (Papa-Uccellarunk, S., L.-J. Zhao, N. Adya, J. V. Cross, B. R. Cullen, I. M. Boros, and C.-Z. Gian: "In vitro selection of DNA elements highly responsive to the human T-cell lymphotropic virus type 1 transcriptional activator, Tax." *Mol. Cell. Biol.* 14:456, 1994; Adya, N., L.-J. Zhao, W. Huang, I. Boros, and C.-Z. Gian: "Expansion of CREB's DNA recognition specificity by Tax results from interaction with Ala-Ala-Arg at positions 282-284 near the conserved DNA-binding domain of CREB." *Proc. Natl. Acad. Sci. USA* 91:5642, 1994).

**SUMMARY OF THE INVENTION**

The present invention is based on the finding that certain nucleic acids containing unmethylated cytosine-guanine (CpG) dinucleotides activate lymphocytes in a subject and redirect a subject's immune response from a Th2 to a Th1 (e.g., by inducing monocycte cells and other cells to produce Th1 cytokines, including II-12, IFN-γ and GM-CSF). Based on this finding, the invention features, in one aspect, novel immunostimulatory nucleic acid compositions. In one embodiment, the invention provides an isolated immunostimulatory nucleic acid sequence containing a CpG motif represented by the formula:

\[ 5'N_X_bCGX_bN_y, \]

wherein at least one nucleotide separates consecutive CpGs; \( X_b \) is adenine, guanine, or thymine; \( X_y \) is cytosine or thymine; \( N \) is any nucleotide and \( N_b^+N_y \) is from about 0-26 bases with the proviso that \( N_b \) and \( N_y \) do not contain a CCGG or CGG trimmer; and the nucleic acid sequence is from about 8-30 bases in length.

In another embodiment, the invention provides an isolated immunostimulatory nucleic acid sequence contains a CpG motif represented by the formula:

\[ 5'N_X_bCGX_bN_y, \]

wherein at least one nucleotide separates consecutive CpGs; \( X_b \) is selected from the group consisting of GpT, GpG, GpA, ApT and ApA; \( X_y \) is selected from the group consisting of TpT or CpT; \( N_b \) is any nucleotide and \( N_y^+N_b \) is from about 0-26 bases with the proviso that \( N_b \) and \( N_y \) do not contain a CCGG or CGG trimmer; and the nucleic acid sequence is from about 8-30 bases in length.

In another embodiment, the invention provides a method of stimulating immune activation by administering the nucleic acid sequences of the invention to a subject, preferably a human. In a preferred embodiment, the immune activation effects predominantly a Th1 pattern of immune activation.

In another embodiment, the nucleic acid sequences of the invention stimulate cytokine production. In particular, cytokines such as II-6, II-12, IFN-γ, TNF-α and GM-CSF are produced via stimulation of the immune system using the nucleic acid sequences described herein. In another aspect, the nucleic acid sequences of the invention stimulate the lytic activity of natural killer cells (NK) and the proliferation of T cells.

In another embodiment, the nucleic acid sequences of the invention are useful as an artificial adjuvant for use during antibody generation in a mammal such as a mouse or a human.

In another embodiment, autoimmune disorders are treated by inhibiting a subject's response to CpG mediated leukocyte activation. The invention provides administration of inhibitors of endosomal acidification such as bafilomycin a, chloroquine, and monensin to ameliorate autoimmune disorders. In particular, systemic lupus erythematosus is treated in this manner.

The nucleic acid sequences of the invention can also be used to treat, prevent or ameliorate other disorders (e.g., a tumor or cancer or a viral, fungal, bacterial or parasitic infec-
tion). In addition, the nucleic acid sequences can be administered to stimulate a subject's response to a vaccine. Furthermore, by redirecting a subject's immune response from Th2 to Th1, the claimed nucleic acid sequences can be used to treat or prevent an asthmatic disorder. In addition, the claimed nucleic acid molecules can be administered to a subject in conjunction with a particular allergen as a type of desensitization therapy to treat or prevent the occurrence of an allergic reaction associated with an asthmatic disorder.

Further, the ability of the nucleic acid sequences of the invention described herein to induce leukemic cells to enter the cell cycle supports their use in treating leukemia by increasing the sensitivity of chronic leukemia cells followed by conventional ablative chemotherapy, or by combining the nucleic acid sequences with other immunotherapies.

Other features and advantages of the invention will become more apparent from the following detailed description and claims.

BRIEF DESCRIPTION OF THE FIGURES

FIGS. 1A-C are graphs plotting dose-dependent IL-6 production in response to various DNA sequences in T cell depleted spleen cell cultures. FIG. 1A. E. coli DNA (●) and calf thymus DNA (■) sequences and LPS (at 10x the concentration of E. coli and calf thymus DNA) (○). FIG. 1B. Control phosphodiester oligodeoxynucleotide (ODN) 5'-ATGGAGGCTCCAGGTGTC (SEQ ID NO: 114) (■) and two phosphodiester CpG ODN 5'-ATGACGATCTGGTGTC (SEQ ID NO: 2) (○) and 5'-TCCATGACGTCTTAGCT (SEQ ID NO: 3) (●). FIG. 1C. Control phosphorothioate ODN 5'-GCTAGATGGTACG (SEQ ID NO: 4) (■) and two phosphorothioate CpG ODN 5'-GAGAACGTCTCGAGCTG (SEQ ID NO: 5) (○) and 5'-GCTAGACGTCTTAGCT (SEQ ID NO: 6) (●). Data present the mean ±standard deviation of triplicates.

FIG. 2 is a graph plotting IL-6 production induced by CpG DNA in vivo as determined 1-8 hrs after injection. Data represent the mean from duplicate analyses of sera from two mice. BALB/c mice (two mice/group) were injected iv with 100 µl of PBS (□) or 200 µg of CpG phosphorothioate ODN 5'-TCCATGACGTCTTAGCT (SEQ ID NO: 7) (■) or non-CpG phosphorothioate ODN 5'-TCCATGACGTCTTAGCT (SEQ ID NO: 8) (○).

FIG. 3 is an autoradiograph showing IL-6 mRNA expression as determined by reverse transcription polymerase chain reaction in liver, spleen, and thymus at various time periods after in vivo stimulation of BALB/c mice (two mice/group) injected iv with 100 µl of PBS, 200 µg of CpG phosphorothioate ODN 5'-TCCATGACGTCTTAGCT (SEQ ID NO: 7) or non-CpG phosphorothioate ODN 5'-TCCATGACGTCTTAGCT (SEQ ID NO: 8).

FIG. 4A is a graph plotting dose-dependent inhibition of CpG-induced IgM production by anti-IL-6. Splenic B-cells from DBA/2 mice were stimulated with CpG ODN 5'-TCCAGACGTCTTAGCT (SEQ ID NO: 9) in the presence of the indicated concentrations of neutralizing anti-IL-6 (●) or isotype control Ab (○) and IgM levels in culture supernatants determined by ELISA. In the absence of CpG ODN, the anti-IL-6 Ab had no effect on IgM secretion (■).

FIG. 4B is a graph plotting the stimulation index of CpG-induced splenic B cells cultured with anti-IL-6 and CpG S-ODN 5'-TCCATGACGTCTTAGCT (SEQ ID NO: 7) (●) or anti-IL-6 antibody only (■). Data present the mean ± standard deviation of triplicates.

FIG. 5 is a bar graph plotting chlamydomphila acetyltansferase (CAT) activity in WEHI-231 cells transfected with a promoter-less CAT construct (pCAT), positive control plasmid (R5V), or IL-6 promoter-CAT construct alone or cultured with CpG 5'-TCCATGACGTCTTAGCT (SEQ ID NO: 7) or non-CpG 5'-TCCATGACGTCTTAGCT (SEQ ID NO: 8) phosphorothioate ODN at the indicated concentrations. Data present the mean of triplicates.

FIG. 6 is a schematic overview of the immune effects of the immunostimulatory unmethylated CpG containing nucleic acids, which can directly activate both B cells and monocytic cells (including macrophages and dendritic cells) as shown. The immunostimulatory oligonucleotides do not directly activate purified NK cells, but render them competent to respond to IL-12 with a marked increase in their IFN-γ secretion by NK cells, the immunostimulatory nucleic acid promoters a Th1 type immune response to direct activation of proliferation of cytokine secretion by highly purified T cells has been found. However, the induction of Th1 cytokine secretion by the immunostimulatory oligonucleotides promotes the development of a cytotoxic lymphocyte response.

FIG. 7 is an autoradiograph showing NFκB mRNA induction in monocytes treated with E. coli (EC) DNA (containing unmethylated CpG motifs), control (CT) DNA (containing no unmethylated CpG motifs) and lipopolysaccharide (LPS) at various measured times, 15 and 30 minutes after contact.

FIG. 8A shows the results from a flow cytometry study using mouse B cells with the dihydrodoradmine 123 dye to determine levels of reactive oxygen species. The dye only sample in Panel A of the figure shows the background level of cells positive for the dye at 28.6%. This level of reactive oxygen species was greatly increased to 80% in the cells treated for 20 minutes with PMA and ionomycin, a positive control (Panel B). The cells treated with the CpG oligo (TCCATGACGTCTTAGCT SEQ ID NO: 10) also showed an increase in the level of reactive oxygen species such that more than 50% of the cells became positive (Panel D). However, cells treated with an oligonucleotide that lacked a CpG motif (TCCATGACGTCTTAGCT SEQ ID NO: 8) did not show this significant increase in the level of reactive oxygen species (Panel E).

FIG. 8B shows the results from a flow cytometry study using mouse B cells in the presence of chloroquine with the dihydrodoradmine 123 dye to determine levels of reactive oxygen species. Chloroquine slightly lowers the background level of reactive oxygen species in the cells such that the untreated cells in Panel A have only 4.3% that are positive. Chloroquine completely abolishes the induction of reactive oxygen species in the cells treated with CpG DNA (Panel B) but does not reduce the level of reactive oxygen species in the cells treated with PMA and ionomycin (Panel E).

FIG. 9 is a graph plotting lung lavage cell count over time. The graph shows that when the mice are initially injected with Schistosoma mansoni eggs “egg”, which induces a Th2 immune response, and subsequently inhale Schistosoma mansoni egg antigen “SEA” (open circle), many inflammatory cells are present in the lungs. However, when the mice are initially given CpG oligo (SEQ ID NO: 10) along with egg, the inflammatory cells in the lung are not increased by subsequent inhalation of SEA (open triangles).

FIG. 10 is a graph plotting lung lavage eosinophil count over time. Again, the graph shows that when the mice are initially injected with egg and subsequently inhale SEA (open circle), many eosinophils are present in the lungs. However, when the mice are initially given CpG oligo (SEQ ID NO: 10)
along with egg, the inflammatory cells in the lung are not increased by subsequent inhalation of the SEA (open triangles).

FIG. 11 is a bar graph plotting the effect on the percentage of macrophage, lymphocyte, neutrophil and eosinophil cells induced by exposure to saline alone; egg, then SEA; egg and SEQ ID NO: 10, then SEA; and egg and control oligo (SEQ ID NO: 8), then SEA. When the mice are treated with the control oligo at the time of the initial exposure to the egg, there is little effect on the subsequent influx of eosinophils into the lungs after inhalation of SEA. Thus, when mice inhale the eggs on days 14 or 21, they develop an acute inflammatory response in the lungs. However, giving a CpG oligo along with the eggs at the time of initial antigen exposure on days 0 and 7 almost completely abolishes the increase in eosinophils when the mice inhale the egg antigen on day 14.

FIG. 12 is a bar graph plotting eosinophil count in response to injection of various amounts of the protective oligo SEQ ID NO: 10.

FIG. 13 is a graph plotting interleukin 4 (IL-4) production (pg/ml) in mice over time in response to injection of saline; egg, then SEA; or SEQ ID NO: 10 and egg, then SEA. The graph shows that administration of an oligonucleotide containing an unmethylated CpG motif can actually redirect the cytokine response of the lung to production of IL-12, indicating a Th1 type of immune response.

FIG. 14 is a bar graph plotting interleukin 12 (IL-12) production (pg/ml) in mice over time in response to injection of saline; egg, then SEA; or SEQ ID NO: 10 and egg, then SEA. The graph shows that administration of an oligonucleotide containing an unmethylated CpG motif can also redirect the cytokine response of the lung to production of IFN-γ, indicating a Th1 type of immune response.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

As used herein, the following terms and phrases shall have the meanings set forth below:

An “allergen” refers to a substance that can induce an allergic asthmatic response in a susceptible subject. The list of allergens is enormous and can include pollens, insect venom, animal dander dust, fungal spores and drugs (e.g., penicillin). Examples of natural, animal and plant allergens include proteins specific to the following genera: Canine (Canis familiaris); Dermatophagoides (e.g., Dermatophagoides farinae); Felis (Felis domesticus); Ambrosia (Ambrosia artemisiifolia); Lulium (e.g., Lolium perenne or Lolium multiflorum); Cryptomeria (Cryptomeria japonica); Alternaria (Alternaria alternata); Alder; Alnus (Alnus glutinosa); Betula (Betula verrucosa); Quercus (Quercus alba); Olea (Olea europea); Artemisia (Artemisia vulgaris); Plantago (e.g., Plantago lanceolata); Parietaria (e.g., Parietaria officinalis or Parietaria judaica); Blatella (e.g., Blatella germanica); Apis (e.g., Apis mellifera); Cupressus (e.g., Cupressus sempervirens, Cupressus arizonica and Cupressus macrocarpa); Juniperus (e.g., Juniperus sabina, Juniperus virginiana, Juniperus communis and Juniperus ashei); Thuja (e.g., Thuja orientalis); Chamaecyparis (e.g., Chamaecyparis obtusa); Periplaneta (e.g., Periplaneta americana); Agropyron (e.g., Agropyron repens); Secale (e.g., Secale cereale); Triticum (e.g., Triticum aestivum); Daetmlys (e.g., Daetmlys glomerata); Festuca (e.g., Festuca elatior); Poa (e.g., Poa pratensis or Poa compressa); Avena (e.g., Avena salina); Holcus (e.g., Holcus lanatus); Anthoxanthum (e.g., Anthoxanthum odoratum); Arrhenatherum (e.g., Arrhenatherum elatius); Agris (e.g., Agris alba); Phleum (e.g., Phleum pratense); Phalaris (e.g., Phalaris arundinacea); Psalidium (e.g., Psalidium notatum); Sorghum (e.g., Sorghum halepensis) and Bromus (e.g., Bromus inermis).

An “allergy” refers to acquired hypersensitivity to a substance (allergen). Allergic conditions include eczema, allergic rhinitis or coriza, hay fever, bronchial asthma, uticaria (hives) and food allergies, and other atopic conditions.

“Asthma” refers to a disorder of the respiratory system characterized by inflammation, narrowing of the airways and increased reactivity of the airways to inhaled agents. Asthma is frequently, although not exclusively associated with atopic or allergic symptoms.

An “immune system deficiency” shall mean a disease or disorder in which the subject’s immune system is not functioning in normal capacity or in which it would be useful to boost a subject’s immune response for example to eliminate a tumor or cancer (e.g., tumors of the brain, lung (e.g., small cell and non-small cells), ovary, breast, prostate, colon, as well as other carcinomas and sarcomas) or an infection in a subject.

Examples of infectious virus include: Retroviridae (e.g., human immunodeficiency viruses, such as HIV-1, also referred to as HTLV-I, III, LAV or HTLV-III/LAV, or HIV-III, and other isolates, such as HIV-LP; Picornaviridae (e.g., polio viruses, hepatitis A virus; enteroviruses, human coxsackie viruses, rhinoviruses, echoviruses; Caliciviridae (e.g., strains that cause gastroenteritis); Togaviridae (e.g., equine encephalitis viruses, rubella viruses); Flaviridae (e.g., dengue viruses, encephalitis viruses, yellow fever viruses; Coronaviridae (e.g., coronaviruses); Rhabdoviridae (e.g., vesicular stomatitis viruses, rabies viruses; Filoviridae (e.g., ebola viruses); Paramyxoviridae (e.g., parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus); Orthomyxoviridae (e.g., influenza viruses; Bunyaviridae (e.g., Hantaan virus, bunga viruses, phleboviruses and Nairo viruses); Arena viridae (hemorrhagic fever virus); Reoviridae (e.g., reoviruses, orbiviruses and rotaviruses); Birnaviridae; Hepadnaviridae (Hepatitis B virus; Paroviridae (paroviruses); Papovaviridae (papilloma viruses, polyoma viruses); Adenoviridae (most adenoviruses); Herperviridae (herpes simplex virus (HSV) 1 and 2, varicella zoster virus, cytomegalovirus (CMV), herpes viruses); Poxviridae (variola viruses, vaccinia viruses, pox viruses); and Iridoviridae (e.g., African swine fever virus); and unclassified viruses (e.g., the etiological agents of Spongiform encephalopathies, the agent of delta hepatitis (thought to be a defective satellite of hepatitis B virus), the agents of non-A, non-B hepatitis (class 1—internally transmitted; class 2—parenterally transmitted (i.e., Hepatitis C); Norwalk and related viruses, and astroviruses).

Examples of infectious bacteria include: Helicobacter pylori, Borrelia burgdorferi, Legionella pneumophilia, Mycobacteria sps (e.g., M. tuberculosis, M. avium, M. Intra-cellulare, M. kansai, M. gordonae), Staphylococcus aureus, Neisseria gonorrhoeae, Neisseria meningitidis, Listeria monocyctogenes, Streptococcus pyogenes (Group A Streptococcus), Streptococcus agalactiae (Group B Streptococcus), Streptococcus (viridans group), Streptococcus faecalis, Streptococcus bovis, Streptococcus (anaerobic sps.), Streptococcus pneumoniae, pathogenic Campylobacter sp., Enterococcus sp., Haemophilus influenzae, Bacillus anthracis,
corynebacterium diphtheriae, corynebacterium sp., Ery-
sipelothrix rhusiopathiae, Clostridium perfringens, 
Clostridium tetani, Enterobacter aerogenes, Klebsiella pneu-
moniae, Pasteurella multocida, Bacteroides sp., Fusobacterium 
nucleatum, Streptococcus mitis, Treponema pallidum, Treponema pertenue, Leptospira, and Actinomyces israelii.

Examples of infectious fungi include: Cryptococcus neo-
formans, Histoplasma capsulatum, Coccidioides immitis, 
Blastomyces dermatitidis, Chlamydia trachomatis, Candida albicans. Other infectious organisms (i.e., protozoa) include: Plasmodium faliciparum and Toxoplasma gondii.

An “immunostimulatory nucleic acid molecule” refers to a nucleic acid molecule, which contains an unmethylated cytosine, guanine dinucleotide sequence (i.e., “CpG DNA” or DNA containing a cytosine followed by a guanosine and linked by a phosphate bond) and stimulates (e.g., has a mitogenic effect on, or induces or increases cytokine expression by) a vertebrate lymphocyte. An immunostimulatory nucleic acid molecule can be double-stranded or single-stranded. Generally, double-stranded molecules are more stable in vivo, while single-stranded molecules have increased immune activity.

In one preferred embodiment, the invention provides an isolated immunostimulatory nucleic acid sequence containing a CpG motif represented by the formula:

$$5' N\_X_1 CGX_2 N_3'$$

wherein at least one nucleotide separates consecutive CpGs; $X_1$ is adenine, guanine, or cytosine; $X_2$ is cytosine or thymine; and $N_3$ is any nucleotide and $N_3 + N_3$ is from about 0-26 bases with the proviso that $N_3$ and $N_3$ do not contain a CCGG quadmer or more than one CCG or CGG trimer; and the nucleic acid sequence is from about 8-30 bases in length.

In another embodiment the invention provides an isolated immunostimulatory nucleic acid sequence containing a CpG motif represented by the formula:

$$5' N\_X_1 CGX_2 N_3'$$

wherein at least one nucleotide separates consecutive CpGs; $X_1$, $X_2$, and $X_3$ are selected from the group consisting of G, A, or C; $X_1$, $X_2$, and $X_3$ are selected from the group consisting of T, $X_2$, or $X_3$; and $X_3$ and $X_3$ are from about 0-26 bases with the proviso that $N_3$ and $N_3$ do not contain a CCGG quadmer or more than one CCG or CGG trimer; and the nucleic acid sequence is from about 8-30 bases in length.

Preferably, the immunostimulatory nucleic acid sequences of the invention include $X_1$, $X_2$, and $X_3$ selected from the group consisting of G, G, or A, and $X_3$, $X_3$, and $X_3$, is selected from the group consisting of T, $X_2$, or $X_3$ (see Table 5). For facilitating uptake into cells, CpG containing immunostimulatory nucleic acid molecules are preferably in the range of 8 to 30 bases in length. However, nucleic acids of any size (even many kb long) are immunostimulatory if sufficient immunostimulatory motifs are present, since such larger nucleic acids are degraded into oligonucleotides inside of cells. Preferred synthetic oligonucleotides do not include a CCGG quadmer or more than one CCGG trimer at or near the 5’ and/or 3’ terminals and/or the consensus motific CpG motif is not a palindrome. Prolonged immunostimulation can be obtained using stabilized oligonucleotides, where the oligonucleotide incorporates a phosphate backbone modification. For example, the modification is a phosphorothioate or phosphorodithioate modification. More particularly, the phosphate backbone modification occurs at the 5’ end of the nucleic acid for example, at the first two nucleotides of the 5’ end of the nucleic acid. Further, the phosphate backbone modification may occur at the 3’ end of the nucleic acid for example, at the last five nucleotides of the 3’ end of the nucleic acid. Preferably the immunostimulatory CpG DNA is in the range of between 8 to 30 bases in size when it is an oligonucleotide. Alternatively, CpG dimers can be produced on a large scale in plasmids, which after being administered to a subject are degraded into oligonucleotides. Preferred immunostimulatory nucleic acid molecules (e.g., for use in increasing the effectiveness of a vaccine or to treat an immune system deficiency by stimulating an antibody (i.e., humoral) response in a subject) have a relatively high stimulation index with regard to B cell, monocyte and/or natural killer cell responses (e.g., cytokine, proliferative, lytic or other responses).

The nucleic acid sequences of the invention stimulate cytokine production in a subject for example. Cytokines include but are not limited to II-6, II-12, IFN-g, TNF-c, and GM-CSF. Exemplary sequences include: TCCATTGCCTATTGCATT (SEQ ID NO: 37), TCCATGTCGTGGATTGATT (SEQ ID NO: 38), TCGTCGTTTTGAGTTTTGAGTTT (SEQ ID NO: 46).

The nucleic acid sequences of the invention are also useful for stimulating natural killer cell (NK) lytic activity in a subject such as a human. Specific, but non-limiting examples of such sequences include: TCGTCTGTTGGATTGATT (SEQ ID NO: 47), TCGTCGTTTTTGAGTTTTGAGTTT (SEQ ID NO: 46), TCGTCGTTTTGAGTTTTGAGTTT (SEQ ID NO: 48), GCGTCGTTTTTGAGTTTTGAGTTT (SEQ ID NO: 51), and TCGTCGTTTTGAGTTTTGAGTTT (SEQ ID NO: 50). The nucleic acid sequences of the invention are useful for stimulating B cell proliferation in a subject such as a human. Specific, but non-limiting examples of such sequences include: TCCATTGCCTATTGCATT (SEQ ID NO: 52), TCGTCGTTTTGAGTTTTGAGTTT (SEQ ID NO: 53), TCGTCGTTTTGAGTTTTGAGTTT (SEQ ID NO: 54), TCGTCGTTTTGAGTTTTGAGTTT (SEQ ID NO: 55), TCGTCGTTTTGAGTTTTGAGTTT (SEQ ID NO: 46), TCGTCGTTTTGAGTTTTGAGTTT (SEQ ID NO: 49) and TCGTCGTTTTGAGTTTTGAGTTT (SEQ ID NO: 50).

In another aspect, the nucleic acid sequences of the invention are useful as an adjuvant for use during antibody production in a mammal. Specific, but non-limiting examples of such sequences include: TCCATGACGTTTCTGACAGTT (SEQ ID NO: 10), GTCGTT (SEQ ID NO: 57), GTCGCT (SEQ ID NO: 58), TGTCGCT (SEQ ID NO: 101) and TGTCGCT (SEQ ID NO: 102). Furthermore, the claimed nucleic acid sequences can be administered to treat or prevent the symptoms of an asthmatic disorder by redirecting a subject’s immune response from Th2 to Th1. An exemplary sequence includes TCCATGACGTTTCTGACAGTT (SEQ ID NO: 10).

The stimulation index of a particular immunostimulatory CpG DNA can be tested in various immune cell assays. Preferably, the stimulation index of the immunostimulatory CpG DNA with regard to B-cell proliferation is at least about 5, preferably at least about 10, more preferably at least about 15 and most preferably at least about 20 as determined by incorporation of 1H uridine in a murine B cell culture, which has been contacted with a 20 μM of ODN for 20 h at 37° C. and has been pulsed with 1 μCi of 3H uridine; and harvested and counted 4 h later as described in detail in Example 1. For use in vivo, for example to treat an immune system deficiency by stimulating a cell-mediated (local) immune response in a subject, it is important that the immunostimulatory CpG
DNA be capable of effectively inducing cytokine secretion by monocyte cells and/or Natural Killer (NK) cell lytic activity.

Preferred immunostimulatory CpG dinucleotides should effect at least about 500 pg/ml of TNF-α, 15 pg/ml IFN-γ, 70 pg/ml of GM-CSF 275 pg/ml of IL-6, 200 pg/ml IL-12, depending on the therapeutic indication as determined by the assays described in Example 12. Other preferred immunostimulatory CpG DNAs should effect at least about 10%, more preferably at least about 15% and most preferably at least about 20% YAC-1 cell specific lysis or at least about 30, more preferably at least about 35 and most preferably at least about 40% 2C11 cell specific lysis as determined by the assay described in detail in Example 4.

A “nucleic acid” or “DNA” means multiple nucleotides (i.e., molecules comprising a sugar (e.g., ribose or deoxyribose) linked to a phosphate group and to an exchangeable organic base, which is either a substituted pyrimidine (e.g., cytosine (C), thymine (T) or uracil (U)) or a substituted purine (e.g., adenine (A) or guanine (G))). As used herein, the term refers to ribonucleotides as well as oligodeoxynucleotides. The term shall also include polynucleotides (i.e., a polynucleotide minus the phosphate) and any other organic base containing polymer. Nucleic acid molecules can be obtained from existing nucleic acid sources (e.g. genomic or cDNA), but are preferably synthetic (e.g., produced by oligonucleotide synthesis).

A “nucleic acid delivery complex” shall mean a nucleic acid molecule associated with (e.g., ionically or covalently bound to; or encapsulated within) a targeting means (e.g., a molecule that results in higher affinity binding to target cell (e.g., B-cell and natural killer (NK) cell) surfaces and/or increased cellular uptake by target cells). Examples of nucleic acid delivery complexes include nucleic acids associated with: a sterol (e.g., a ligand recognized by target cell specific receptor). Preferred complexes must be sufficiently stable in vivo to prevent significant uncoupling prior to internalization by the target cell. However, the complex should be cleavable under appropriate conditions within the cell so that the nucleic acid is released in a functional form.

“Palindromic sequences” shall mean an inverted repeat (i.e., a sequence such as ABCDE/DCB/A’ in which A and A’ are bases capable of forming the usual Watson-Crick base pairs. In vivo, such sequences may form double stranded structures.

A “stabilized nucleic acid molecule” shall mean a nucleic acid molecule that is relatively resistant to in vivo degradation (e.g., via an exo- or endo-nuclease). Stabilization can be a function of length or secondary structure. Unmethylated CpG containing nucleic acid molecules that are tens to hundreds of kbs long are relatively resistant to in vivo degradation. For shorter immunostimulatory nucleic acid molecules, secondary structure can stabilize and increase their effect. For example, if the 3’ end of a nucleic acid molecule has self-complementarity to an upstream region, so that it can fold back and form a sort of stem loop structure, then the nucleic acid molecule becomes stabilized and therefore exhibits more activity.

Preferred stabilized nucleic acid molecules of the instant invention have a modified backbone. For use in immune stimulation, especially preferred stabilized nucleic acid molecules are phosphorothioate (i.e., at least one of the phosphate oxygens of the nucleic acid molecules is replaced by sulfur) or phosphorodithioate modified nucleic acid molecules. Moreover, the phosphate backbone modification occurs at the 5’ end of the nucleic acid for example, at the first two nucleotides of the 5’ end of the nucleic acid. Further, the phosphate backbone modification may occur at the 3’ end of the nucleic acid for example, at the last five nucleotides of the 3’ end of the nucleic acid. In addition to stabilizing nucleic acid molecules, as reported further herein, phosphorothioate-modified nucleic acid molecules (including phosphorodithioate-modified) can increase the extent of immune stimulation of the nucleic acid molecule, which contains an unmethylated CpG dinucleotide as shown herein. "Immune Stimulation By Phosphorothioate Oligonucleotide Analogs" also reports on the non-sequence specific immunostimulatory effect of phosphorothioate modified oligonucleotides. As reported herein, unmethylated CpG containing nucleic acid molecules having a phosphorothioate backbone have been found to preferentially activate B-cell activity, while unmethylated Cpg containing nucleic acid molecules having a phosphodiester backbone have been found to preferably activate monocytic (macrophages, dendritic cells and monocytes) and NK cells. Phosphorothioate CpG oligonucleotides with preferred human motifs are also strong activators of monocytic and NK cells.

Other stabilized nucleic acid molecules include: nontoxic DNA analogs, such as alkyl- and aryl-phosphates (in which the charged phosphonate oxygen is replaced by an alkyl or aryl group), phosphodiester and alkylphosphotriesters, in which the charged oxygen moiety is alkylated. Nucleic acid molecules which contain a diol, such as tetraethyleneglycol or hexaethyleneglycol, at either or both termini have also been shown to be substantially resistant to nuclease degradation. A “subject” shall mean a human or vertebrate animal including a dog, cat, horse, cow, pig, sheep, goat, chicken, monkey, rat, and mouse.

As used herein, the term “vector” refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. Preferred vectors are those capable of autonomous replication and expression of nucleic acids to which they are linked (e.g., an episome). Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as “expression vectors.” In general, expression vectors of utility in recombinant DNA techniques are often in the form of “plasmids” which refer generally to circular double stranded DNA loops which, in their vector form, are not bound to the chromosome. In the present specification, “plasmid” and “vector” are used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

Certain Unmethylated CpG Containing Nucleic Acides Have B Cell Stimulatory Activity As Shown In vitro and In vivo

In the course of investigating the lymphocyte stimulatory effects of two antisense oligonucleotides specific for endogenous retroviral sequences, using protocols described in the attached Examples 1 and 2, it was surprisingly found that two out of twenty-four “controls” (including various scrambled, sense, and mismatch controls for a panel of “antisense” ODN) also mediated B cell activation and IgM secretion, while the other “controls” had no effect.

Two observations suggested that the mechanism of this B cell activation by the “control” ODN may not involve anti-sense effects 1) comparison of vertebrate DNA sequences listed in GenBank showed no greater homology than that seen with non-stimulatory ODN and 2) the two controls showed no hybridization to Northern blots with 10 µg of spleen poly A+ RNA. Resynthesis of these ODN on a different synthesizer or extensive purification by polyacrylamide gel electrophoresis or high pressure liquid chromatography gave identical stimu-
lation, eliminating the possibility of an impurity. Similar stimulation was seen using B cells from C3H/HeJ mice, eliminating the possibility that lipopolysaccharide (LPS) contamination could account for the results.

The fact that two "control" ODN caused B cell activation similar to that of the two "antisense" ODN raised the possibility that all four ODN were stimulating B cells through some non-antisense mechanism involving a sequence motif that was absent in all of the other nonstimulatory control ODN. In comparing these sequences, it was discovered that all of the four stimulatory ODN contained CpG dinucleotides that were in a different sequence context from the nonstimulatory control.

To determine whether the CpG motif present in the stimulatory ODN was responsible for the observed stimulation, over 300 ODN ranging in length from 5 to 42 bases that contained methylated, unmethylated, or no CpG dinucleotides in various sequence contexts were synthesized. These ODNs, including the two original "controls" (ODN 1 and 2) and two originally synthesized as "antisense" (ODN 3D and 3M; Krieg, A. M. J. Immunol. 143:2448 (1989)), were then examined for in vitro effects on spleen cells (representative sequences are listed in Table 1). Several ODN that contained CpG dinucleotides induced B cell activation and IgM secretion; the magnitude of this stimulation typically could be increased by adding more CpG dinucleotides (Table 1; compare ODN 2 to 2a or 3D to 3Da and 3 Db). Stimulation did not appear to result from an antisense mechanism or impurity. ODN caused no detectable proliferation of γδ or other T cell populations.

Mitogenic ODN sequences uniformly became nonstimulatory if the CpG dinucleotide was mutated (Table 1; compare ODN 1 to 1a; 3D to 3Dc; 3M to 3Ma; and 4 to 4a) or if the cytosine of the CpG dinucleotide was replaced by 5-methylcytosine (Table 1; ODN 1b, 2b, 3Dd, and 3Mb). Partial methylation of CpG motifs caused a partial loss of stimulatory effect (compare 2a to 2c, Table 1). In contrast, methylation of other cytosines did not reduce ODN activity (ODN 1c, 2d, 3De and 3Mc). These data confirmed that CpG motif is the essential element present in ODN that activate B cells.

In the course of these studies, it became clear that the bases flanking the CpG dinucleotide played an important role in determining the murine B cell activation induced by an ODN. The optimal stimulatory motif was determined to consist of a CpG flanked by two 5' purines (preferably a CpA dinucleotide) and two 3' pyrimidines (preferably a TpT or TpC dinucleotide). Mutations of ODN to bring the CpG motif closer to this ideal improved the motif reduced stimulation (e.g., Table 1, compare ODN 3D to 3Df; 4 to 4b, 4c and 4d). On the other hand, mutations outside the CpG motif did not reduce stimulation (e.g., Table 1, compare ODN to 1d; 3D to 3Dg, 3M to 3Me). For activation of human cells, the best flanking bases are slightly different (See Table 5).

Of those tested, ODNs shorter than 8 bases were nonstimulatory (e.g., Table 1, ODN 4e). Among the forty-eight 8 base ODN tested, a highly stimulatory sequence was identified as TCAACGTG (SEQ. ID. NO: 90) (ODN4) which contains the self complementary "palindromic" AAGCTT (SEQ. ID. NO: 105). In further optimizing this motif, it was found that ODN containing Gs at both ends showed increased stimulation, particularly if the ODN were rendered nuclease resistant by phosphorothioate modification of the terminal internucleotide linkages. ODN 1585 (GGGCTAACCATTGAGGCGG (SEQ.ID NO: 12)), in which the first two and last five internucleotide linkages are phosphorothioate modified caused an average 25.4 fold increase in mouse spleen cell proliferation compared to an average 3.2 fold increase in proliferation induced by ODN 1638, which has the same sequence as ODN 1585 except that the 10 Gs at the two ends are replaced by 10 As. The effect of the G-rich ends is cis; addition of an ODN with poly G ends but no CpG motif to cells along with 1638 gave no increased proliferation. For nucleic acid molecules longer than 8 base pairs, non-palindromic motifs containing an unmethylated CpG were found to be more immunostimulatory.

<table>
<thead>
<tr>
<th>CpG</th>
<th>ODN</th>
<th>Sequence (5' to 3')</th>
<th>3H</th>
<th>IgM Production</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(SEQ ID NO: 89)</td>
<td>GCTAGACTATTACACCT</td>
<td>6.1 ± 0.8</td>
<td>17.9 ± 3.6</td>
</tr>
<tr>
<td>1a</td>
<td>(SEQ ID NO: 4)</td>
<td>TTTTTTTTTTTTTTTTTTTTTTTTT</td>
<td>1.2 ± 0.2</td>
<td>1.7 ± 0.5</td>
</tr>
<tr>
<td>1b</td>
<td>(SEQ ID NO: 13)</td>
<td>Z     Z     Z     Z     Z     Z     Z     Z     Z     Z</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>1c</td>
<td>(SEQ ID NO: 14)</td>
<td>Z     Z     Z     Z     Z     Z     Z     Z     Z     Z</td>
<td>10.3 ± 4.4</td>
<td>9.5 ± 1.8</td>
</tr>
<tr>
<td>1d</td>
<td>(SEQ ID NO: 16)</td>
<td>AT     Z     Z     Z     Z     Z     Z     Z     Z     Z</td>
<td>13.0 ± 2.3</td>
<td>18.3 ± 9.7</td>
</tr>
<tr>
<td>2</td>
<td>(SEQ ID NO: 1)</td>
<td>ATTTAAGTACCTACACCT</td>
<td>2.9 ± 0.2</td>
<td>13.6 ± 2.0</td>
</tr>
<tr>
<td>2a</td>
<td>(SEQ ID NO: 16)</td>
<td>C     C     C     C     C     C     C     C     C     C</td>
<td>7.7 ± 0.8</td>
<td>24.2 ± 3.2</td>
</tr>
<tr>
<td>2b</td>
<td>(SEQ ID NO: 16)</td>
<td>Z     Z     Z     Z     Z     Z     Z     Z     Z     Z</td>
<td>1.6 ± 0.5</td>
<td>2.8 ± 2.2</td>
</tr>
<tr>
<td>2c</td>
<td>(SEQ ID NO: 17)</td>
<td>Z     Z     Z     Z     Z     Z     Z     Z     Z     Z</td>
<td>3.1 ± 0.6</td>
<td>7.3 ± 1.4</td>
</tr>
<tr>
<td>2d</td>
<td>(SEQ ID NO: 16)</td>
<td>C     C     C     C     C     C     C     C     C     C</td>
<td>7.4 ± 1.4</td>
<td>27.7 ± 5.4</td>
</tr>
<tr>
<td>2e</td>
<td>(SEQ ID NO: 19)</td>
<td>C     C     C     C     C     C     C     C     C     C</td>
<td>5.6 ± 2.0</td>
<td>ND</td>
</tr>
<tr>
<td>3D</td>
<td>(SEQ ID NO: 20)</td>
<td>GAGAAGCTGAGACAATCGC</td>
<td>4.9 ± 0.5</td>
<td>19.9 ± 3.6</td>
</tr>
<tr>
<td>3Da</td>
<td>(SEQ ID NO: 21)</td>
<td>C     C     C     C     C     C     C     C     C     C</td>
<td>6.6 ± 1.5</td>
<td>33.9 ± 6.8</td>
</tr>
<tr>
<td>3Db</td>
<td>(SEQ ID NO: 22)</td>
<td>C     C     C     C     C     C     C     C     C     C</td>
<td>10.1 ± 2.8</td>
<td>25.4 ± 0.8</td>
</tr>
<tr>
<td>3De</td>
<td>(SEQ ID NO: 23)</td>
<td>C     C     C     C     C     C     C     C     C     C</td>
<td>1.0 ± 0.1</td>
<td>1.2 ± 0.5</td>
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<tr>
<td>3Dc</td>
<td>(SEQ ID NO: 24)</td>
<td>Z     Z     Z     Z     Z     Z     Z     Z     Z     Z</td>
<td>1.2 ± 0.2</td>
<td>1.0 ± 0.4</td>
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<tr>
<td>3De</td>
<td>(SEQ ID NO: 25)</td>
<td>Z     Z     Z     Z     Z     Z     Z     Z     Z     Z</td>
<td>4.4 ± 1.2</td>
<td>18.8 ± 4.4</td>
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<tr>
<td>3Df</td>
<td>(SEQ ID NO: 26)</td>
<td>Z     Z     Z     Z     Z     Z     Z     Z     Z     Z</td>
<td>1.6 ± 0.1</td>
<td>7.7 ± 0.4</td>
</tr>
<tr>
<td>3Dg</td>
<td>(SEQ ID NO: 27)</td>
<td>Z     Z     Z     Z     Z     Z     Z     Z     Z     Z</td>
<td>6.1 ± 1.5</td>
<td>18.6 ± 1.5</td>
</tr>
<tr>
<td>3M</td>
<td>(SEQ ID NO: 20)</td>
<td>TCTCACTCGTCCTGCTCA</td>
<td>4.1 ± 0.2</td>
<td>23.2 ± 4.9</td>
</tr>
<tr>
<td>3Ma</td>
<td>(SEQ ID NO: 29)</td>
<td>C     C     C     C     C     C     C     C     C     C</td>
<td>0.9 ± 0.1</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td>3Mb</td>
<td>(SEQ ID NO: 30)</td>
<td>Z     Z     Z     Z     Z     Z     Z     Z     Z     Z</td>
<td>1.3 ± 0.3</td>
<td>1.5 ± 0.6</td>
</tr>
<tr>
<td>3Mc</td>
<td>(SEQ ID NO: 31)</td>
<td>Z     Z     Z     Z     Z     Z     Z     Z     Z     Z</td>
<td>5.4 ± 1.5</td>
<td>8.5 ± 2.6</td>
</tr>
</tbody>
</table>
TABLE 1—continued

<table>
<thead>
<tr>
<th>CPG ODN</th>
<th>Sequence (5' to 3')</th>
<th>$^3$H</th>
<th>IgM Production</th>
</tr>
</thead>
<tbody>
<tr>
<td>3Hd</td>
<td>(SEQ ID NO: 37)</td>
<td>A...T</td>
<td>17.2 ± 9.4</td>
</tr>
<tr>
<td>3He</td>
<td>(SEQ ID NO: 32)</td>
<td>C...A</td>
<td>3.6 ± 0.2</td>
</tr>
<tr>
<td>4a</td>
<td>(SEQ ID NO: 90)</td>
<td>TCAAGTT</td>
<td>6.1 ± 1.4</td>
</tr>
<tr>
<td>4b</td>
<td>(SEQ ID NO: 91)</td>
<td>G..GC</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>4c</td>
<td>(SEQ ID NO: 92)</td>
<td>G..GC</td>
<td>4.5 ± 0.2</td>
</tr>
<tr>
<td>4d</td>
<td>(SEQ ID NO: 93)</td>
<td>T..CT</td>
<td>2.7 ± 1.0</td>
</tr>
<tr>
<td>4e</td>
<td>(SEQ ID NO: 94)</td>
<td>T..AA</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>4f</td>
<td>(SEQ ID NO: 106)</td>
<td>C...C</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>4g</td>
<td>(SEQ ID NO: 95)</td>
<td>C...C</td>
<td>3.9 ± 1.4</td>
</tr>
<tr>
<td>4h</td>
<td>(SEQ ID NO: 107)</td>
<td>C...C</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>LPS</td>
<td></td>
<td></td>
<td>1.2 ± 0.2</td>
</tr>
</tbody>
</table>

Stimulation indexes are the means and std. dev. derived from at least 3 separate experiments, and are compared to wells cultured with no added ODN. ND = not done.

Cpd dinucleotides are underlined.

Dots indicate identity; dashes indicate deletion.

$^3$ = 5 methyl cytosine.

Other octamer ODN containing a 6 base palindromic with a TpC dinucleotide at the 5' end were also active (e.g., Table 1, ODN 4b, 4c). Other dinucleotides at the 5' end gave reduced stimulation (e.g., ODN 4f, all six possible dinucleotides were tested). The presence of a 3' dinucleotide was insufficient to compensate for the lack of a 5' dinucleotide (e.g., Table 1, ODN 4g). Disruption of the palindromic eliminated stimulation in octamer ODN (e.g., Table 1, ODN 4h), but palindromes were not required in longer ODN.

The kinetics of lymphocyte activation were investigated using mouse B cells. When the cells were pulsed at the same time as ODN addition and harvested just four hours later, there was already a two-fold increase in $^3$H uridine incorporation. Stimulation peaked at 12-48 hours and then decreased. After 24 hours, no intact ODN were detected, perhaps accounting for the subsequence full in stimulation when purified B cells with or without anti-IgM (at a subminitogenic dose) were cultured with Cpg ODN, proliferation was found to synergistically increase about 10-fold by the two mitogens in combination after 48 hours. The magnitude of stimulation was concentration dependent and consistently exceeded that of LPS under optimal conditions for both. Oligonucleotides containing a nuclease resistant phosphorothioate backbone were approximately two hundred times more potent than unmodified oligonucleotides.

Cell cycle analysis was used to determine the proportion of B cells activated by Cpg-ODN. Cpg-ODN induced cycling in more than 95% of B cells. Splenic B lymphocytes sorted by flow cytometry into CD23-(marginal zone) and CD23+(follicular) subpopulations were equally responsive to ODN-induced stimulation, as were both resting and activated populations of B cells isolated by fractionation over Percoll gradients. These studies demonstrated that Cpg-ODN induced essentially all B cells to enter cell cycle.

Immunostimulatory Nucleic Acid Molecules Block Murine B Cell Apoptosis

Certain B cell lines, such as WEHI-231, are induced to undergo growth arrest and/or apoptosis in response to crosslinking of their antigen receptor by anti-IgM (Jakway, J. P. et al., “Growth regulation of the B lymphoma cell line WEHI-231 by anti-immunoglobulin, lipopolysaccharide and other bacterial products” J. Immunol. 137: 2225 (1986); Tsubata, T., J. Wu and T. Honjo: B-cell apoptosis induced by antigen receptor crosslinking is blocked by a T-cell signal through CD40.” Nature 365: 645 (1993)). WEHI-231 cells are rescued from this growth arrest by certain stimuli such as LPS and by the CD40 ligand. ODN containing the CpG motif were also found to protect WEHI-231 from anti-IgM induced growth arrest, indicating that accessory cell populations are not required for the effect. Subsequent work indicates that Cpg ODN induce Bcl-x and myc expression, which may account for the protection from apoptosis. Also, Cpg nucleic acids have been found to block apoptosis in human cells. This inhibition of apoptosis is important since it should enhance and prolong immune activation by Cpg DNA.

Identification of the Optimal CpG Motif for Induction of Murine IL-6 and IgM Secretion and B Cell Proliferation

To evaluate whether the optimal B cell stimulatory CpG motif was identical with the optimal CpG motif for IL-6 secretion, a panel of ODNs in which the bases flanking the CpG dinucleotide were progressively substituted was studied. This ODN panel was analyzed for effects on B cell proliferation, Ig production, and IL-6 secretion, using both splenic B cells and CHL2.LX cells. As shown in Table 2, the optimal stimulatory motif contains an unmethylated CpG flanked by two 5' purines and two 3' pyrimidines. Generally a mutation of 5' purines to C were especially deleterious, but changes in 5' purines to T or 3' pyrimidines to purines had less marked effects. Based on analyses of these and scores of other ODN, it was determined that the optimal CpG motif for induction of IL-6 secretion is TGACGTT (SEQ. ID. NO: 108), which is identical with the optimal mitogenic and IgM-inducing CpG motif (Table 2). This motif was more stimulatory than any of the palindromic containing sequences studied (1639, 1707 and 1708).

Induction of Murine Cytokine Secretion by CpG Motifs in Bacterial DNA or Oligonucleotides

As described in Example 9, the amount of IL-6 secreted by spleen cells after CpG DNA stimulation was measured by ELISA. T cell depleted spleen cell cultures rather than whole spleen cells were used for in vitro studies following preliminary studies showing that T cells contribute little or nothing to the IL-6 produced by CpG DNA-stimulated spleen cells. As shown in Table 3, IL-6 production was markedly increased in
cells cultured with \( E. \) coli DNA but not in cells cultured with calf thymus DNA. To confirm that the increased IL-6 production observed with \( E. \) coli DNA was not due to contamination by other bacterial products, the DNA was digested with DNAase prior to analysis. DNAase pretreatment abolished IL-6 production induced by \( E. \) coli DNA (Table 3). In addition, spleen cells from LPS-nonresponsive C3H/HeJ mice produced similar levels of IL-6 in response to bacterial DNA. To analyze whether the IL-6 secretion induced by \( E. \) coli DNA was mediated by the unmethylated CpG dinucleotides in bacterial DNA, methylated \( E. \) coli DNA and a panel of synthetic ODN were examined. As shown in Table 3, CpG ODN significantly induced IL-6 secretion (ODN 5a, 5b, 5c) while CpG methylated \( E. \) coli DNA, or ODN containing methylated CpG (ODN 5f) or no CpG (ODN 5d) did not. Changes at sites other than CpG dinucleotides (ODN 5b) or methylation of other cytosines (ODN 5g) did not reduce the effect of CpG ODN. Methylation of a single CpG in an ODN with three CpGs resulted in a partial reduction in the stimulation (compare ODN 5c to 5e; Table 3).

### TABLE 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IL-6 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>calf thymus DNA</td>
<td>≤10</td>
</tr>
<tr>
<td>calf thymus DNA + DNAase</td>
<td>≤10</td>
</tr>
<tr>
<td>( E. ) coli DNA</td>
<td>1169.5 ± 94.1</td>
</tr>
<tr>
<td>( E. ) coli DNA + DNAase</td>
<td>≤10</td>
</tr>
<tr>
<td>CpG methylated ( E. ) coli DNA</td>
<td>≤10</td>
</tr>
<tr>
<td>LPS</td>
<td>280.1 ± 17.1</td>
</tr>
</tbody>
</table>

Medi \( \alpha \) (no DNA) | ≤10 |

<table>
<thead>
<tr>
<th>ODNs</th>
<th>SEQ. ID. No.</th>
<th>AGG</th>
<th>TGGACTCTCAGCAGCCTTCC</th>
<th>1096.4 ± 372.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>5b</td>
<td>SEQ. ID. No.19</td>
<td>AGG</td>
<td>1224.5 ± 126.2</td>
<td></td>
</tr>
<tr>
<td>5c</td>
<td>SEQ. ID. No.15</td>
<td>GGG</td>
<td>1783.0 ± 189.5</td>
<td></td>
</tr>
<tr>
<td>5d</td>
<td>SEQ. ID. No.124</td>
<td>AGG</td>
<td>1951.1 ± 114.4</td>
<td></td>
</tr>
<tr>
<td>5e</td>
<td>SEQ. ID. No.116</td>
<td>AGG</td>
<td>1862.3 ± 87.26</td>
<td></td>
</tr>
</tbody>
</table>

CpG Motifs can be used as an Artificial Adjuvant.

Non-specific simulators of the immune response are known as adjuvants. The use of adjuvants is essential to induce a strong antibody response to soluble antigens (Harlow and Lane, *Antibodies: A Laboratory manual*, Cold Spring Harbor, N.Y. Current Edition: hereby incorporated by reference). The overall effect of adjuvants is dramatic and their importance cannot be overemphasized. The action of an adjuvant allows much smaller doses of antigen to be used and generates antibody responses that are more persistent. The non-specific activation of the immune response often can spell the difference between success and failure in obtaining an immune response. Adjuvants should be used for first injections unless there is some very specific reason to avoid this. Most adjuvants incorporate two components. One component is designed to protect the antigen from rapid catabolism (e.g., liposomes or synthetic surfactants (Hunter et al. 1981)). Liposomes are only effective when the immunogen is incorporated into the outer lipid layer; entrapped molecules are not seen by the immune system. The other component is a substance that will stimulate the immune response nonspecifically. These substances act by raising the level of lymphokines. Lymphokines stimulate the activity of antigen-processing cells directly and cause a local inflammatory reaction at the site of injection. Early work relied entirely on heat-killed bacteria (Dienes 1936) or lipopolysaccharide (LPS) (Johnson et al. 1956). LPS is reasonably toxic, and, through analysis of its structural components, most of its properties as an adjuvant have been shown to be in a portion known as lipid A. Lipid A is available in a number of synthetic and natural forms that are much less toxic than LPS, but still retains most of the better adjuvant properties of parental LPS molecule. Lipid A compounds are often delivered using liposomes.

Recently an intense drive to find potent adjuvants with more acceptable side effects has led to the production of new synthetic adjuvants. The present invention provides the sequence 1826 TCCATGACGTTCCTGACGT (SEQ ID NO: 10), which is an adjuvant including CpG containing nucleic acids. The sequence is a strong immune activating sequence and is a superb adjuvant, with efficacy comparable or superior to complete Freund’s, but without apparent toxicity.

Titrations of Induction of Murine IL-6 Secretion by CpG motifs Bacterial DNA and CpG ODN induced IL-6 production in T cell depleted murine spleen cells in a dose-dependent manner, but vertebrate DNA and non-CpG ODN did not (FIG. 1). IL-6 production plateaued at approximately 50 ng/ml of bacterial DNA or 40 μM of CpG O-ODN. The maximum levels of IL-6 induced by bacterial DNA and CpG ODN were 1-1.5 ng/ml and 2-4 ng/ml respectively. These levels were significantly greater than those seen after stimulation by LPS (0.35 ng/ml) (FIG. 1A). To evaluate whether CpG ODN with a nucleoside-resistant DNA backbone would also induce IL-6 production, S-ODN were added to T cell depleted murine spleen cells. CpG S-ODN also induced IL-6 production in a dose-dependent manner to approximately the same level as CpG O-ODN while non-CpG S-ODN failed to induce IL-6 (FIG. 1C). CpG S-ODN at a concentration of 0.04 μM could induce maximal IL-6 production in these cells.

This result indicated that the nucleoside-resistant DNA backbone modification retains the sequence specific ability of CpG DNA to induce IL-6 secretion and that CpG S-ODN are more than 80-fold more potent than CpG O-ODN in this assay system.
Induction of Murine IL-6 secretion by CpG DNA in vivo

To evaluate the ability of bacterial DNA and CpG ODN to induce IL-6 secretion in vivo, BALB/c mice were injected iv. with 100 µg of E. coli DNA, calf thymus DNA, or CpG or non-stimulatory S-ODN and bled 2 hr after stimulation. The level of IL-6 in the sera from the E. coli DNA injected group was approximately 13 ng/ml while IL-6 was not detected in the sera from calf thymus DNA or PBS injected groups (Table 4). CpG S-ODN also induced IL-6 secretion in vivo. The IL-6 level in the sera from CpG S-ODN injected groups was approximately 20 ng/ml. In contrast, IL-6 was not detected in the sera from non-stimulatory S-ODN stimulated group (Table 4).

**TABLE 4**

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>IL-6 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>&lt;50</td>
</tr>
<tr>
<td>E. coli DNA</td>
<td>1425 ± 3414</td>
</tr>
<tr>
<td>Calf Thymus DNA</td>
<td>&lt;50</td>
</tr>
<tr>
<td>CpG S-ODN</td>
<td>20715 ± 606</td>
</tr>
<tr>
<td>(5'-CGTACAGTGGTTAAGCTG3')</td>
<td>(SEQ. ID. No:48)</td>
</tr>
<tr>
<td>non-Cpg S-ODN</td>
<td>&lt;50</td>
</tr>
<tr>
<td>(5'-CGTACAGTGGTTAAGCTG3')</td>
<td>(SEQ. ID. No:49)</td>
</tr>
</tbody>
</table>

Mice (2 mice/group) were i.p. injected with 100 µl of PBS, 200 µg of E. coli DNA or calf thymus DNA, or 500 µg of CpG S-ODN or non-Cpg control S-ODN. Mice were bled 2 hr. after injection and 1:10 dilution of each serum was analyzed by IL-6 ELISA. Sensitivity limit of IL-6 ELISA was 9 pg/ml. Sequences of the CpG S-ODN is 5'-CGTACAGTGGTTAAGCTG3' (SEQ. ID. No:48) and of the non-stimulatory S-ODN is 5'-CGTACAGTGGTTAAGCTG3' (SEQ. ID. No:49).

Note that although there is a CpG in sequence 48, it is too close to the 3’ end to effect stimulation, as explained herein. Data represent mean ± SD of duplicates. The experiment was done at least twice with similar results.

Kinetics of Murine IL-6 Secretion after Stimulation by CpG Motifs In vivo

To evaluate the kinetics of induction of IL-6 secretion by CpG DNA in vivo, BALB/c mice were injected iv. with CpG or control non-Cpg S-ODN. Serum IL-6 levels were significantly increased within 1 hr and peaked at 2 hr. to a level of approximately 9 ng/ml in the CpG S-ODN injected group (FIG. 2). IL-6 protein in sera rapidly decreased after 4 hr and returned to basal level by 12 hr after stimulation. In contrast to CpG DNA stimulated groups, no significant increase of IL-6 was observed in the sera from the non-stimulatory S-ODN or PBS injected groups (FIG. 2).

| TABLE 5 |

Induction of human PBMC cytokine secretion by CpG oligos

<table>
<thead>
<tr>
<th>ODN</th>
<th>Sequence (5'-3')</th>
<th>IL-6 6h</th>
<th>TNF-α 6h</th>
<th>IFN-γ 6h</th>
<th>GM-CSF 6h</th>
<th>IL-12 6h</th>
</tr>
</thead>
<tbody>
<tr>
<td>512</td>
<td>TCGATCGTGGTTAAGCTG3'</td>
<td>500</td>
<td>140</td>
<td>15.6</td>
<td>70</td>
<td>250</td>
</tr>
<tr>
<td>SEQ ID NO:28</td>
<td>1637......C...</td>
<td>550</td>
<td>16</td>
<td>7.8</td>
<td>15.6</td>
<td>35</td>
</tr>
<tr>
<td>SEQ ID NO:29</td>
<td>1615......A...</td>
<td>600</td>
<td>145</td>
<td>7.8</td>
<td>45</td>
<td>250</td>
</tr>
<tr>
<td>SEQ ID NO:100</td>
<td>1614......A...</td>
<td>550</td>
<td>31</td>
<td>0</td>
<td>50</td>
<td>250</td>
</tr>
<tr>
<td>SEQ ID NO:102</td>
<td>1636......A...</td>
<td>325</td>
<td>250</td>
<td>35</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>SEQ ID NO:103</td>
<td>1634......C...</td>
<td>300</td>
<td>400</td>
<td>40</td>
<td>85</td>
<td>200</td>
</tr>
<tr>
<td>SEQ ID NO:104</td>
<td>1619......A...</td>
<td>275</td>
<td>450</td>
<td>200</td>
<td>60</td>
<td>&gt;500</td>
</tr>
<tr>
<td>SEQ ID NO:105</td>
<td>1618......A...</td>
<td>300</td>
<td>60</td>
<td>15.6</td>
<td>15.6</td>
<td>62</td>
</tr>
<tr>
<td>SEQ ID NO:7</td>
<td>1639......A...</td>
<td>625</td>
<td>220</td>
<td>15.6</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>SEQ ID NO:3</td>
<td>1707......A...</td>
<td>300</td>
<td>70</td>
<td>17</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SEQ ID NO:8</td>
<td>1708......CAG...</td>
<td>270</td>
<td>10</td>
<td>17</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Identity: CpG dinucleotides are underlined.

**Note:** Measured by ELISA using Quantikine kits from R & D Systems (pg/ml)

Data are presented as the level of cytokine above that in wells with no added oligodeoxynucleotide.
CpG DNA Induces Cytokine Secretion by Human PBMC Specifically Monocytes

The same panels of ODN used for studying mouse cytokine expression were used to determine whether human cells also are induced by CpG motifs to express cytokine (or proliferate), and to identify the CpG motif(s) responsible. Oligonucleotide 1619 (GTCGTT; residues 6-11 of ODN, ID: NO: 105) was the best inducer of TNF-α and IFN-γ secretion, and was closely followed by a nearly identical motif in oligonucleotide 1634 (GTCGCT; residues 6-11 of ODN, ID: NO: 104) (Table 5). The motifs in oligodeoxynucleotides 1637 and 1614 (GCGCGT; residues 6-11 of ODN, ID: NO: 29) and (GACGCT; residues 6-11 of ODN, ID: NO: 102) led to strong IL-6 secretion with relatively little induction of other cytokines. Thus, it appears that human lymphocytes, like murine lymphocytes, secrete cytokines differentially in response to CpG dinucleotides, depending on the surrounding bases. Moreover, the motifs that stimulate murine cells best differ from those that are most effective with human cells. Certain CpG oligodeoxynucleotides are poor at activating human cells (oligodeoxynucleotides 1707, 1708, which contain the palindromic forms of sequences GACGCT residues 6-11 of ODN, ID: NO: 88 and CAGCGT residues 6-11 of ODN, ID: NO: 106, respectively).

The cells responding to the DNA appear to be monocytes, since the cytokine secretion is abolished by treatment of the cells with L-locy1-L-locy3-methyl ester (L-LIME), which is selectively toxic to monocytes (but also to cytotoxic T lymphocytes and NK cells), and does not affect B cell Ig secretion (Table 6). The cells surviving L-LIME treatment had ~95% viability by trypan blue exclusion, indicating that the lack of a cytokine response among these cells did not simply reflect a nonspecific death of all cell types. Cytokine secretion in response to E. coli (EC) DNA requires unmethylated CpG motifs, since it is abolished by methylation of the EC DNA (next to the bottom row, Table 6). LPS contamination of the DNA cannot explain the results since the level of contamination was identical in the native and methylated DNA, and since addition of twice the highest amount of contaminating LPS had no effect (not shown).

<p>| TABLE 6 |
| Cpg DNA induces cytokine secretion by human PBMC |</p>
<table>
<thead>
<tr>
<th>DNA</th>
<th>TNF-α (pg/ml)</th>
<th>IL-6 (pg/ml)</th>
<th>IFN-γ (pg/ml)</th>
<th>RANTES (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC DNA (50 μg/ml)</td>
<td>900</td>
<td>12,000</td>
<td>700</td>
<td>1560</td>
</tr>
<tr>
<td>EC DNA (5 μg/ml)</td>
<td>850</td>
<td>11,000</td>
<td>400</td>
<td>750</td>
</tr>
<tr>
<td>EC DNA (50 μg/ml)</td>
<td>500</td>
<td>ND</td>
<td>200</td>
<td>0</td>
</tr>
<tr>
<td>EC DNA (0.05 μg/ml)</td>
<td>62.5</td>
<td>15.6</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>EC DNA (50 μg/ml) + L-LIME</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>EC DNA (50 μg/ml)</td>
<td>0</td>
<td>5</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CT DNA (50 μg/ml)</td>
<td>0</td>
<td>600</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

5Levels of all cytokines were determined by ELISA using Quantikine kits from R&D Systems as described in the previous table. Results are representative using PBMC from different donors.

6Cells were pretreated for 15 min with L-locy1-L-locy3-methyl ester (M-LIME) to determine whether the cytokine production under these conditions was from monocytes (or other L-LIME-sensitive cells).

7DNA was methylated using 2 UagI DNA of CpG methytransferase (New England Biolabs) according to the manufacturer's directions, and methylation confirmed by digestion with HpaII and MspI. As a negative control, samples were included containing twice the maximal amount of LPS contained in the highest concentration of EC DNA which failed to induce detectable cytokine production under these experimental conditions.

The loss of cytokine production in the PBMC treated with L-LIME suggested that monocytes may be responsible for cytokine production in response to CpG DNA. To test this hypothesis more directly, the effects of CpG DNA on highly purified human monocytes and macrophages was tested. As hypothesized, CpG DNA directly activated production of the cytokines IL-6, GM-CSF, and TNF-α by human macrophages, whereas non-CpG DNA did not (Table 7).

<p>| TABLE 7 |
| Cpg DNA induces cytokine expression in purified human macrophages |</p>
<table>
<thead>
<tr>
<th>IL-6 (pg/ml)</th>
<th>GM-CSF (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells alone</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CT DNA (50 μg/ml)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EC DNA (50 μg/ml)</td>
<td>2000</td>
<td>15.6</td>
</tr>
</tbody>
</table>

Biological Role of IL-6 in Inducing Murine IgM Production in Response to CpG Motifs

The genetic studies described above revealed that induction of IL-6 secretion, which occurs within 1 hr post CpG stimulation, precedes IgM secretion. Since the optimal CpG motif for ODN inducing secretion of IL-6 is the same as that for IgM (Table 2), whether the CpG motifs independently induce IgM and IL-6 production or whether the IgM production is dependent on prior IL-6 secretion was examined. The addition of neutralizing anti-IL-6 antibodies inhibited in vitro IgM production mediated by CpG ODN in a dose-dependent manner, but a control antibody did not (Fig. 4A). In contrast, anti-IL-6 addition did not affect either the basal level or the CpG-induced B cell proliferation (Fig. 4B).

Increased Transcriptional Activity of the IL-6 Promoter in Response to CpG DNA

The increased level of IL-6 mRNA and protein after CpG DNA stimulation could result from transcriptional or post-transcriptional regulation. To determine if the transcriptional activity of the IL-6 promoter was unregulated in B cells cultured with CpG ODN, a murine B cell line, WEHI-231, which produces IL-6 in response to CpG DNA, was transfected with an IL-6 promoter-CAT construct (pIL-6-CAT) (Pottrats, S. T. et al., 1986; estradiol) inhibits expression of human interleukin-6-promoter-reporter constructs by a receptor-dependent mechanism. J. Clin. Invest. 93:94). CAT assays were performed after stimulation with various concentrations of CpG or non-CpG ODN. As shown in FIG. 5, CpG ODN induced increased CAT activity in dose-dependent manner while non-CpG ODN failed to induce CAT activity. This confirms that CpG induces the transcriptional activity of the IL-6 promoter.

Dependence of B Cell Activation by CpG ODN on the Number of 5' and 3' Phosphorothioate Internucleotide Linkages

To determine whether partial sulfur modification of the ODN backbone would be sufficient to enhance B cell activation, the effects of a series of ODN with the same sequence, but with differing numbers of 5' internucleotide linkages at the 5' end of ODN were required to provide optimal protection of the ODN from degradation by intracellular exo- and endonucleases. Only chimeric ODN containing two 5' phosphorothioate-modified linkages, and a variable number of 3' modified linkages were therefore examined.

The lymphocyte stimulating effects of these ODN were tested at three concentrations (3.3, 10, and 30 μM) by measuring the total levels of RNA synthesis (by 3H uridine incorporation) or DNA synthesis (by 3H thymidine incorporation) in treated spleen cell cultures (Example 10). O-ODN (0/0 phosphorothioate modifications) bearing a CpG motif caused no spleen cell stimulation unless added to the cultures at
concentrations of at least 10 μM (Example 10). However, when this sequence was modified with two S linkages at the 5' end and at least three S linkages at the 3' end, significant stimulation was seen at a dose of 3.3 μM. At this low dose, the level of stimulation showed a progressive increase as the number of S modified bases was increased, until this reached or exceeded six, at which point the stimulation index began to decline. In general, the optimal number of 3' S linkages for spleen cell stimulation was five. Of all three concentrations tested in these experiments, the S-ODN was less stimulatory than the optimal chimeric compounds.

Dependent of CpG-mediated Lymphocyte Activation on the Type of Backbone Modification

Phosphorothioate modified ODN (S-ODN) are far more nucleic acid resistant than phosphodiester modified ODN (O-ODN). Thus, the increased immune stimulation caused by S-ODN and S-O-ODN (i.e., chimeric phosphorothioate ODN in which the central linkages are phosphodiester, but the two 5' and five 3' linkages are phosphorothioate modified) compared to O-ODN may result from the nucleic acid resistance of the former. To determine the role of ODN nucleic acid resistance in immune stimulation by CpG ODN, the stimulatory effects of chimeric ODN in which the 5' and 3' ends were rendered nucleic acid resistant with either methylphosphonate (MP-), methylphosphorothioate (MPS-), phosphorothioate (S-), or phosphorodithioate (S2-) internucleotide linkages were tested (Example 10). These studies showed that despite their nucleic acid resistance, MP-O-ODN were actually less immune stimulatory than O-ODN. However, combining the MP and S modifications by replacing both nonbridging O molecules with 5' and 3' MPS internucleotide linkages restored immune stimulation to a slightly higher level than that triggered by O-ODN.

S-O-ODN were far more stimulatory than O-ODN, and were even more stimulatory than S-ODN, at least at concentrations above 3.3 μM. At concentrations below 3 μM, the S-ODN with the 3M sequence was more potent than the corresponding S-O-ODN, while the S-ODN with the 3D sequence was less potent than the corresponding S-O-ODN (Example 10). In comparing the stimulatory CpG motifs of these two sequences, it was noted that the 3D sequence is a perfect match for the stimulatory motif that the CpG is flanked by two 5' purines and two 3' pyrimidines. However, the bases immediately flanking the CpG in ODN 3D are not optimal; it has a 5' pyrimidine and a 3' purine. Based on further testing, it was found that the sequence requirement for immune stimulation is more stringent for S-ODN than for S-O- or O-ODN. S-ODN with poor matches to the optimal CpG motif cause little or no lymphocyte activation (e.g., Sequence 3D). However, S-ODN with good matches to the motif, most critically at the positions immediately flanking the CpG, are more potent than the corresponding S-O-ODN (e.g., Sequence 3M, Sequences 4 and 6), even though at higher concentrations (greater than 3 μM) the peak effect from the S-O-ODN is greater (Example 10).

S-O-ODN were remarkably stimulatory, and caused substantially greater lymphocyte activation than the corresponding S-ODN or S-O-ODN at every tested concentration.

The increased B cell stimulation seen with CpG ODN bearing S or S2 substitutions could result from any or all of the following effects: nucleic acid resistance, increased cellular uptake, increased protein binding, and altered intracellular localization. However, nucleic acid resistance cannot be the only explanation, since the MP-O-ODN were actually less stimulatory than the O-ODN with CpG motifs. Prior studies have shown that ODN uptake by lymphocytes is markedly affected by the backbone chemistry (Zhao et al. 1993) Comparison of cellular binding and uptake of antisense phosphodiester, phosphorothioate, and mixed phosphorothioate and methylphosphonate oligonucleotides. (Antisense Research and Development 3, 53-66; Zhao et al., 1994) Stage specific oligonucleotide uptake in murine bone marrow B cell precursors. Blood 84, 3660-3666). The highest cell membrane binding and uptake was seen with S-ODN, followed by S-O-ODN, O-ODN, and MP-ODN. This differential uptake correlates with the degree of immune stimulation.

Unmethylated CpG Containing Oligos Have NK Cell Stimulatory Activity

Experiments were conducted to determine whether CpG containing oligonucleotides stimulated the activity of natural killer (NK) cells in addition to B cells. As shown in Table 8, a marked induction of NK activity among spleen cells cultured with CpG ODN 1 and 3Dd was observed. In contrast, there was relatively induction in effectors that had been treated with non-CpG control ODN.

**TABLE 8**

<table>
<thead>
<tr>
<th>ODN</th>
<th>% YAC-1 Specific Lysis*</th>
<th>% 2Cl1 Specific Lysis*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effector: Target</td>
<td>Effector: Target</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>-1.1</td>
<td>15.3</td>
</tr>
<tr>
<td>1 (SEQ ID NO:13)</td>
<td>-1.1</td>
<td>15.3</td>
</tr>
<tr>
<td>3Dd (SEQ ID NO:22)</td>
<td>-1.1</td>
<td>15.3</td>
</tr>
<tr>
<td>Non-CpG ODN</td>
<td>-1.1</td>
<td>15.3</td>
</tr>
</tbody>
</table>

Induction of NK Activity by DNA Containing CpG Motifs, but not by non-CpG DNA.

Bacterial DNA cultured for 18 hrs at 37° C. and then assayed for killing of K562 (human) or Yac-1 (mouse) target cells induced NK lytic activity in both mouse spleen cells depleted of B cells and human PHMC, but vertebrate DNA may be a consequence of its increased level of unmethylated CpG dinucleotides, the activating properties of more than 50 synthetic ODN containing unmethylated, methylated, or no CpG dinucleotides was tested. The results, summarized in Table 9, demonstrate that synthetic ODN can stimulate significant NK activity, as long as they contain at least one unmethylated CpG dinucleotide. No difference was observed in the stimulatory effects of ODN in which the CpG was within a palindrome (such as ODN 1585, which contains the palindrome AAGCGTT; SEQ, ID NO: 105) from those ODN without palindromes (such as 1613 to 1619), with the caveat that optimal stimulation was generally seen with ODN in which the CpG was flanked by two 5' purines or a 5' CpG dinucleotide and two 3' pyrimidines. Kinetic experiments demonstrated that NK activity peaked around 18 hrs. after addition of the ODN. The data indicates that the murine NK response is dependent on the prior activation of monocytes by CpG DNA, leading to the production of IL-12, TNF-α, and IFN-α/β (Example 11).
From all of these studies, a more complete understanding of the immune effects of CpG DNA has been developed, which is summarized in Fig. 6.

Immune activation by CpG motifs may depend on bases flanking the CpG, and the number of spacing of the CpGs present within an ODN. Although a single CpG in an ideal base context can be a very strong and useful immune activator; superior effects can be seen with ODN containing several CpGs with the appropriate spacing and flanking bases. For activation of murine B cells, the optimal CpG motif is TGCAGT (SEQ. ID. NO: 108); residues 11-17 of Seq. ID. No 70.

The following studies where conducted to identify optimal ODN sequences for stimulation of human cells by examining the effects of changing the number, spacing, and flanking bases of CpG dinucleotides.

Identification of Phosphorothioate ODN with Optimal CpG Motifs for Activation of Human NK Cells.

To have clinical utility, ODN must be administered to a subject in a form that protects them against nuclease degradation. Methods to accomplish this with phosphodiester ODN are well known in the art and include encapsulation in lipids or delivery systems such as nanoparticles. This protection can also be achieved using chemical substitutions to the DNA such as modified DNA backbones including those in which the internucleotide linkages are nucleosil resistant. Some modifications may confer additional desirable properties such as increasing cellular uptake. For example, the phosphodiester linkage can be modified via replacement of one of the nonbridging oxygen atoms with a sulfur, which constitutes phosphorothioate DNA. Phosphorothioate ODN have enhanced cellular uptake (Krieg et al., Antisense Res. Dev. 6:133, 1996,) and improved B cell stimulation if they also have a CpG motif. Since NK activation correlates strongly with in vivo adjuvant effects, the identification of phosphorothioate ODN that will activate human NK cells is very important.

The effects of different phosphorothioate ODNs—containing CpG dinucleotides in various base contexts—on human NK activation (Table 10) were examined. ODN 1840, which contained 2 copies of the TGTCGT (SEQ. ID. NO: 102) residues 14-20 of SEQ. ID. NO: 47 motif, had significant NK lytic activity (Table 10). To further identify additional ODNs optimal for NK activation, approximately one hundred ODN containing different numbers and spacing of CpG motifs, were tested with ODN1982 serving as a control. The results are shown in Table 11.

Effective ODNs began with a TC or TG at the 5' end, however, this requirement was not mandatory. ODNs with internal CpG motifs (e.g., ODN 1840) are generally less potent stimulators than those in which a GTGCCT (SEQ. ID. NO: 58) motif immediately follows the 5' TC (e.g., ODN 1967 and 1968). ODN 1968, which has a second GTGCCT (SEQ. ID. NO: 57) motif in its 3' half, was consistently more stimulatory than ODN 1967, which lacks this second motif. ODN 1967, however, was slightly more potent than ODN 1968 in experiments 1 and 3, but not in experiment 2. ODN 2005, which has a third GTGCCT (SEQ. ID. NO: 57) motif, inducing slightly higher NK activity on average than 1968. However, ODN 2006, in which the spacing between the GTGCCT (SEQ. ID. NO: 57) motifs was increased by the addition of two Ts between each motif, was superior to ODN 2005 and to ODN 2007, in which only one of the motifs had the additional of the spacing two Ts. The minimal acceptable spacing between CpG motifs is one nucleotide as long as the ODN has two pyrimidines (preferably T) at the 3' end (e.g., ODN 1967). Surprisingly, joining two GTGCCT (SEQ. ID. NO: 57) motifs end to end with a 5'ST also created a reasonably strong inducer of NK activity (e.g., ODN 2016). The choice of thymine (T) separating consecutive CpG dinucleotides is not absolute, since ODN 2002 induced appreciable NK activation despite the fact that adenine (A) separated its CpGs (i.e., CGACGT; SEQ. ID. NO: 113). It should also be noted that ODNs containing no CpG (e.g., ODN 1982), runs of CpGs, or CpGs in bad sequence contents (e.g., ODN 2010) had no stimulatory effect on NK activation.
### TABLE 10

<table>
<thead>
<tr>
<th>ODN induction of NK Lytic Activity (LU)</th>
<th>SEQ. ID.</th>
<th>LU NO.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ODN Sequence (5'-3')</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cells alone</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1754</td>
<td>0.02</td>
<td>59</td>
</tr>
<tr>
<td>1756</td>
<td>0.05</td>
<td>45</td>
</tr>
<tr>
<td>1761</td>
<td>0.05</td>
<td>60</td>
</tr>
<tr>
<td>1776</td>
<td>0.03</td>
<td>61</td>
</tr>
<tr>
<td>1777</td>
<td>0.05</td>
<td>62</td>
</tr>
<tr>
<td>1776</td>
<td>0.01</td>
<td>63</td>
</tr>
<tr>
<td>1779</td>
<td>0.02</td>
<td>64</td>
</tr>
<tr>
<td>1780</td>
<td>0.29</td>
<td>65</td>
</tr>
<tr>
<td>1781</td>
<td>0.38</td>
<td>66</td>
</tr>
<tr>
<td>1823</td>
<td>0.06</td>
<td>6</td>
</tr>
<tr>
<td>1824</td>
<td>0.01</td>
<td>67</td>
</tr>
<tr>
<td>1825</td>
<td>0.01</td>
<td>68</td>
</tr>
<tr>
<td>1828</td>
<td>0.01</td>
<td>69</td>
</tr>
<tr>
<td>1829</td>
<td>0.42</td>
<td>70</td>
</tr>
<tr>
<td><strong>1830</strong></td>
<td>RANDOM SEQUENCE</td>
<td>0.25</td>
</tr>
<tr>
<td>1834</td>
<td>TCTCCCCAGGCGCGTCAT</td>
<td>0.00</td>
</tr>
<tr>
<td>1836</td>
<td>TCTCCCCAGGCGCGTCAT</td>
<td>0.46</td>
</tr>
<tr>
<td>1840</td>
<td>TCTCCGTGTGTGTAGGTT</td>
<td>2.70</td>
</tr>
<tr>
<td>1841</td>
<td>TCTCCGTGTGTGTAGGTT</td>
<td>1.45</td>
</tr>
<tr>
<td>1842</td>
<td>TCTCCGTGTGTGTAGGTT</td>
<td>0.06</td>
</tr>
<tr>
<td>1851</td>
<td>TCTCCGTGTGTGTAGGTT</td>
<td>2.32</td>
</tr>
</tbody>
</table>

Lytic units (LU) were measured as described [8]. Briefly, PBMC were collected from normal donors and grown over Ficol, then cultured with or without the indicated ODN (which were added to cultures at 6 µg/ml) for 24 hr. Then their ability to lyse 51Cr labeled J52 cells was determined. The results shown are typical of those obtained with several different normal human donors.

This oligo mixture contained a random selection of all 4 bases at each position.

### TABLE 11

Induction of NK LU by Phosphorothioate Cpg ODN with Good Motifs

<table>
<thead>
<tr>
<th>ODN1</th>
<th>Sequence</th>
<th>ID No.</th>
<th>exp. 1</th>
<th>exp. 2</th>
<th>exp. 3</th>
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<tr>
<td>Cells alone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1840</td>
<td>TCTCTGTGGTCTCTGGT</td>
<td>73</td>
<td>2.33</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1860</td>
<td>TCTCTGTGGTCTCTGGT</td>
<td>77</td>
<td>ND</td>
<td>0.48</td>
<td>8.99</td>
</tr>
<tr>
<td>1861</td>
<td>TCTCTGTGGTCTCTGGT</td>
<td>78</td>
<td>4.03</td>
<td>1.23</td>
<td>5.08</td>
</tr>
<tr>
<td>1862</td>
<td>TCTCTGTGGTCTCTGGT</td>
<td>52</td>
<td>ND</td>
<td>1.60</td>
<td>5.74</td>
</tr>
<tr>
<td>1863</td>
<td>TCTCTGTGGTCTCTGGT</td>
<td>79</td>
<td>3.42</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1865</td>
<td>TCTCTGTGGTCTCTGGT</td>
<td>53</td>
<td>0.46</td>
<td>0.42</td>
<td>3.48</td>
</tr>
<tr>
<td>1866</td>
<td>TCTCTGTGGTCTCTGGT</td>
<td>75</td>
<td>2.62</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1867</td>
<td>TCTCTGTGGTCTCTGGT</td>
<td>54</td>
<td>5.82</td>
<td>1.64</td>
<td>8.32</td>
</tr>
<tr>
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<td>TCTCTGTGGTCTCTGGT</td>
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<td>3.77</td>
<td>5.26</td>
<td>6.12</td>
</tr>
<tr>
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<td>0.99</td>
</tr>
<tr>
<td>1890</td>
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<td>80</td>
<td>2.10</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>1891</td>
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<td>81</td>
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<td>ND</td>
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<tr>
<td>2002</td>
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<td>9.79</td>
</tr>
<tr>
<td>2005</td>
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<td>47</td>
<td>ND</td>
<td>4.22</td>
<td>12.75</td>
</tr>
<tr>
<td>2006</td>
<td>TCTCTGTGGTCTCTGGT</td>
<td>56</td>
<td>ND</td>
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<td>12.82</td>
</tr>
<tr>
<td>2007</td>
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<td>49</td>
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<td>2.62</td>
<td>9.36</td>
</tr>
<tr>
<td>2008</td>
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<td>56</td>
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<td>1.37</td>
<td>8.15</td>
</tr>
<tr>
<td>2010</td>
<td>TCTCTGTGGTCTCTGGT</td>
<td>83</td>
<td>ND</td>
<td>0.01</td>
<td>0.05</td>
</tr>
<tr>
<td>2012</td>
<td>TCTCTGTGGTCTCTGGT</td>
<td>48</td>
<td>ND</td>
<td>2.02</td>
<td>11.61</td>
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<td>2013</td>
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<td>84</td>
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<td>0.56</td>
<td>5.22</td>
</tr>
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<td>2014</td>
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<td>ND</td>
<td>5.74</td>
<td>10.89</td>
</tr>
<tr>
<td>2015</td>
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<td>51</td>
<td>ND</td>
<td>4.53</td>
<td>10.13</td>
</tr>
<tr>
<td>2016</td>
<td>TCTCTGTGGTCTCTGGT</td>
<td>95</td>
<td>ND</td>
<td>6.54</td>
<td>8.06</td>
</tr>
</tbody>
</table>

1PBMC essentially as described herein. Results are representative of 6 separate experiments; each experiment represents a different donor.

2This is the methylated version of ODN 1840.

3 5-methylcytosine LU is Lytic units.

ND = not done.

Cpg dinucleotides are underlined for clarity.
Identification of Phosphorothioate ODN with Optimal CpG Motifs for Activation of Human B Cell Proliferation.

The ability of a CpG ODN to induce B cell proliferation is a good measure of its adjuvant potential. Indeed, ODN with strong adjuvant effects generally also induce B cell proliferation. To determine whether the optimal CpG ODN for inducing B cell proliferation are the same as those for inducing NK cell activity, similar panels of ODN (Table 12) were tested. The most consistent stimulation appeared with ODN 2006 (Table 12).

### TABLE 12

<table>
<thead>
<tr>
<th>DN</th>
<th>Sequence (5'-3')</th>
<th>SEQ. ID.</th>
<th>NO. expt. 1</th>
<th>expt. 2</th>
<th>expt. 3</th>
<th>expt. 4</th>
<th>expt. 5</th>
</tr>
</thead>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1841</td>
<td>TCTAGCGGTTCTCCTCAGOTT</td>
<td>74</td>
<td>3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
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<td>3.6</td>
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<td>3.7</td>
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</tr>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
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<td>2.0</td>
<td>4.9</td>
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<tr>
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<td>2.8</td>
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<td>3.2</td>
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<tr>
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<td>TCAAGGCCTCTTTGCTCAGOTT</td>
<td>51</td>
<td>5</td>
<td>1.8</td>
<td>2.6</td>
<td>4.5</td>
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<tr>
<td>2016</td>
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<td>1.1</td>
<td>1.7</td>
<td>2.7</td>
<td>7.3</td>
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</table>

1Cells = human spleen cells stored at -70°C. after surgical harvest or PBMC collected from normal donors and exposed to TcR.
Cells were cultured in 96 well U-bottom microtiter plates with or without the indicated ODN (which were added to cultures at 6 μM).
N = 12 experiments.
Cells were cultured for 4-7 days, pulsed with 1 μCi of 3H thymidine for 18 hr. before harvest and scintillation counting.
Stimulation index = the ratio of cpm in wells without ODN to that in wells that had been stimulated throughout the culture period with the indicated ODN (there were no further additions of ODN after the cultures were set up).
ND = not done.
Identification of Phosphorothioate ODN that Induce Human IL-12 Secretion

The ability of a CpG ODN to induce IL-12 secretion is a good measure of its adjuvant potential, especially in terms of its ability to induce a Th1 immune response, which is highly dependent on IL-12. Therefore, the ability of a panel of phosphorothioate ODN to induce IL12 secretion from human PBMC in vitro (Table 13) was examined. These experiments showed that in some human PBMC, most CpG ODN could induce IL-12 secretion (e.g., exp. 1). However, other donors responded to just a few CpG ODN (e.g., exp. 2). ODN 2006 was a consistent inducer of IL12 secretion from most subjects (Table 13).

<p>| TABLE 13 |
| Induction of Human IL-2 Secretion by Phosphorothioate CpG ODN |</p>
<table>
<thead>
<tr>
<th>IL-12 (pg/ml)</th>
<th>ODN1</th>
<th>Sequence (5' - 3')</th>
<th>SEQ ID</th>
<th>exp. t</th>
<th>exp. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells alone</td>
<td>1942</td>
<td>TCTTGTGTCCCTCGTTGTT</td>
<td>52</td>
<td>19</td>
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<tr>
<td></td>
<td>1965</td>
<td>TTCTGTTTTTTTCGTTGTT</td>
<td>53</td>
<td>36</td>
<td>0</td>
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<tr>
<td></td>
<td>1967</td>
<td>TCTCGTTCTGCCCTCTTTT</td>
<td>54</td>
<td>41</td>
<td>0</td>
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<tr>
<td></td>
<td>1968</td>
<td>TCTCGTTCTCCCTGTTGTT</td>
<td>55</td>
<td>24</td>
<td>0</td>
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<tr>
<td></td>
<td>2005</td>
<td>GTCGTTCTGGTGTTGTTGTT</td>
<td>47</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2006</td>
<td>GTCGTTCTGGTGTTGTTGTT</td>
<td>46</td>
<td>29</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>2014</td>
<td>GTCGTTCTGGTGTTGTTGTT</td>
<td>50</td>
<td>28</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2015</td>
<td>GTCGTTCTGGTGTTGTTGTT</td>
<td>51</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2016</td>
<td>GTCGTTCTGGTGTTGTTGTT</td>
<td>65</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

*PBMC were collected from normal donors and spun over ficoll, then cultured at 190 cells/well in 96 well microtiter plates with or without the indicated ODN which were added to cultures at 1ug/ml. Supernatants were collected at 24 hr. and tested for IL-12 levels by ELISA as described methods. A standard curve was run in each experiment, which represents a different donor.

Identification of B cell and Monocyte/NK Cell-Specific Oligonucleotides

As shown in FIG. 6, CpG DNA can directly activate highly purified B cells and monocytes as well as many similarities in the mechanism through which CpG DNA activates these cell types. For example, both require NFkB activation as explained further below.

In further studies of different immune effects of CpG DNA, it was found that there is more than one type of CpG motif. Specifically, oligo 1668, with the best mouse B cell motif, is a strong inducer of both B cell and natural killer (NK) cell activation, while oligo 1758 is a weak B cell activator, but still induces excellent NK responses (Table 14).

<p>| TABLE 14 |
| Different CpG Motifs Stimulate Optimal Murine B Cell and NK Activation |</p>
<table>
<thead>
<tr>
<th>ODN1</th>
<th>Sequence (5' - 3')</th>
<th>B Cell Activation</th>
<th>NK Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1668</td>
<td>TCCATAGAGCTCTGAGAGCT</td>
<td>42,849</td>
<td>2.52</td>
</tr>
</tbody>
</table>

CpG di nucleotides are underlined: oligonucleotides are synthesized with phosphorothioate modified backbones to improve their nucleic resistance. Measured by % thymidine incorporation after 48 hr culture with oligodeoxynucleotide at a 200 nM concentration as described in Example 1. Measured in lytic units.

Teleological Basis of Immunostimulatory Nucleic Acids

Vertebrate DNA is highly methylated and CpG dinucleotides are under represented. However, the stimulatory CpG motif is common in microbial genomic DNA, but quite rare in vertebrate DNA. In addition, bacterial DNA has been reported to induce B cell proliferation and immunoglobulin (Ig) production, while mammalian DNA does not (Messina, J.P. et al., J. Immunol. 147:1759 (1991)). Experiments further described in Example 3, in which methylation of bacterial DNA with CpG methylase was found to abolish mitogenicity, demonstrates that the difference in CpG status is the cause of B cell stimulation by bacterial DNA. This data supports the following conclusion: that unmethylated CpG dinucleotides present within bacterial DNA are responsible for the stimulatory effects of bacterial DNA.

Teleologically, it appears likely that lymphocyte activation by the CpG motif represents an immune defense mechanism that can thereby distinguish bacterial from host DNA. Host DNA, which would commonly be present in many anatomic regions and areas of inflammation due to apoptosis (cell death), would generally induce little or no lymphocyte activation due to CpG suppression and methylation. However, the presence of bacterial DNA containing unmethylated CpG motifs can cause lymphocyte activation precisely in infected anatomic regions, where it is beneficial. This novel activation pathway provides a rapid alternative to T cell dependent antigen specific B cell activation. Since the CpG pathway synergizes with B cell activation through the antigen receptor, B cells bearing antigen receptor specific for bacterial antigens would receive an activation signal through cell membrane Igs and a second signal from bacterial DNA, and would therefore tend to be preferentially activated. The interrelationship of this pathway with other pathways of B cell activation provide a physiologic mechanism employing a polyclonal antigen to induce antigen-specific responses.

However, it is likely that B cell activation would not be totally nonspecific. B cells bearing antigen receptors specific for bacterial products could receive one activation signal through cell membrane Igs, and a second from bacterial DNA, thereby more vigorously triggering antigen specific immune responses. As with other immune defense mechanisms, the response to bacterial DNA could have undesirable consequences in some settings. For example, autoimmune responses to self antigens would also tend to be preferentially triggered by bacterial infections, since autoantigens could also provide a second activation signal to autoactive B cells triggered by bacterial DNA. Indeed the induction of autoimmunity by bacterial infections is a common clinical observation. For example, the autoimmune disease systemic lupus erythematosus, which is i) characterized by the production of anti-DNA antibodies; ii) induced by drugs which inhibit
DNA methyltransferase (Cornaccia, E. J. et al., *J. Clin. Invest.* 92:38 (1993)); and iii) associated with reduced DNA methylation (Richardson, B., et al., *Arth. Rheum* 55:647 (1992)), is likely triggered at least in part by activation of DNA-specific B cells through stimulatory signals provided by Cpg motifs, as well as by binding of bacterial DNA to antigen receptors.

Further, sepsis, which is characterized by high morbidity and mortality due to massive and nonspecific activation of the immune system may be initiated by bacterial DNA and other products released from dying bacteria that reach concentrations sufficient to directly activate many lymphocytes. Further evidence of the role of Cpg DNA in the sepsis syndrome is described in Cowdyer, J., et al., (1996) the *Journal of Immunology* 156:4570-4575.

Unlike antigens that trigger B cells through their surface Ig receptor, Cpg-ODN did not induce any detectable Ca<sup>2+</sup> flux, changes in protein tyrosine phosphorylation, or IP3 generation. Flow cytometry with FITC-conjugated ODN with or without a Cpg motif was performed as described in Zhao, Q. et al., (*Antisense Research and Development* 3:53-66 (1993)), and showed equivalent membrane binding, cellular uptake, efflux, and intracellular localization. This suggests that there may not be cell membrane proteins specific for Cpg ODN. Rather than acting through the cell membrane, that data suggests that unmethylated Cpg containing oligonucleotides require cell uptake for activity: ODN covalently linked to a solid Teflon support were nonstimulatory, as were biotinylated ODN immobilized on either avidin beads or avidin coated petri dishes. Cpg ODN conjugated to either FITC or biotin retained full mitogenic properties, indicating no steric hindrance.

Recent data indicate the involvement of the transcription factor NFκB as a direct or indirect mediator of the Cpg effect. For example, within 15 minutes of treating B cells or monocytes with Cpg DNA, the level of NFκB binding activity is increased (FIG. 7). However, it is not increased by DNA that does not contain Cpg motifs. In addition, it was found that two different inhibitors of NFκB activation, PTD and gilitoxin, completely block the lymphocyte stimulation by Cpg DNA as measured by B cell proliferation or monocyte cell cytokine secretion, suggesting that NFκB activation is required for both cell types.

There are several possible mechanisms through which NFκB can be activated. These include through activation of various protein kinases, or through the generation of reactive oxygen species. No evidence for protein kinase activation induced immediately after Cpg DNA treatment of B cells or monocytes has been found, and inhibitors of protein kinase A, protein kinase C, and protein tyrosine kinases had no effects on the Cpg induced activation. However, Cpg DNA causes a rapid induction of the production of reactive oxygen species in both B cells and monocytes, as detected by the sensitive fluorescent dye dihydorhodamine 123 as described in Royall, J.A., and Ischiropoulos, H. (*Archives of Biochemistry and Biophysics* 302:348-355 (1993)). Moreover, inhibitors of the generation of these reactive oxygen species completely block the induction of NFκB and the later induction of cell proliferation and cytokine secretion by Cpg DNA.

Work backwards, the next question was how Cpg DNA leads to the generation of reactive oxygen species so quickly. Previous studies by the inventors demonstrated that oligonucleotides and plasmid or bacterial DNA are taken up by cells into endosomes. These endosomes rapidly become acidified inside the cell. To determine whether this acidification step may be important in the mechanism through which Cpg DNA activates reactive oxygen species, the acidification step was blocked with specific inhibitors of endosome acidification including chloroquine, monensin, and batflomycin, which work through different mechanisms. FIG. 8A shows the results from a flow cytometry study using mouse B cells with the dihydorhodamine 123 dye to determine levels of reactive oxygen species. The dye only sample in Panel A of the figure shows the background level of cells positive for the dye at 28.6%. As expected, this level of reactive oxygen species was greatly increased to 80% in the cells treated for 20 minutes with PMA and Ionomycin, a positive control (Panel B). The cells treated with the Cpg oligo also showed an increase in the level of reactive oxygen species such that more than 50% of the cells became positive (Panel D). However, cells treated with an oligonucleotide with the identical sequence except that the Cpg was switched did not show this significant increase in the level of reactive oxygen species (Panel E).

In the presence of chloroquine, the results are very different (FIG. 8B). Chloroquine slightly lowers the background level of reactive oxygen species in the cells such that the untreated cells in Panel A have only 4.3% that are positive. Chloroquine completely abolishes the induction of reactive oxygen species in the cells treated with Cpg DNA (Panel B) but does not reduce the level of reactive oxygen species in the cells treated with PMA and Ionomycin (Panel E). This demonstrates that unlike the PMA plus Ionomycin, the generation of reactive oxygen species following treatment of B cells with Cpg DNA requires that the DNA undergo an acidification step in the endosomes. This is a completely novel mechanism of leukocyte activation. Chloroquine, monensin, and batflomycin also appear to block the activation of NFκB by Cpg DNA as well as the subsequent proliferation and induction of cytokine secretion.

**Chronic Immune Activation by Cpg DNA and Autoimmune Disorders**

B cell activation by Cpg DNA synergizes with signals through the B cell receptor. This makes the possibility that DNA-specific B cells may be activated by the concurrent binding of bacterial DNA to their antigen receptor, and by the co-stimulatory Cpg-mediated signals. In addition, Cpg DNA induces B cells to become resistant to apoptosis, a mechanism thought important for preventing immune responses to self-antigens, such as DNA. Indeed, exposure to bDNA can trigger anti-DNA Ab production. Given this potential ability of Cpg DNA to promote autoimmunity, it is therefore noteworthy that patients with the autoimmune disease systemic lupus erythematosus have persistently elevated levels of circulating plasma DNA which is enriched in hypomethylated CpGs. These findings suggest a possible role for chronic immune activation by Cpg DNA in lupus etiopathogenesis. A class of medications effective in the treatment of lupus is antimalarial drugs, such as chloroquine. While the therapeutic mechanism of these drugs has been unclear, they are known to inhibit endosomal acidification. Leukocyte activation by Cpg DNA is not mediated through binding to a cell surface receptor, but requires cell uptake, which occurs via adsorptive endocytosis into an acidified chloroquine-sensitive intracellular compartment. This suggested the hypothesis that leukocyte activation by Cpg DNA may occur in association with acidified endosomes, and might even be pH dependent. To test this hypothesis specific inhibitors of DNA acidification were applied to determine whether B cells or monocytes could respond to Cpg DNA if endosomal acidification was prevented.
The earliest leukocyte activation event that was detected in response to Cpg DNA was the production of reactive oxygen species (ROS), which is induced within five minutes in primary spleen cells and both B and monocyte cell lines. Inhibitors of endosomal acidification including chloroquine, bafilomycin A, and monensin, which have different mechanisms of action, blocked the Cpg-induced generation of ROS, but had no effect on ROS generation mediated by PMA, or ligation of CD40 or IgM. These studies show that ROS generation is a common event in leukocyte activation through diverse pathways. This ROS generation is generally independent of endosomal acidification, which is required only for the ROS response to Cpg DNA. ROS generation in response to Cpg is not inhibited by the NfκB inhibitor giltoxin, confirming that it is not secondary to NfκB activation.

To determine whether endosomal acidification of Cpg DNA was also required for its other immune stimulatory effects were performed. Both LPS and Cpg DNA induce similar rapid NfκB activation, increases in proto-oncogene mRNA levels, and cytokine secretion. Activation of NfκB by Cpg DNA depended on Cpg motifs since it was not induced by bDNA treated with Cpg methylase, nor by ODN in which bases were switched to disrupt the Cpgs. Supershift experiments using specific antibodies indicated that the activated NfκB complexes included the p50 and p65 components. Unexpectedly, NfκB activation in LPS- or Cpg-treated cells was accompanied by the degradation of IκBα and IκBβ. However, inhibitors of endosomal acidification selectively blocked all of the Cpg-induced but none of the LPS-induced cellular activation events. The very low concentration of chloroquine (<10 μM) that has been determined to inhibit Cpg-mediated leukocyte activation is noteworthy since it is well below that required for antimalarial activity and other reported immune effects (e.g., 100-1000 μM). These experiments support the role of a pH-dependent signaling mechanism in mediating the stimulatory effects of Cpg DNA.

Table 15: Specific blockade of Cpg-induced TNF-α and IL-12 expression by inhibitors of endosomal acidification or NfκB activation

<table>
<thead>
<tr>
<th>Activators</th>
<th>TNF-α (250 μM)</th>
<th>IL-12 (25 μg/mL)</th>
<th>Chloroquine (10 μM)</th>
<th>Mornesin (50 μM)</th>
<th>NAC (50 μM)</th>
<th>TPCK (50 μM)</th>
<th>Gliotoxin (0.1 μg/mL)</th>
<th>Bigliotoxin (0.1 μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cpg</td>
<td>455</td>
<td>17,114</td>
<td>71</td>
<td>116</td>
<td>28</td>
<td>6</td>
<td>49</td>
<td>777</td>
</tr>
<tr>
<td>ODN</td>
<td>901</td>
<td>22,485</td>
<td>1370</td>
<td>4051</td>
<td>1025</td>
<td>1248</td>
<td>491</td>
<td>4706</td>
</tr>
<tr>
<td>LPS</td>
<td>462</td>
<td>169,416</td>
<td>8,071</td>
<td>179,034</td>
<td>6,520</td>
<td>1,480</td>
<td>23</td>
<td>84</td>
</tr>
</tbody>
</table>

Table 15 legend: IL-12 and TNF-α assays: The murine monocyte cell line J774 (1 x 10^6 cells/ml) for IL-12 or 1 x 10^6 cells/ml for TNF-α, were cultured with or without the indicated inhibitors at the concentrations shown for 2 hr and then stimulated with the LPS oligodeoxynucleotide (DNA) 1826 (TTGAGACCTTAGCTGTTGTTGC TTGACGGC) at 30 μg/mL or LPS (10 μg/ml) for 2 hr (IL-12) and 24 hr (IL-12) at which time the supernatant was harvested. ELISA for IL-12 or TNF-α (pg/ml) was performed on the supernatants essentially as described. A. K. Krieg, A. K. Y. S. Matson, T. J. Waldschmidt, G. H. Bishop, R. Teadale, G. Korzyb and D. Klinman, Nature347, 546 (1990); A. K. Krieg, N. Matson and A. M. Krieg, J. Immunol., 157, 5394-5402 (1996); Krieg, A. M., J. Lab. Clin. Med., 128, 128-133 (1996). Cells cultured with ODN that lacked Cpg motifs did not induce cytokine secretion. Specific inhibition of Cpg responses was observed with the inhibitors tested. Experiments using primary spleen cells on the LPS lines CH12.SX and WEHI-231.25 μg/ml of chloroquine is equivalent to 5 μM. Other inhibitors of NfκB activity including PDTC and calpain inhibitors I and II gave similar results to the inhibitors shown. The results shown are representative of those obtained in six different experiments.

Excessive immune activation by Cpg motifs may contribute to the pathogenesis of the autoimmune disease systemic lupus erythematosus, which is associated with elevated levels of circulating hemoplastic Cpg DNA. Chloroquine and related antimalarial compounds are effective therapeutic agents for the treatment of systemic lupus erythematosus and some other autoimmune diseases, although their mechanism of action has been obscure. Our demonstration of the ability of extremely low concentrations of chloroquine to specifically inhibit Cpg-mediated leukocyte activation suggests a possible new mechanism for its beneficial effect. It is noteworthy that lupus recurrences frequently are thought to be triggered by microbial infection. Levels of bDNA present in infected tissues can be sufficient to induce a local inflammatory response. Together with the likely role of Cpg DNA as a mediator of the sepsis syndrome and other diseases our studies suggest possible new therapeutic applications for the antimalarial drugs that act as inhibitors of endosomal acidification.

Cpg-induced ROS generation could be an incidental consequence of cell activation, or a signal that mediates this activation. The ROS scavenger N-acetyl-L-cysteine (NAC) blocks Cpg-induced NfκB activation, cytokine production, and B cell proliferation, suggesting a causal role for ROS generation in these pathways. These data are compatible with previous evidence supporting a role for ROS in the activation of NfκB. UWEL-231 B cells (5 x 10⁶ cells/ml) were preincubated for 30 minutes with or without chloroquine (5 μg/ml [-10 μM]) or giltoxin (0.2 μg/ml). Cell aliquots were then cultured as above for 10 minutes in RPMI medium with or without a Cpg ODN (1826) or non-Cpg ODN (1119) at 1 μM or phorbol myristate acetate (PMA) plus ionomycin (100 ng/ml). Cells were then stained with dihydroeharidine-123 and analyzed for intracellular ROS production by flow cytometry as described (A. K. Krieg, A. K. Y. S. Matson, T. J. Waldschmidt, G. H. Bishop, R. Teadale, G. Korzyb and D. Klinman, J. Immunol., 157, 5394-5402 (1996); Krieg, A. M., J. Lab. Clin. Med., 128, 128-133 (1996). J 1774 cells, a monocytic cell line, showed similar pH-dependent Cpg induced ROS responses. In contrast, Cpg DNA did not induce the generation of extracellular ROS, nor any detectable neutrophil ROS. The concentrations of chloroquine (and those used with the other inhibitors of endosomal acidification) prevented acidification of the internalized Cpg DNA using fluorescein-conjugated ODN as described by Tonkinson, et al. (Nucl. Acids Res. 22, 4208 (1994); A. M. Krieg, J. Am. Med. Assoc. 276, 651 (1996)).
ence or absence of pyridoxine dithiocarbamate (PDTC), an inhibitor of iNOS phosphorylation. These inhibitors of NFkB activation completely blocked the CpG-induced expression of proto-oncogene and cytokine mRNA and protein, demonstrating the essential role of NFkB as a mediator of these events. None of the inhibitors reduced cell viability under the experimental conditions used in these studies. A J774, a murine macrophage cell line, was cultured in the presence of calf thymus (CT), E. coli (EC), or methylated E. coli (mEC) DNA (methylated with Cpg methylase as described) at 5 μg/ml or a Cpg oligodeoxynucleotide (ODN 1826; Table 15) or a non-Cpg ODN (ODN 1745; TCTATGACGTTCCT-GAGTCT, SEQ. ID. NO: 8) at 0.75 μM for 1 hr, following which the cells were lysed and nuclear extracts prepared. A double stranded ODN containing a consensus NFxB site was 5’ radiolabeled and used as a probe for EMSA essentially as described (J. D. Dinagam, R. M. Lebovitz and R. G. Roeder, Nucleic Acids Res. 11, 1475 (1983); M. Briskin, M. Damore, R. Law, G. Lee, P. W. Kincade, C. H. Sibbey, M. Kuehl and R. Wall, Mol. Cell. Biol. 10. 422 (1990)). The position of the p50/p65 heterodimer was determined by supershifting with specific Ab to p65 and p50 (Santa Cruz Biotechnology, Santa Cruz, Calif.). Chloroquine inhibition of Cpg-induced but not LPS-induced NFkB activation was established using J774 cells. The cells were preincubated for 2 hr in the presence or absence of chloroquine (20 μg/ml) and then stimulated as above for 1 hr with either EC DNA, Cpg ODN, non-Cpg ODN or LPS (1 μg/ml). Similar chloroquine sensitive Cpg-induced activation of NFkB was seen in a B cell line, WEHI-231 and primary spleen cells. These experiments were performed three times over a range of chloroquine concentrations 2.5 to 20 μg/ml with similar results.

It was also established that Cpg-stimulated mRNA expression requires endosomal acidification and NFkB activation in B cells and monocytes. J774 cells (2x10^5 cells/ml) were cultured 2 hr in the presence or absence of chloroquine (2.5 μg/ml (≤5 μM)) or N-tosyl-L-phenylalanine chloromethyl ketone (TPCK; 50 μM), a serine/threonine protease inhibitor that prevents iNOS proteolysis and thus blocks NFkB activation. Cells were then stimulated with the addition of E. coli DNA (EC: 50 μg/ml), calf thymus DNA (CT: 50 μg/ml), LPS (10 μg/ml), Cpg ODN (1826; 1 μM), or control non-Cpg ODN (1911; 1 μM) for 3 hr. WEHI-231 B cells (5x10^5 cells/ml) were cultured in the presence or absence of guloitin (0.1 μg/ml) or bisglututin (0.1 μg/ml) for 2 hrs and then stimulated with a Cpg ODN (1826), or control non-Cpg ODN (1911; TCTATGACGTTCCTGAGTCT, SEQ. ID. NO:97) at 0.5 μM for 8 hr. In both cases, cells were harvested and RNA was prepared using RNAzol following the manufacturer's protocol. Multi-probe RNase protection assay was performed as described (A.-K. Yi, P. Hombbeck, D. E. Laflenz and A. M. Krieg, J Immunol., 157, 4918-4925 (1996). Comparable amounts of RNA were loaded into each lane by using ribosomal mRNA as a loading control (L.32). These experiments were performed three times with similar results.

The results indicate that leukocytes respond to Cpg DNA through a novel pathway involving the pH-dependent generation of intracellular ROS. The pH dependent step may be the transport or processing of the Cpg DNA, the ROS generation, or some other event. ROS are widely thought to be second messengers in signaling pathways in diverse cell types, but have not previously been shown to mediate a stimulatory signal in B cells.

Presumably, there is a protein in or near the endosomes that specifically recognizes DNA containing CpG motifs and leads to the generation of reactive oxygen species. To detect any protein in the cell cytoplasm that may specifically bind Cpg DNA, electrophoretic mobility shift assays (EMSA) were used with 5’ radioactively labeled oligonucleotides with or without Cpg motifs. A band was found that appears to represent a protein binding specifically to single stranded oligonucleotides that have Cpg motifs, but not to oligonucleotides that lack Cpg motifs or to oligonucleotides in which the Cpg motif has been methylated. This binding activity is blocked if excess of oligonucleotides that contain the NFkxB binding site was added. This suggests that an NFkB or related protein is a component of a protein or protein complex that binds the stimulatory Cpg oligonucleotides.

No activation of CREB/ATF proteins was found at time points where NFkB was strongly activated. These data therefore do not provide proof the NFkB proteins actually bind to the Cpg nucleic acids, but rather that the proteins are required in some way for the Cpg activity. It is possible that a CREB/ATF or related protein may interact in some way with NFkB proteins or other proteins thus explaining the remarkable similarity in the binding motifs for CREB proteins and the optimal Cpg motif. It remains possible that the oligos bind to a CREB/ATF or related protein, and that this leads to NFkB activation.

Alternatively, it is very possible that the Cpg nucleic acids may bind to one of the TRAF proteins that bind to the cytoplasmic region of CD40 and mediate NFkB activation when CD40 is cross-linked. Examples of such TRAF proteins include TRAF-2 and TRAF-5.

Method for Making Immunosuppressant Nucleic Acids

For use in the instant invention, nucleic acids can be synthesized de novo using any of a number of procedures well known in the art. For example, the b-cyanoethyl phosphoramidite method (S. L. Beaucage and M. H. Caruthers, (1981) Tet. Let. 22:1859; nucleoside H-phosphonate method (Garrett et al., 1986) Tet. Let. 27:4051-4054; Frohlicher et al., 1986) Nucl. Acid. Res 14:5399-5407; Garegg et al., 1986) Tet. Let. 27:4055-4058; Guffey et al., (1988) Tet. Let. 29:2619-2622). These chemistries can be performed by a variety of automated oligonucleotide synthesizers available in the market. Alternatively, oligonucleotides can be prepared from existing nucleic acid sequences (e.g. genomic or cDNA) using known techniques, such as those employing restriction enzymes, exonucleases or endonucleases.

For use in vivo, nucleic acids are preferably relatively resistant to degradation (e.g. via endo- and exo-nucleases). Secondary structures, such as stem loops, can stabilize nucleic acids against degradation. Alternatively, nucleic acid stabilization can be accomplished via phosphate backbone modifications. A preferred stabilized nucleic acid has at least a partial phosphorothioate modified backbone. Phosphorothioates may be synthesized using automated techniques employing either phosphoramidate or H-phosphonate chemistries. Aryl- and alkyl-phosphonates can be made e.g. as described in U.S. Pat. No. 4,469,863; and alkylphosphonates (in which the charged oxygen moiety is alkylated as described in U.S. Pat. No. 5,023,243 and European Patent No. 092,574) can be prepared by automated solid phase synthesis using commercially available reagents. Methods for making other DNA backbone modifications and substitutions have been described (Uhlmann, E. And Peyman, A. (1990) Chem. Rev. 90:544; Goodchild, J. (1990) Bioconjugate Chem. 1: 165). 2’-O-methyl nucleic acids with Cpg motifs also cause immune activation, as do ethoxy-modified Cpg nucleic acids. In fact, no backbone modifications have been found that completely abolish the Cpg effect, although it is greatly reduced by replacing the C with a 5-methyl C.

For administration in vivo, nucleic acids may be associated with a molecule that results in higher affinity binding to target
cell (e.g., B-cell, monocyte cell and natural killer (NK) cell) surfaces and/or increased cellular uptake by target cells to form a “nucleic acid delivery complex”. Nucleic acids can be ionically, or covalently associated with appropriate molecules using techniques which are well known in the art. A variety of coupling or crosslinking agents can be used e.g., Protein A, carbodiimide, and N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP). Nucleic acids can alternatively be encapsulated in liposomes or virosomes using well-known techniques.

Therapeutic Uses of Immunostimulatory Nucleic Acid Molecules

Based on their immunostimulatory properties, nucleic acid molecules containing at least one unmethylated CpG dinucleotide can be administered to a subject in vivo to treat an “immune system deficiency”. Alternatively, nucleic acid molecules containing at least one unmethylated CpG dinucleotide can be contacted with lymphocytes (e.g., B cells, monocytes or NK cells) obtained from a subject having an immune system deficiency ex vivo and activated lymphocytes can then be re-implanted in the subject.

As reported herein, in response to unmethylated CpG containing nucleic acid molecules, an increased number of spleen cells secrete IL-6, IL-12, IFN-γ, IFN-α, IFN-β, IL-1, IL-3, IL-10, TNF-α, TNF-β, GM-CSF, RANTES, and probably others. The increased IL-6 expression was found to occur in B cells, CD4+T cells and monocytes.

Immunostimulatory nucleic acid molecules can also be administered to a subject in conjunction with a vaccine to boost a subject’s immune system and thereby effect a better response from the vaccine. Preferably the immunostimulatory nucleic acid molecule is administered slightly before or at the same time as the vaccine. A conventional adjuvant may optionally be administered in conjunction with the vaccine, which is minimally comprised of an antigen, as the conventional adjuvant may further improve the vaccination by enhancing antigen absorption.

When the vaccine is a DNA vaccine at least two components determine its efficacy. First, the antigen encoded by the vaccine determines the specificity of the immune response. Second, if the backbone of the plasmid contains CpG motifs, its functions as an adjuvant for the vaccine. Thus, CpG DNA acts as an effective “danger signal” and causes the immune system to respond vigorously to new antigens in the area. This mode of action presumably results primarily from the stimulatory local effects of CpG DNA on dendritic cells and other “professional” antigen presenting cells, as well as from the co-stimulatory effects on B cells.

Immunostimulatory oligonucleotides and unmethylated CpG containing vaccines which directly activate lymphocytes and co-stimulate an antigen-specific response, are fundamentally different from conventional adjuvants (e.g., aluminum precipitates), which are inert when injected alone and are thought to work through absorbing the antigen and thereby presenting it more effectively to immune cells. Further, conventional adjuvants only work for certain antigens, only induce an antibody (humoral) immune response (Th2), and are very poor at inducing cellular immune responses (Th1). For many pathogens, the humoral response contributes little to protection, and can even be detrimental.

In addition, an immunostimulatory oligonucleotide can be administered prior to and with or after administration of a chemotherapy or immunotherapy to increase the responsiveness of the malignant cells to subsequent chemotherapy or immunotherapy or to speed the recovery of the bone marrow through induction of restorative cytokines such as GM-CSF.

CpG nucleic acids also increase natural killer cell lytic activity and antibody dependent cellular cytotoxicity (ADCC). Induction of NK activity and ADCC may likewise be beneficial in cancer immunotherapy, alone or in conjunction with other treatments.

Another use of the described immunostimulatory nucleic acid molecules is in desensitization therapy for allergies, which are generally caused by IgE antibody generation against harmless allergens. The cytokines that are induced by unmethylated CpG nucleic acids are predominantly of a class called “Th1” which is most marked by a cellular immune response and is associated with IL-12 and IFN-γ. The other major type of immune response is termed a Th2 immune response, which is associated with more of an antibody immune response and with the production of IL-4, IL-5 and IL-10. In general, it appears that allergic diseases are mediated by Th2 type immune responses and atopic diseases are mediated by Th1 immune response. Based on the ability of the immunostimulatory nucleic acid molecules to shift the immune response in a subject from a Th2 (which is associated with production of IgE antibodies and allergy) to a Th1 response (which is protective against allergic reactions), an effective dose of an immunostimulatory nucleic acid (or a vector containing a nucleic acid) alone or in conjunction with an allergen can be administered to a subject to treat or prevent an allergy.

Nucleic acids containing unmethylated CpG motifs may also have significant therapeutic utility in the treatment of asthma. Th2 cytokines, especially IL-4 and IL-5 are elevated in the airways of asthmatic subjects. These cytokines promote important aspects of the asthmatic inflammatory response, including IgE isotype switching, eosinophil chemotaxis and activation, and mast cell growth. Th1 cytokines, especially IFN-γ and IL-12, can suppress the formation of Th2 clones and production of Th2 cytokines.

As described in detail in the following Example 12, oligonucleotides containing an unmethylated CpG motif (i.e., TCCATGACGTTTCCTGACGT; SEQ ID NO. 10) but not a control oligonucleotide (TCCATGACGTTTCCTGAGCT; SEQ ID NO. 8) prevented the development of an inflammatory cellular infiltrate and eosinophilia in a murine model of asthma. Furthermore, the suppression of eosinophilic inflammation was associated with a suppression of a Th2 response and induction of a Th1 response.

For use in therapy, an effective amount of an appropriate immunostimulatory nucleic acid molecule alone or formulated as a delivery complex can be administered to a subject by any mode allowing the oligonucleotide to be taken up by the appropriate target cells (e.g., B-cells and monocyte cells). Preferred routes of administration include injection (subcutaneous, intravenous, parenteral, intraperitoneal, intrathecal, etc.). The injection can be in a bolus or a continuous infusion.

A nucleic acid alone or as a nucleic acid delivery complex can be administered in conjunction with a pharmaceutically acceptable carrier. As used herein, the phrase “pharmaceutically acceptable carrier” is intended to include substances that can be coadministered with a nucleic acid or a nucleic acid delivery complex and allows the nucleic acid to perform its indicated function. Examples of such carriers include solutions, solvents, dispersion media, delay agents, emulsions and the like. The use of such media for pharmaceutically active substances are well known in the art. Any other conventional carrier suitable for use with the nucleic acids falls within the scope of the instant invention.
The term “effective amount” of a nucleic acid molecule refers to the amount necessary or sufficient to realize a desired biologic effect. For example, an effective amount of a nucleic acid containing at least one unmethylated CpG for treating an immune system deficiency could be that amount necessary to eliminate a tumor, cancer, or bacterial, viral or fungal infection. An effective amount for use as a vaccine adjuvant could be the amount useful for boosting a subjects immune response to a vaccine. An “effective amount” for treating asthma can be that amount; useful for redirecting a Th2 type of immune response that is associated with asthma to a Th1 type of response. The effective amount for any particular application can vary depending on such factors as the disease or condition being treated, the particular nucleic acid being administered (e.g. the number of unmethylated CpG motifs or their location in the nucleic acid), the size of the subject, or the severity of the disease or condition. One of ordinary skill in the art can empirically determine the effective amount of a particular oligonucleotide without necessitating undue experimentation.

The present invention is further illustrated by the following Examples, which in no way should be construed as further limiting. The entire contents of all of the references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by reference.

EXAMPLES

Example 1
Effects of ODNs on B Cell Total RNA Synthesis and Cell Cycle

B cells were purified from spleens obtained from 6-12 week old specific pathogen free DBA/2 or BXXSB mice (bred in the University of Iowa animal care facility; no substantial strain differences were noted) that were depleted of T cells with anti-Thy-1.2 and complement and centrifugation over lymphocyte Mc(Cedarlane Laboratories, Hornby, Ontario, Canada) (“B cells”). B cells contained fewer than 1% CD4+ or CD8+ cells. 8x10^6 B cells were dispersed in triplicate into 96 well microtiter plates in 100 µl RPMI containing 10% FBS (heat inactivated to 65°C, for 30 min.), 50 µM 2-mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamate. 20 µM ODN were added at the start of culture for 20 h at 37°C, cells pulsed with 1 µCi of ^3H thymidine, and harvested and counted 4 hr later. Ig secreting B cells were enumerated using the ELISA spot assay after culture of whole spleen cells with ODN at 20 µM for 48 hr. Data, reported in Table 1, represents the stimulation index compared to cell cultured without ODN. ^3H thymidine incorporation assays showed similar results, but with some nonspecific inhibition by thymidine released from degraded ODN (Matson, S and A. M. Krieg (1992) Nonspecific suppression of ^3H-thymidine incorporation by control oligonucleotides. Antisense Research and Development 2:325).

Example 2
Effects of ODN on Production of IgM from B Cells

Single cell suspensions from the spleens of freshly killed mice were treated with anti-Thy, anti-CD4, and anti-CD8 and complement by the method of Leibson et al. [J. Exp. Med. 154:1681 (1981)]. Resting B cells (102% T cell contamination) were isolated from the 63-70% band of a discontinuous Percoll gradient by the procedure of DeFranco et al., J. Exp. Med. 155:1523 (1982). These were cultured as described above in 30 µg/ml LPS for 48 hr. The number of B cells actively secreting IgM was maximal at this time point, as determined by ELISpot assay (Klinman, D. M. et al. J. Immunol 144:506 (1990)). In that assay, B cells were incubated for 6 hrs on anti-lg coated microtiter plates. The lg they produced (99% IgM) was detected using phosphatase-labeled anti-lg (Southern Biotechnology Associated, Birmingham, Ala.). The antibodies produced by individual B cells were visualized by addition of BCIP (Sigma Chemical Co., St. Louis Mo.) which forms an insoluble blue precipitate in the presence of phosphatase. The dilution of cells producing 20-40 spots/well was used to determine the total number of antibody-secreting B cells/sample. All assays were performed in triplicate (data reported in Table 1). In some experiments, culture supernatants were assayed for IgM by ELISA, and showed similar increased in response to CpG-ODN.

Example 3
B cell Stimulation by Bacterial DNA

DBA/2 B cells were cultured with no DNA or 50 µg/ml of (Microcoscos lysodeikticus; by NZB/N mouse spleen; and c) NSF/N mouse spleen genomic DNAs for 48 hours, then pulsed with ^3H thymidine for 4 hours prior to cell harvest. Duplicate DNA samples were digested with DNASE I for 30 minutes at 37°C, prior to addition to cell cultures. E.coli DNA also induced an 8.8 fold increase in the number of IgM secreting B cells by 48 hours using the ELISAspot assay.

DBA/2 B cells were cultured with either no additive, 50 µg/ml LPS or the ODN 1; 1a; 4; or 4a at 20 µM. Cells were cultured and harvested at 4, 8, 24 and 48 hours. BXXSB cells were cultured as in Example 1 with 5, 10, 20, 40 or 80 µM of ODN 1; 1a; 4; or 4a or LPS. In this experiment, wells with no ODN had 3833 cpm. Each experiment was performed at least three times with similar results. Standard deviations of the triplicate wells were <5%.

Example 4
Effects of ODN on Natural Killer (NK) Activity

10x10^6 C57BL/6 spleen cells were cultured in two ml RPMI (supplemented as described for Example 1) with or without 40 µM CpG or non-CpG ODN for forty-eight hours. Cells were washed, and then used as effector cells in a short term 51Cr release assay with YAC-1 and 2C11, two NK sensitive target cell lines (Dallas, Z. K. et al. (1993) J. Immunol. 150:17). Effector cells were added at various concentrations to 1x10^5 Cr-labeled target cells in V-bottom microtiter plates in 0.2 ml, and incubated in 5% CO₂ for 4 hr. At 37°C Plates were then centrifuged, and an aliquot of the supernatant was assayed for radioactivity. Percent specific lysis was determined by calculating the ratio of the 51Cr released in the presence of effector cells minus the 51Cr released when the target cells were cultured alone, over the total counts released after cell lysis in 2% acetic acid minus the 51Cr cpm released when the cells are cultured alone.

Example 5
In vivo Studies with CpG Phosphorothioate ODN

Mice were weighted and injected IP with 0.25 ml of sterile PBS or the indicated phosphorothioate ODN dissolved in
PBS. Twenty hours later, spleen cells were harvested, washed, and stained for flow cytometry using phycoerythrin conjugated 6B2 to gate on B cells in conjunction with biotin conjugated anti Ly-6A/E or anti-λ5b (Pharmaningen, San Diego, Calif.) or anti-IgM (Hardy, R. R. et al., J. Exp. Med 159;1169 (1984). Two mice were studied for each condition and analyzed individually.

**Example 6**

Titration of Phosphorothioate ODN for B Cell Stimulation

B cells were cultured with phosphorothioate ODN with the sequence of control ODN 1a or the CpG ODN 1d and 3Db and then either pulsed after 20 hr with 3H thymidine or after 44 hr with 3H thymidine before harvesting and determining cpm.

**Example 7**

Rescue of B Cells From Apoptosis

WEHI-231 cells (5x10^5/well) were cultured for 1 hr. at 37°C, in the presence or absence of LPS or the control ODN 1a or the CpG ODN 1d and 3Db before addition of anti-IgM (1 μg/mL). Cells were cultured for a further 20 hr. Before a 4 hr. Pulse with 2 μCi/well 3H thymidine. In this experiment, cells with no ODN or anti-IgM gave 90.4x10^3 cpn of 3H thymidine incorporation by addition of anti-IgM. The phosphodiester ODN shown in Table 1 gave similar protection, though some nonspecific suppression due to ODN degradation. Each experiment was repeated at least 3 times with similar results.

**Example 8**

In vivo Induction of Murine IL-6

DBA/2 female mice (2 mos. old) were injected IP with 500 g CpG or control phosphorothioate ODN. At various time points after injection, the mice were bled. Two mice were studied for each time point. IL-6 was measured by ELISA and IL-6 concentration was calculated by comparison to a standard curve generated using recombinant IL-6. The sensitivity of the assay was 10 pg/ml. Levels were undetectable after 8 hrs.

**Example 9**

Systemic Induction of Murine IL-6 Transcription

Mice and cell lines. DBA/2, BALB/c, and C3H/HeJ mice at 5-10 wk of age were used as a source of lymphocytes. All mice were obtained from The Jackson Laboratory (Bar Harbor, Me.), and bred and maintained under specific pathogen-free conditions in the University of Iowa Animal Care Unit. The mouse B cell line CH12.L1 was kindly provided by Dr. G. Bishop (University of Iowa, Iowa City).

Cell preparation. Mice were killed by cervical dislocation. Single cell suspensions were prepared aseptically from the spleens from mice. T cell depleted mouse splenocytes were prepared by using anti-Thy-1.2 and complement and centrifugation over lymphocyte M (Cedarlane Laboratories, Hornby, Ontario, Canada) as described (Krieg, A. M. et al., 1989) A role for endogenous retroviral sequences in the regulation of lymphocyte activation. J. Immunol 143:2448).

ODN and DNA. Phosphodiester oligonucleotides (O-ODN) and the backbone modified phosphorothioate oligonucleotides (S-ODN) were obtained from the DNA Core Facility at the University of Iowa or from Operon Technologies (Alameda, Calif.). E. coli DNA (Strain B) and calf thymus DNA were purchased from Sigma (St. Louis, Mo.). All DNA and ODN were purified by extraction with phenol: chloroform:isoamyl alcohol (25:24:1) and/or ethanol precipitation. E. coli and calf thymus DNA were single stranded prior to use by boiling for 10 min. followed by cooling on ice for 5 min. For some experiments, E. coli and calf thymus DNA were digested with DNase I (2 U/μg of DNA) at 37°C for 2 hr in 1x SSC with 5 mM MgCl2. To methylate the cytosine in CpG dinucleotide in E. coli DNA, E. coli DNA was treated with CpG methylase (M. SssI; 2 U/μg of DNA) in NEBuffer 2 supplemented with 160 μM S-adenosyl methionine and incubated overnight at 37°C. Methylated DNA was purified as above. Efficiency of methylation was confirmed by Hpa II digestion followed by analysis by gel electrophoresis. All enzymes were purchased from New England Biolabs (Beverly, Mass.). LPS level in ODN was less than 12.5 ng/mg and E. coli and calf thymus DNA contained less than 2.5 ng of LPS/mg of DNA by Limulus assay.

Cell Culture. All cells were cultured at 37°C in 5% CO2 humidifier incubator maintained in RPMI-1640 supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS), 1.5 mM L-glutamine, 50 μg/mL, CpG or non-CpG phosphodiester ODN (O-ODN) (20 μM), phosphorothioate ODN (S-ODN) (0.5 μM), or E. coli or calf thymus DNA (50 μg/mL) at 37°C for 24 hr. (for IL-6 production) or 5 days (for IgM production). Concentrations of stimulants were chosen based on preliminary studies with titrations. In some instances, cells were treated with CpG O-ODN along with various concentrations (1-10 μg/mL) of neutralizing rat IgG1 antibody against murine IL-6 (hybridoma MP5-20F3) or control rat IgG1 mAB to E. coli b-galactosidase (hybridoma GL 113; ATCC, Rockville, Md.) (20) for 5 days. At the end of incubation, culture supematant fractions were analyzed by ELISA as below.

In vivo induction of IL-6 and IgM. BALB/c mice were injected intravenously (iv) with PBS, calf thymus DNA (200 μg/100 μL PBS/mouse), E. coli DNA (200 μg/100 μL PBS/mouse), or CpG or non-CpG S-ODN (200 μg/100 μL PBS/mouse). Mice (two/group) were bled by retroorbital puncture and sacrificed by cervical dislocation at various time points. Liver, spleen, thymus, and bone marrow were removed by RNA was prepared from those organs using RNAzol B (Tel-Test, Friendswood, Tex.) according to the manufacturers protocol.

ELISA. Flat-bottomed Immun 1 plates (Dynatech Laboratories, Inc., Chantilly, Va.) were coated with 100 μL/well of anti-mouse IL-6 mAb (MP5-20F3) (2 μg/mL) or anti-mouse IgM μ-chain specific (5 μg/mL; Sigma, St. Louis, Mo.) in carbonate-bicarbonate, pH 9.6 buffer (15 mM Na2CO3, 35 mM NaHCO3) overnight at 4°C. The plates were then washed with TBS (0.5 mM MgCl2, 0.6 mM KCl, 1.47 mM KH2PO4, 0.14 M NaCl, 6.6 mM K2HPO4, 0.5% Tween 20) and blocked with 10% FCS and TBS for 2 hr at room temperature and then washed again. Culture supernatants, mouse sera, recombinant mouse IL-6 (Pharmigen, San Diego, Calif.) or purified mouse IgM (Calbiochem, San Diego, Calif.) were appropriately diluted in 10% FCS and incubated in triplicate wells for 6 hr at room temperature. The plates were washed and 100 μL/well of biotinylated rat anti-mouse IL-6 monoclonal antibodies (MP5-32C11, Pharmingen, San Diego, Calif.) (1 μg/mL in 10% FCS) or biotinylated anti-mouse Ig (Sigma, St. Louis, Mo.) were added and incubated for 45 min at room temperature following washes with TBS. Horseradish peroxidase (HRP) conjugated avidin
Oligodeoxynucleotide Modifications Determine the Magnitude of B Cell Stimulation by CpG Motifs

ODNs were synthesized on an Applied Biosystems Inc. (Foster City, Calif.) model 380A, 380B, or 394 DNA synthesizer using standard procedures (Beaucage and Caruthers (1981) Deoxynucleoside phosphoramidites—A new class of key intermediates for deoxypolyribonucleotide synthesis. Tetrahedron Letters 22, 1859-1862.). Phosphodiester ODNs were synthesized using standard beta-cyanoethyl phosphoramidite chemistry. Phosphorothioate linkages were introduced by oxidizing the phosphate linkage with elemental sulfur instead of the standard iodine oxidation. The four common nucleoside phosphoramidites were purchased from Applied Biosystems. All phosphodiester and phosphorothioate containing ODNs were protected by treatment with concentrated ammonia at 55°C for 12 hours. The ODNs were purified by gel exclusion chromatography and lyophilized to dryness prior to use. Phosphorothioate linkages were introduced by using deoxynucleoside S-(2-benzoylmercaptoethyl) pyrrolidino thiophosphoramidites (Wissler, W. T. et al., (1993) Methods in Molecular Biology: Protocols for Oligonucleotides and Analogs-Synthesis and Properties, Agrawal, S. (Ed.), Humana Press, 191-206.). Dithioate containing ODNs were deprotected by treatment with concentrated ammonia at 55°C for 12 hours followed by reverse phase HPLC purification.

In order to synthesize oligomers containing methlyphosphonoothioates or methylphosphonates as well as phosphodiesters at any desired internucleotide linkage, two different synthetic cycles were used. The major synthetic differences in these two cycles are the coupling reagent where dialkylaminomethylphosphonates are used and the phosphoramidite phosphonates due to the slower kinetics of coupling (Schwarz, H. K. et al., (1984) Synthesis of deoxycytidylate methylphosphonates via a phosphoramidite approach. Tetrahedron Lett. 25, 1437-1440). After the coupling step has been completed, the methylphosphodiester is treated with the sulfurizing reagent (5% elemental sulfur, 100 millimolar N,N-dimethylaminopyridine in carbon disulfide/pyridine/triethylamine), four consecutive times for 450 seconds each to produce methylphosphonoothioates. To produce methylphosphonate linkages, the methylphosphodiester is treated with standard oxidizing reagent (0.1 M iodine in tetrahydrofuran/2,6-lutidine/water).

The silica gel bound oligomer was treated with distilled pyridine/concentrated ammonia, 1:1, (v/v) for four days at 4 degrees centigrade. The supernatant was dried in vacuo, dissolved in water and chromatographed on a G50/50 Sephadex column.

As used herein, O-ODN refers to ODN which are phosphodiester; S-ODN are completely phosphorothioate modified; S-O-ODN are chimeric ODN in which the central linkages are phosphodiester, but the two 5’ and five 3’ linkages are phosphorothioate modified; 2’-O-ODN are chimeric ODN in which the central linkages are phosphodiester, but the two 5’ and five 3’ linkages are phosphorothioate modified; and MP-O-ODN are chimeric ODN in which the central linkages are phosphodiester, but the two 5’ and five 3’ linkages are methylphosphate modified. The ODN segments studied (with CpG dimers indicated by underlining) include:

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5
ODN were synthesized on an Applied Biosystems Inc. (Foster City, Calif.) model 380A, 380B, or 394 DNA synthesizer using standard procedures (Beaucage and Caruthers (1981) Deoxynucleoside phosphoramidites—A new class of key intermediates for deoxypolyribonucleotide synthesis. Tetrahedron Letters 22, 1859-1862.). Phosphodiester ODNs were synthesized using standard beta-cyanoethyl phosphoramidite chemistry. Phosphorothioate linkages were introduced by oxidizing the phosphate linkage with elemental sulfur instead of the standard iodine oxidation. The four common nucleoside phosphoramidites were purchased from Applied Biosystems. All phosphodiester and phosphorothioate containing ODNs were protected by treatment with concentrated ammonia at 55°C for 12 hours. The ODNs were purified by gel exclusion chromatography and lyophilized to dryness prior to use. Phosphorothioate linkages were introduced by using deoxynucleoside S-(2-benzoylmercaptoethyl) pyrrolidino thiophosphoramidites (Wissler, W. T. et al., (1993) Methods in Molecular Biology: Protocols for Oligonucleotides and Analogs-Synthesis and Properties, Agrawal, S. (Ed.), Humana Press, 191-206.). Dithioate containing ODNs were deprotected by treatment with concentrated ammonia at 55°C for 12 hours followed by reverse phase HPLC purification.

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These sequences are representative of literally hundreds of CpG and non-CpG ODN that have been tested in the course of these studies.

Mice. DBA/2, or BXSB mice obtained from The Jackson Laboratory (Bar Harbor, Me.), and maintained under specific pathogen-free conditions were used as a source of lymphocytes at 3-10 wk of age with essentially identical results.

Cell proliferation assay. For cell proliferation assays, mouse spleen cells (5x10^6 cells/100 μl/well) were cultured at 37°C in a 5% CO2, humidified incubator in RPMI-1640 supplemented with 10% (v/v) heat inactivated fetal calf serum (heated to 65°C) for experiments with O-ODN, or 5% (v/v) for experiments using only modified ODN). 1.5 μM L-glutamine, 50 μM 2-mercaptoethanol, 100 μM/mL penicillin and 100 μG/mL streptomycin for 24 hr or 48 hr as indicated. 1 μC of 3H uridine or thymidine (as indicated) was added to each well, and the cells harvested after a standard 4 hours of culture. Filters were counted by scintillation counting. Standard deviations of the triplicate wells were <5%. The results are presented in FIGS. 6-8.

Example 11

Induction of NK Activity

Phosphodiester ODN were purchased from Operon Technologies (Alameda, Calif.). Phosphorothioate ODN were purchased from the DNA core facility, University of Iowa, or from The Midland Certified Reagent Company (Midland Tex.). E. coli strain B) DNA and calf thymus DNA were purchased from Sigma (St. Louis, Mo.). All DNA and ODN were purified by extraction with phenol:chloroform:isoamyl alcohol (25:24:1) and/or ethanol precipitation. The LPS level in ODN was less than 12.5 ng/mg and E. coli and calf thymus DNA contained less than 2.5 ng of LPS/mg of DNA by Limulus assay.

Viruses-free, 4-6 week old, DBA/2, C57BL/6 (B6) and congenitally thymic BALB/C mice were obtained on contract through the Veterans Affairs from the National Cancer Institute (Bethesda, Md.). C57BL/6 SCID mice were bred in the SPF barrier facility at the University of Iowa Animal Care Unit.

Human peripheral mononuclear blood leukocytes (PBMC) were obtained as previously described (Ballas, Z. K. et al., (1990) J. Allergy Clin. Immunol. 85:453; Ballas, Z. K. and W. Rasmussen (1990) J. Immunol. 145:1039; Ballas, Z. K. and W. Rasmussen (1993). J. Immunol. 150:17). Human or murine cells were cultured at 5x10^6/well, at 37°C in a 5% CO2 humidified atmosphere in 24-well plates (Ballas, Z. K. et al., (1990) J. Allergy Clin Immunol. 85:453; Ballas, Z. K. and W. Rasmussen (1990) J. Immunol. 145:1039; and Ballas, Z. K. and W. Rasmussen (1993). J. Immunol. 150:17), with medium alone or with CpG or non- CpG ODN at the indicated concentrations, or with E. coli or calf thymus (50 μg/ml) at 37°C for 24 h. All cultures were harvested at 18 h, and the cells were used as effectors in a standard 4 h 51Cr-release assay against K562 (human) or YAC-1 (mouse) target cells as previously described. For calculation of lytic units (LU), 1 LU was defined as the number of cells needed to effect 30% specific lysis. Where indicated, neutralizing antibodies against IFN-β (Lee Biomolecular, San Diego, Calif.) or IL-12 (C15.1, C15.6, C17.8, and C17.15; provided by Dr. Giorgio Trinchieri, The Wistar Institute, Philadelphia, Pa.) or their isotype controls were added at the initiation of cultures to a concentration of 10 μg/mL. For anti-IL-12 additional, 10 μG of each of the 4 MAB (or isotype controls) were added simultaneously. Recombinant human IL-2 was used at a concentration of 100 U/mL.

Example 12

Prevention of the Development of an Inflammatory Cellular Infiltrate and Eosinophilia in a Murine Model of Asthma

6-8 week old C57BL/6 mice (from The Jackson Laboratory, Bar Harbor, Me.) were immunized with 5,000 Schistosoma mansoni eggs by intraperitoneal (i.p.) injection on days 0 and 7. Schistosoma mansoni eggs contain an antigen (Schistosoma mansoni egg antigen (SEA)) that induces a Th2 immune response (e.g. production of IgE antibody). IgE antibody production is known to be an important cause of asthma.

The immunized mice were then treated with oligonucleotides (30 μg in 200 μl saline by i.p. injection), which either contained an unmethylated CpG motif (i.e., TCCATGACGTTCCTGACGT; SEQ ID NO. 10) or did not (i.e., control, TCCATGACGTTCCTGAGTC; SEQ ID NO. 8). Soluble SeEA (10 μg in 25 μl of saline) was administered by intranasal instillation on days 14 and 21. Saline was used as a control.

Mice were sacrificed at various times after airway challenge. Whole lung lavage was performed to harvest airway and alveolar inflammatory cells. Cytokine levels were measured from lavage fluid by ELISA. RNA was isolated from whole lung for Northern analysis and RT-PCR studies using CsCl gradients. Lungs were inflated and perfused with 4% paraformaldehyde for histologic examination.

FIG. 9 shows that when the mice are initially injected with the eggs i.p., and then inhale the egg antigen (open circle), many inflammatory cells are present in the lungs. However, when the mice are initially given a nucleic acid containing an unmethylated CpG motif along with the eggs, the inflammatory cells in the lung are not increased by subsequent inhalation of the egg antigen (open triangles).

FIG. 10 shows that the same results are obtained when eosinophils present in the lung lavage are measured. Eosinophils are the type of inflammatory cell most closely associated with asthma.

FIG. 11 shows that when the mice are treated with a control oligo at the time of the initial exposure to the egg, there is little effect on the subsequent influx of eosinophils into the lungs after inhalation of SEA. Thus, when mice inhale the eggs on days 14 or 21, they develop an acute inflammatory response in the lungs. However, giving a CpG oligo along with the eggs at the time of initial antigen exposure on days 0 and 7 almost completely abolishes the increase in eosinophils when the mice inhale the egg antigen on day 14.

FIG. 12 shows that very low doses of oligonucleotide (<10 μg) can give this protection.

FIG. 13 shows that the resultant inflammatory response correlates with the levels of the Th2 cytokine IL-4 in the lung.

FIG. 14 shows that administration of an oligonucleotide containing an unmethylated CpG motif can actually redirect
the cytokine response of the lung to production of IL-12, indicating the Th1 type of immune response.

Fig. 15 shows that administration of an oligonucleotide containing an unmethylated CpG motif can also redirect the cytokine response of the lung to production of IFN-γ, indicating a Th1 type of immune response.

Example 13

CpG Oligonucleotides Induce Human PBMC to Secrete Cytokines.

Human PBMC were prepared from whole blood by standard centrifugation over Ficoll hypaque. Cells (5x10^6/ml) were cultured in 10% autologous serum in 95 well microtiter plates with CpG or control oligodeoxynucleotides (24 μg/ml for phosphodiester oligonucleotides; 6 μg/ml for nuclease resistant phosphorothioate oligonucleotides) for 4 hr in the case of TNF-α or 24 hr. For the other cytokines before supernatant harvest and assay, measured by ELISA using Quantikine kits or reagents from R&D Systems (pg/ml) or cytokine ELISA kits from BioSource (for IL-12 assay), Assays were performed as per the manufacturer’s instructions. Data are presented in Table 6 as the level of cytokine above that in wells with no added oligodeoxynucleotide.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

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<400> SEQUENCE: 99

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<210> SEQ ID NO 100
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<400> SEQUENCE: 100

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<210> SEQ ID NO 101
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<211> LENGTH: 7
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<400> SEQUENCE: 102

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7

<210> SEQ ID NO 103
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<400> SEQUENCE: 103

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7

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<212> TYPE: DNA
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8

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<212> TYPE: DNA
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 105

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6

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<212> TYPE: DNA
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 106

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7

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<212> TYPE: DNA
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 107

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8
SEQ ID NO 108
LENGTH: 7
TYPE: DNA
ORGANISM: Artificial Sequence
FEATURE:
OTHER INFORMATION: Synthetic Oligonucleotide

SEQUENCE: 108
tgacgtt

SEQ ID NO 109
LENGTH: 6
TYPE: DNA
ORGANISM: Artificial Sequence
FEATURE:
OTHER INFORMATION: Synthetic Oligonucleotide

SEQUENCE: 109
gccggt

SEQ ID NO 110
LENGTH: 6
TYPE: DNA
ORGANISM: Artificial Sequence
FEATURE:
OTHER INFORMATION: Synthetic Oligonucleotide

SEQUENCE: 110
gacggt

SEQ ID NO 111
LENGTH: 6
TYPE: DNA
ORGANISM: Artificial Sequence
FEATURE:
OTHER INFORMATION: Synthetic Oligonucleotide

SEQUENCE: 111
gacgct

SEQ ID NO 112
LENGTH: 6
TYPE: DNA
ORGANISM: Artificial Sequence
FEATURE:
OTHER INFORMATION: Synthetic Oligonucleotide

SEQUENCE: 112
cacggtg

SEQ ID NO 113
LENGTH: 7
TYPE: DNA
ORGANISM: Artificial Sequence
FEATURE:
OTHER INFORMATION: Synthetic Oligonucleotide

SEQUENCE: 113
cgacggtt

SEQ ID NO 114
LENGTH: 20
TYPE: DNA
ORGANISM: Artificial Sequence
FEATURE:
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atggaagtc cagtggttc 20

<210> SEQ ID NO 115
<211> LENGTH: 20
<212> TYPE: DNA
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<400> SEQUENCE: 115
atggaacttc cagcgttttc 20

<210> SEQ ID NO 116
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<220> FEATURE:
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<400> SEQUENCE: 116
atggaacttc gagmgggttc 20

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<223> OTHER INFORMATION: m5c

<400> SEQUENCE: 117
gctagangtt agtgt 15

<210> SEQ ID NO 118
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 118
catttccacg attttecc 16

<210> SEQ ID NO 119
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 119
togtcgctg ctgctccttc t 21

<210> SEQ ID NO 120
<211> LENGTH: 21
<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide
We claim:
1. A method for treating cancer, comprising:
   administering to a human subject an effective amount for
   treating cancer of a CpG immunostimulatory oligonucleotide
   having at least one unmethylated CpG dinucleotide, wherein
   at least one nucleotide of the stabilized CpG immunostimulatory
   oligonucleotide has a phosphate backbone modification and
   wherein the oligonucleotide is 8 to 100 nucleotides in length,
   wherein the phosphate backbone modification is a phosphorothioate
   modification, further comprising administering an antigen.
   2. The method of claim 1, further comprising administering
      a chemotherapeutic agent.
   3. The method of claim 1, further comprising administering
      a cancer immunotherapeutic agent.
   4. The method of claim 1, wherein the cancer is brain
      cancer.
   5. The method of claim 1, wherein the cancer is lung
      cancer.
   6. The method of claim 1, wherein the cancer is ovarian
      cancer.
   7. The method of claim 1, wherein the cancer is breast
      cancer.
   8. The method of claim 1, wherein the cancer is prostate
      cancer.

9. The method of claim 1, wherein the cancer is colon
   cancer.
10. The method of claim 1, wherein the cancer is leukemia.
11. The method of claim 1, wherein the cancer is carcinoma.
12. The method of claim 1, wherein the cancer is sarcoma.
13. The method of claim 1, wherein the CpG immunostimulatory
    oligonucleotide comprises:

\[ 5'X_1X_2CGX_3X_43' \]

wherein \( X_1, X_2 \) and \( X_3, X_4 \) are nucleotides.

14. The method of claim 13, wherein \( X_1, X_4 \) are nucleotides
    selected from the group consisting of: TpT and TpC.
15. The method of claim 13, wherein \( X_1, X_2 \) are GpA and
    \( X_3, X_4 \) are TpT.
16. The method of claim 13, wherein \( X_1, X_2 \) are both
    purines and \( X_3, X_4 \) are both pyrimidines.
17. The method of claim 13, wherein \( X_1, X_2 \) are GpA and
    \( X_3, X_4 \) are both pyrimidines.
18. The method of claim 13, wherein the oligonucleotide is
    8 to 40 nucleotides in length.
19. The method of claim 13, wherein \( 5'X_1X_2CGX_3X_43' \) is
    not palindromic.
20. The method of claim 1, wherein the CpG immunostimulatory
    oligonucleotide includes at least two CpG motifs.
21. The method of claim 20, wherein at least one of the at least two CpG motifs is not palindromic.

22. The method of claim 1, wherein the oligonucleotide is administered prior to a chemotherapy.

23. The method of claim 1, wherein the oligonucleotide is administered subcutaneously.

24. A method for treating non small cell lung carcinoma (NSCLC) in a human subject, comprising administering to a human subject having NSCLC an effective amount to treat NSCLC of an immunostimulatory oligonucleotide that includes at least one unmethylated CpG dinucleotide, wherein the immunostimulatory oligonucleotide includes a phosphate backbone modification and, wherein the oligonucleotide is 8 to 100 nucleotides in length, wherein the phosphate backbone modification is a phosphorothioate modification, further comprising administering an antigen.

25. The method of claim 24, further comprising administering a chemotherapeutic agent.

26. The method of claim 24, further comprising administering an immunotherapeutic agent.

27. The method of claim 24, wherein the oligonucleotide is 8 to 40 nucleotides in length.

28. The method of claim 24, wherein the CpG immunostimulatory oligonucleotide includes at least two CpG motifs.

29. The method of claim 28, wherein at least one of the at least two CpG motifs is not palindromic.

30. The method of claim 24, wherein the oligonucleotide is administered subcutaneously.