IMMUNOSTIMULATORY NUCLEIC ACID MOLECULES

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ABSTRACT
Nucleic acids containing unmethylated CpG dinucleotides and therapeutic utilities based on their ability to stimulate an immune response and to redirect a Th2 response to a Th1 response in a subject are disclosed. Methods for treating atopic diseases, including atopic dermatitis, are disclosed.

4 Claims, 19 Drawing Sheets
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FIG. 3
FIG. 5

- pCAT
- RSV
- IL-6
- IL-6 + CpG 0.25 uM 0.5 uM
- IL-6 + non-CpG 0.25 uM 0.5 uM

cpm CAT ACTIVITY (THOUSANDS)
FIG. 6

- B CELL
  - IL-6 IMMUNOGLOBULIN
  - PROLIFERATE EXPRESS CLASS II MHC, B7-1, B7-2

- NK CELL
- MONOCYTE MACROPHAGE DENDRITIC CELL
  - IL-1
  - IL-6
  - IL-10
  - RANTES
  - GM-CSF
  - TNF-β
  - MIP-1α
  - IL-12
  - TNF-α
  - IFN-α
  - IFN-β
  - IFN-γ

CpG

+ + + +
1 IMMUNOSTIMULATORY NUCLEIC ACID MOLECULES

RELATED APPLICATIONS


GOVERNMENT SUPPORT

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

BACKGROUND OF THE INVENTION

DNA Binds to Cell Membranes and is Internalized


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 Con A showed enhanced ODN uptake by T but not B cells (Krieg, A. M., F. Gmelig-Mayling, M. F. Gourley, W. J. Kisch, L. A. Chrisey, and A. D. Steinberg. 1991. “Uptake of oligodeoxynucleotides by lymphoid cells is heterogeneous and inducible”. Antisense Research and Development 1:161).

2 Immune Effects of Nucleic Acids


Several observations suggest that certain DNA structures may also have the potential to activate lymphocytes. For

The C/EBP/ATF Family of Transcription Factors and Their Role in Replication

The cAMP response element binding protein (CREB) and activating transcription factor (ATF) or C/EBP/ATF family of transcription factors is a ubiquitously expressed class of transcription factors of which 11 members have so far been cloned (reviewed in 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., and N. Masson: “Transcriptional regulation by CREB and its relatives”. Biochem. Biophys. Acta 1174:221, 1993). They all belong to the basic region/leucine zipper (bZIP) 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 cAMP response element, the CRE, the consensus form of which is the unmethylated sequence TGACGTC (binding is abolished if the Cπ6 is methylated) (Iguchi-Ariga, S. M., and W. Schaffner: “Cπ6 methylation of the cAMP-responsive enhancer/promoter sequence TGACGTC abolishes specific factor binding as well as transcriptional activation”. Genes & Develop. 3:612, 1989).


SUMMARY OF THE INVENTION

The instant 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 monocyte cells and other cells to produce Th1 cytokines, including IL-12, IFN-γ and GM-CSF). Based on this finding, the invention features, in one aspect, novel immunostimulatory nucleic acid compositions.

In a preferred embodiment, the immunostimulatory nucleic acid contains a consensus mitogenic CpG motif represented by the formula:

\[ 5' \text{X1CGXX3}3' \]

wherein \( X_1 \) is selected from the group consisting of A or G and \( X_2 \) is C or T.

In a particularly preferred embodiment an immunostimulatory nucleic acid molecule contains a consensus mitogenic CpG motif represented by the formula:

\[ 5' \text{X1X2CGXX3}3' \]

wherein C and G are unmethylated; and \( X_1, X_2, X_3, \) and \( X_4 \) are nucleotides.

Enhanced immunostimulatory activity of human cells occurs where \( X_1 = X_2 \) is selected from the group consisting of GpI, GpG, GpA or ApA and/or \( X_3, X_4 \) is selected from the group consisting of TpT, CpT and GpT (Table 5). For facilitating uptake into cells, CpG containing immunostimulatory nucleic acid molecules are preferably in the range of 8 to 40 base pairs in size. 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 CGG trimucleotide sequence at or near the 5' and/or 3' terminals and/or the consensus mitogenic CpG motif is not a palindrome. Prolonged immunostimulation can be obtained using stabilized oligonucleotides, particularly phosphorothioate stabilized oligonucleotides.

In a second aspect, the invention features useful therapies, which are based on the immunostimulatory activity of the nucleic acid molecules. For example, the immunostimulatory nucleic acid molecules can be used to treat, prevent or ameliorate an immune system deficiency (e.g., a tumor or cancer or a viral, fungal, bacterial or parasitic infection in a subject). In addition, immunostimulatory nucleic acid molecules can be administered to stimulate a subject’s response to a vaccine.

Further, by redirecting a subject’s immune response from Th2 to Th1, the instant claimed nucleic acid molecules can be administered to treat or prevent the symptoms of asthma. In addition, the instant claimed nucleic acid molecules can be administered in conjunction with a particular allergen to a subject as a type of desensitization therapy to treat or prevent the occurrence of an allergic reaction.

Further, the ability of immunostimulatory nucleic acid molecules to induce leukemic cells to enter the cell cycle supports the use of immunostimulatory nucleic acid molecules in treating leukemia by increasing the sensitivity of
chronic leukemia cells and then administering conventional ablative chemotherapy, or combining the immunostimulatory nucleic acid molecules with another immunotherapy.

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

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1A-C are graphs plotting dose-dependent IL-6 production in response to various DNA sequences in T cell depleted spleen cell cultures. A. E. coli DNA (●), and calf thymus DNA (■) sequences and LPS (at 10x the concentration of E. coli and calf thymus DNA) (○). B. Control phosphodiester oligodeoxynucleotide (ODN) 3'-ATGGAAAGTCCAGTGTTCTC3' (SEQ ID NO:1) (■) and two phosphodiester CpG ODN 3'-ATGACCTACGTGATCTTC3' (SEQ ID NO:2) (○) and 3'-TCCAAGACGTCCAGTGCT3' (SEQ ID NO:3) (●) C. Control phosphorothioate ODN 3'-GCTAGATGTGCAGG3' (SEQ ID NO:4) (■) and two phosphorothioate CpG ODN 3'-GAGAACGTCCAGTGCT3' (SEQ ID NO:5) (●) and 3'-GCATACGTGCT3' (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±standard deviation 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 3'-TCAATGACGTCCAGTGCT3' (SEQ ID NO:7) (■) or non-CpG phosphorothioate ODN 3'-TCAATGACGTCCAGTGCT3' (SEQ ID NO:8) (○). FIG. 3 is an autoradiograph showing IL-6 mRNA expression 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 3'-TCAATGACGTCCAGTGCT3' (SEQ ID NO:7) or non-CpG phosphorothioate ODN 3'-TCAATGACGTCCAGTGCT3' (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 3'-TCAATGACGTCCAGTGCT3' (SEQ ID NO:9) in the presence of the indicated concentrations of neutralizing anti-IL-6 (●) or isotype control Ab (○). 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 3'-TCAATGACGTCCAGTGCT3' (SEQ ID NO:7) (○) or anti-IL-6 control antibody only (■). Data present the mean±standard deviation of triplicates.

FIG. 5 is a bar graph plotting chloramphenicol acetyltransferase (CAT) activity in WEHI-231 cells transfected with a promoterless CAT construct (pCAT), positive control plasmid (RSV), or IL-6 promoter-CAT construct alone or cultured with CpG 5'-TCAATGACGTCCAGTGCT3' (SEQ ID NO:7) or non-CpG 5'-TCAATGACGTCCAGTGCT3' (SEQ ID NO:8) phosphorothioate ODN at the indicated concentrations. Data present the mean±standard deviation 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 monocyteic 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 production. By inducing IL-12 production and the subsequent increased IFN-γ secretion by NK cells, the immunostimulatory nucleic acids promote a Th1 type immune response. No 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 NFXb3 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 dithyrdohydroamine 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 (TCATGACGTCCAGTGCT3' 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 with the identical sequence except that the CpGs were switched (TCCATTGACGTCCAGTGCT3' SEQ ID NO:11) 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 dithyrdohydroamine 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:11, then SEA; and egg and control oligo (SEQ ID NO:11), 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 egg, then SEA (open diamond); egg and SEQ ID NO: 10, then SEA (open circle); or saline, then saline (open square). The graph shows that the resultant inflammatory response correlates with the levels of the Th2 cytokine IL-4 in the lung.

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 actually redirect the cytokine response of the lung to production of IL-12, indicating a Th1 type of immune response.

FIG. 15 is a bar graph plotting interferon gamma (IFN-γ) production (pg/mL) in mice over time in response to injection of saline; egg, then saline; 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 INF-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 or asthmatic response in a susceptible subject. The list of allergens is enormous and can include pollens, insect venoms, 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); Lolium (e.g., Lolium perenne or Lolium multiflorum); Cryptomeria (Cryptomeria japonica); Alleraria (Alleraria 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); Blattella (e.g., Blattella germanica); Apis (e.g., Apis mellifera); Cupressus (e.g., Cupressus sempervirens; Cupressus arizonica and Cupressus macrocarpa); Juniperus (e.g., Juniperus sabinaoides; Juniperus communis and Juniperus ashei); Thuja (e.g., Thuja orientalis); Chamaecyparis obtusa; Periplaneta (e.g., Periplaneta americana); Agropyron (e.g., Agropyron repens); Secale (e.g., Secale cereale); Triticum (e.g., Triticum aestivum); Daucus (e.g., Daucus glomerata); Festuca (e.g., Festuca elatior); Poa (e.g., Poa pratensis or Poa compressa); Avena (e.g., Avena sativa); Holcus (e.g., Holcus lanatus); Anthemis (e.g., Anthemis odoratum); Arrhenatherum (e.g., Arrhenatherum elatius); Agrostis (e.g., Agrostis alba); Phleum (e.g., Phleum pratense); Phalaris (e.g., Phalaris arundinacea); Papadum (e.g., Papadum 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 coryza, hay fever, bronchial asthma, urticaria (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 cell), 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-III, LAV or HTLV-III/LAV, or HIV-III; and other isolates, such as HIV-1P; 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 virus; Flaviviridae (e.g., dengue viruses, encephalitis viruses, yellow fever viruses); Coronaviridae (e.g., coronavirus); 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 viruses, bunyaviruses, phleboviruses and Nairo viruses); Arena viridae (hemorrhagic fever viruses); Reoviridae (e.g., reoviruses, orbiviruses and rotaviruses); Birnaviridae; Hepadnaviridae (Hepatitis B virus); Paroviridae (paroviruses); Papovaviridae (papilloma viruses, polyoma viruses); Adenoviridae (most adenoviruses); Herpesviridae (herpes simplex viruses (HSV) 1 and 2, varicella zoster virus, cytomegalovirus (CMV), herpes viruses’); Poxviridae (viralota 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 pyloris, Borelia burgdorferi, Legionella pneumophila, Mycobacteria spp. (e.g., M. tuberculosis, M. avium, M. intracellularis, M. kansasii, M. gordonae), Staphylococcus aureus, Neisseria gonorrhoeae, Neisseria meningitidis, Listeria monocytogenes, Streptococcus pyogenes (Group A Streptococcus), Streptococcus agalactiae (Group B Streptococcus), Streptococcus viridans group, Streptococcus faecalis, Streptococcus bovis, Streptococcus (anaerobic spp.), Streptococcus pneumoniae, pathogenic Campylobacter sp., Enterococcus sp., Haemophilus influenzae, Bacillus anthracis, Corynebacterium diphtheriae, Corynebacterium sp., Erysipelothrix rhusiopathiae, Clostridium perfringens, Clostridium tetani, Enterobacter aerogenes, Klebsiella pneumoniae, Pasteurella multocida, Bacteroides sp., Fusobacterium nucleatum, Streptococcus mitis, Treponema pallidum, Treponema pertenum, Leptospira, and Actinomycetes israelii.

Examples of infectious fungi include: Cryptococcus neoformans, Histoplasma capsalata, Coccidioides immitis, Blastomyces dermatitidis, Chlamydia trachomatis, Candida albicans. Other infectious organisms (i.e., protists) include: Plasmodium falciparum 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 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 a preferred embodiment, the immunostimulatory nucleic acid contains a consensus mitogenic CpG motif represented by the formula:

\[ \text{X}_1 \text{CGX}_2 \text{X}_3 \]

wherein \( \text{X}_1 \) is selected from the group consisting of A, G and T; and \( \text{X}_3 \) is C or T.

In a particularly preferred embodiment, immunostimulatory nucleic acid molecules are between 2 to 100 base pairs in size and contain a consensus mitogenic CpG motif represented by the formula:

\[ \text{X}_1 \text{X}_2 \text{CGX}_3 \text{X}_4 \]

wherein C and G are unmethylated, \( \text{X}_1 \), \( \text{X}_2 \), \( \text{X}_3 \), and \( \text{X}_4 \) are nucleotides.

For economic reasons, preferably the immunostimulatory CpG DNA is in the range of between 8 to 40 base pairs in size if it is synthesized as an oligonucleotide. Alternatively, CpG dinucleotides 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 [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 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 \(^3^H\) uridine in a murine B cell culture, which has been contacted with a 20 \( \mu \)M of ODN for 20 h at 37\(^\circ\) C, and has been pulsed with 1 \( \mu \)Ci of \(^3^H\) 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 monocytes and/or Natural Killer (NK) cell lytic activity.

Preferred immunostimulatory CpG nucleic acids should effect at least about 500 pg/ml of TNF-\alpha, 15 pg/ml INF-\gamma, 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. Preferred immunostimulatory CpG DNAs should effect at least about 10\(^\circ\), more preferably at least about 15\(^\circ\) and most preferably at least about 20\(^\circ\) YAC-1 cell specific lysis or at least about 30\(^\circ\), more preferably at least about 35 and most preferably at least about 40\(^\circ\) 2C11 cell specific lysis as determined by the assay described in Example 4.

A "nucleic acid" or "DNA" shall mean 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 steroid (e.g., cholesterol), a lipid (e.g., a cationic lipid, virosome or liposome), or a target cell specific binding agent (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 sequence" shall mean an inverted repeat (i.e. a sequence such as ABCDEDEDCB'BA' 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 exon- or endonucleases). 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 modified nucleic acid molecules (i.e. at least one of the phosphate oxygens of the nucleic acid molecule is replaced by sulfur). Preferably the phosphate modification occurs at or near the 5' and/or 3' end of the nucleic acid molecule. In addition to stabilizing nucleic acid molecules, as reported further herein, phosphorothioate-modified nucleic acid molecules (including phosphorothioate-modified) can increase the extent of immune stimulation of the nucleic acid molecule, which contains an unmethylated CpG dinucleotide as shown herein. International Patent Application Publication Number: WO 95/26204 entitled "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 preferentially activate monocyte (macrophages, dendritic cells and monocytes) and NK cells. Phosphorothioate CpG oligo-
nucleotides with preferred human motifs are also strong activators of monocyte and NK cells.

Other stabilized nucleic acid molecules include: nonionic DNA analogs, such as alkyl- and aryl-phosphonates (in which the charged phosphate oxygen is replaced by an alkyl or aryl group), phosphodiesters and alkylphosphothioesters, in which the charged oxygen moiety is alkylated. Nucleic acid molecules which contain a diol, such as tetrabutylenglycol or hexamethyleneglycol, 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, mouse, etc.

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 Acids 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 stimulation, 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 was not increased by adding more CpG dinucleotides (Table 1; compare ODN 2 to 2a or 3D to 3Da and 3Db). Stimulation did not appear to result from an antisense mechanism or impurity. ODN caused no detectable proliferation of B 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 3De; 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, 3bD, and 3b5). Partial methylation of CpG motifs caused a partial loss of stimulatory effect (compare 2b 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 a 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 GpA dinucleotide) and two 3' pyrimidines (preferably a TpC or CpT dinucleotide). Mutations of ODN to bring the CpG motif closer to this ideal improved stimulation (e.g., Table 1, compare ODN 2 to 2c; 3M to 3Md) while mutations that disturbed the motif reduced stimulation (e.g., Table 1, compare ODN 3D to 3De; 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 1 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 not stimulatory (e.g., Table 1, ODN 4e). Among the forty-eight 8 base ODN tested, the most stimulatory sequence identified was TCAACGGTT (ODN 4) which contains the self-complementary "palindrome" AAGCTT. 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 (5' GGCGTCAACGGTTGACGGG 3' (SEQ 1D 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.

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 sixteen 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 4b), but palindromes were not required in longer ODN.
# Table 1

Oligonucleotide Stimulation of Mouse B Cells

<table>
<thead>
<tr>
<th>ODN</th>
<th>Sequence (5' to 3')</th>
<th>(^7)H Uridine</th>
<th>IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (SEQ ID NO:13)</td>
<td>GCTAGACCCTTAAGCTT</td>
<td>6.1 ± 0.8</td>
<td>17.9 ± 3.6</td>
</tr>
<tr>
<td>1a (SEQ ID NO:4)</td>
<td>...T......</td>
<td>1.2 ± 0.2</td>
<td>1.7 ± 0.5</td>
</tr>
<tr>
<td>1b (SEQ ID NO:14)</td>
<td>...Z......</td>
<td>1.2 ± 0.1</td>
<td>1.6 ± 0.0</td>
</tr>
<tr>
<td>1c (SEQ ID NO:15)</td>
<td>...Z......</td>
<td>10.3 ± 4.4</td>
<td>9.5 ± 1.9</td>
</tr>
<tr>
<td>1d (SEQ ID NO:16)</td>
<td>...AT......GAGC</td>
<td>13.0 ± 2.3</td>
<td>18.3 ± 7.5</td>
</tr>
<tr>
<td>2 (SEQ ID NO:17)</td>
<td>ATUGRAAGTCCAGCSCTTCTC</td>
<td>2.9 ± 0.2</td>
<td>13.6 ± 2.0</td>
</tr>
<tr>
<td>2a (SEQ ID NO:18)</td>
<td>...C...CTC...G...</td>
<td>7.7 ± 0.8</td>
<td>24.2 ± 3.2</td>
</tr>
<tr>
<td>2b (SEQ ID NO:19)</td>
<td>...Z...CTC...Z...</td>
<td>1.6 ± 0.5</td>
<td>2.8 ± 2.2</td>
</tr>
<tr>
<td>2c (SEQ ID NO:20)</td>
<td>...Z...CTC...G...</td>
<td>3.1 ± 0.6</td>
<td>7.3 ± 1.4</td>
</tr>
<tr>
<td>2d (SEQ ID NO:21)</td>
<td>...C...CTC...G......Z...</td>
<td>7.4 ± 1.4</td>
<td>27.7 ± 5.4</td>
</tr>
<tr>
<td>2e (SEQ ID NO:22)</td>
<td></td>
<td>5.6 ± 2.0</td>
<td>ND</td>
</tr>
<tr>
<td>3 (SEQ ID NO:23)</td>
<td>GAGAAACCTGGACCTTCCAT</td>
<td>4.9 ± 0.5</td>
<td>19.9 ± 3.6</td>
</tr>
<tr>
<td>3a (SEQ ID NO:24)</td>
<td>...C...</td>
<td>6.6 ± 1.5</td>
<td>33.9 ± 6.8</td>
</tr>
<tr>
<td>3b (SEQ ID NO:25)</td>
<td>...C...G...</td>
<td>10.1 ± 2.8</td>
<td>25.4 ± 0.8</td>
</tr>
<tr>
<td>3c (SEQ ID NO:26)</td>
<td>...C.A...</td>
<td>1.0 ± 0.1</td>
<td>1.2 ± 0.5</td>
</tr>
<tr>
<td>3d (SEQ ID NO:27)</td>
<td>...Z...</td>
<td>1.2 ± 0.2</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>3e (SEQ ID NO:28)</td>
<td>...Z...</td>
<td>4.4 ± 1.2</td>
<td>18.6 ± 4.4</td>
</tr>
<tr>
<td>3f (SEQ ID NO:29)</td>
<td>...A...</td>
<td>1.6 ± 0.1</td>
<td>7.7 ± 0.4</td>
</tr>
<tr>
<td>3g (SEQ ID NO:30)</td>
<td>...C.C...G.ACTG...</td>
<td>6.1 ± 1.5</td>
<td>19.6 ± 1.5</td>
</tr>
<tr>
<td>3h (SEQ ID NO:31)</td>
<td>TCCATUTCCCTGATUCCCT</td>
<td>4.1 ± 0.2</td>
<td>23.2 ± 4.9</td>
</tr>
<tr>
<td>3i (SEQ ID NO:32)</td>
<td>...CT...</td>
<td>0.9 ± 0.1</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td>3j (SEQ ID NO:33)</td>
<td>...Z...</td>
<td>1.3 ± 0.3</td>
<td>1.5 ± 0.6</td>
</tr>
<tr>
<td>3k (SEQ ID NO:34)</td>
<td>...Z...</td>
<td>5.4 ± 1.5</td>
<td>8.5 ± 2.6</td>
</tr>
<tr>
<td>3l (SEQ ID NO:35)</td>
<td>...A...T...</td>
<td>17.2 ± 9.4</td>
<td>ND</td>
</tr>
<tr>
<td>3m (SEQ ID NO:36)</td>
<td>...C.A...</td>
<td>3.6 ± 0.2</td>
<td>14.2 ± 5.2</td>
</tr>
<tr>
<td>4 (SEQ ID NO:37)</td>
<td>TCAACGTT</td>
<td>6.1 ± 1.4</td>
<td>19.2 ± 5.2</td>
</tr>
<tr>
<td>4a (SEQ ID NO:38)</td>
<td>...GC...</td>
<td>1.1 ± 0.2</td>
<td>1.5 ± 1.1</td>
</tr>
<tr>
<td>4b (SEQ ID NO:39)</td>
<td>...GC...</td>
<td>4.5 ± 0.2</td>
<td>9.6 ± 3.4</td>
</tr>
<tr>
<td>4c (SEQ ID NO:40)</td>
<td>...TCGA...</td>
<td>2.7 ± 1.0</td>
<td>ND</td>
</tr>
<tr>
<td>4d (SEQ ID NO:41)</td>
<td>...TTAA...</td>
<td>1.3 ± 0.2</td>
<td>ND</td>
</tr>
<tr>
<td>4e (SEQ ID NO:42)</td>
<td>...C...</td>
<td>1.3 ± 0.2</td>
<td>1.1 ± 0.5</td>
</tr>
<tr>
<td>4f (SEQ ID NO:43)</td>
<td>...C...</td>
<td>3.9 ± 1.4</td>
<td>ND</td>
</tr>
<tr>
<td>4g (SEQ ID NO:44)</td>
<td>...C...CT</td>
<td>1.4 ± 0.3</td>
<td>ND</td>
</tr>
</tbody>
</table>
The kinetics of lymphocyte activation were investigated using mouse spleen 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 subsequent fall in stimulation when purified B cells with or without anti-IgM (at a submitogenic 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 nucleoside resistant phosphorothiate 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. Spleenic 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 induce essentially all B cells to enter the cell cycle.

Immunostimulatory Nucleic Acid Molecules Block Murine B Cell Apoptosis
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 DNase prior to analysis. DNase pretreatment abolished IL-6 production induced by *E. coli* DNA (Table 3). In addition, spleen cells from LPS-nonresponsive C3H/HeJ mouse 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>
<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 + DNase</td>
<td>≤10</td>
</tr>
<tr>
<td><em>E. coli</em> DNA</td>
<td>1169.5 ± 94.1</td>
</tr>
<tr>
<td><em>E. coli</em> DNA + DNase</td>
<td>≤10</td>
</tr>
<tr>
<td>CpG methylated <em>E. coli</em> DNA</td>
<td>≤10</td>
</tr>
<tr>
<td>LPS</td>
<td>280.1 ± 17.1</td>
</tr>
<tr>
<td>Media (no DNA)</td>
<td>≤10</td>
</tr>
<tr>
<td>ODN 5a SEQ ID NO:1</td>
<td>ATCGACTCTCCAGCTTCTC</td>
</tr>
<tr>
<td>ODN 5b SEQ ID NO:2</td>
<td>. . . . . . . . . . . . . . . . .</td>
</tr>
<tr>
<td>ODN 5c SEQ ID NO:3</td>
<td>. . . . . . . . . . . . . . . . .</td>
</tr>
<tr>
<td>ODN 5d SEQ ID NO:4</td>
<td>. . . . . . . . . . . . . . . . .</td>
</tr>
<tr>
<td>ODN 5e SEQ ID NO:5</td>
<td>. . . . . . . . . . . . . . . . .</td>
</tr>
<tr>
<td>ODN 5f SEQ ID NO:6</td>
<td>. . . . . . . . . . . . . . . . .</td>
</tr>
<tr>
<td>ODN 5g SEQ ID NO:7</td>
<td>. . . . . . . . . . . . . . . . .</td>
</tr>
</tbody>
</table>

T-cell depleted spleen cells from DBA/2 mice were stimulated with phosphodiester modified oligonucleotides (5 ODN (20 μM), calf thymus DNA (50 μg/ml) or *E. coli* DNA (50 μg/ml) with or without enzyme treatment, or LPS (10 μg/ml) for 24 hr. Data represent the mean (pg/ml) ± SD of triplicates. CpG dinucleotides are underlined and dots indicate identity. Z indicates 5-methylcytosine.

**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 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 to the optimal CpG motif for IL-6 secretion, a panel of ODN 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 CH12.LX cells. As shown in Table 2, the optimal stimulatory motif is composed of an unmethylated
CpG flanked by two 5′ purines and two 3′ pyrimidines. Generally a mutation of either 5′ purine to pyrimidine or 3′ pyrimidine to purine significantly reduced its effects. Changes in 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, which is identical with the optimal mitogenic and IgM-inducing CpG motif (Table 2). This motif was more stimulatory than any of the palindromes containing sequences studied (1639, 1707 and 1708).

Trituration 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. 1A). IL-6 production plateaued at approximately 50 pg/ml of bacterial DNA or 40 μg/ml of CpG O-ODN. The maximum levels of IL-6 induced by bacterial DNA and CpG ODN were 11.5 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 nuclelease-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 0.05 μM could induce maximal IL-6 production in these cells. This result indicated that the nuclelease-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 S-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>13858 ± 3143</td>
</tr>
<tr>
<td>Calf Thymus DNA</td>
<td>&lt;50</td>
</tr>
</tbody>
</table>

Secretion of Murine IL-6 induced by CpG DNA stimulation in vivo.  

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>IL-6 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CpG S-ODN</td>
<td>20715 ± 606</td>
</tr>
<tr>
<td>non-CpG S-ODN</td>
<td>&lt;50</td>
</tr>
</tbody>
</table>

Mice (2 mice/group) were iv. 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 5 pg/ml. Sequences of the CpG O-ODN is 5′CGAGTGATGAGCTT′ (SEQ ID NO:48) and of the non-stimulatory S-ODN is 5′CTAGTGATGAGCTT′ (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).

Tissue Distribution and Kinetics of IL-6 mRNA Expression Induced by CpG Motifs in vivo.

As shown in Fig. 2, the level of serum IL-6 increased rapidly after CpG DNA stimulation. To investigate the possible tissue origin of this serum IL-6, and the kinetics of IL-6 gene expression in vivo after CpG DNA stimulation, BALB/c mice were injected iv with CpG or non-CpG S-ODN and RNA was extracted from liver, spleen, thymus, and bone marrow at various time points after stimulation. As shown in Fig. 3A, the level of IL-6 mRNA in liver, spleen, and thymus was increased within 30 min. after injection of CpG S-ODN. The liver IL-6 mRNA peaked at 2 hr post-injection and rapidly decreased and reached basal level 8 hr after stimulation (Fig. 3A). Splenic IL-6 mRNA peaked at 2 hr after stimulation and then gradually decreased (Fig. 3A). Thymus IL-6 mRNA peaked at 1 hr post-injection and then gradually decreased (Fig. 3A). IL-6 mRNA was significantly increased in bone marrow within 1 hr after CpG S-ODN injection but then returned to basal level. In response to CpG S-ODN, liver, spleen and thymus showed more substantial increases in IL-6 mRNA expression than the bone marrow.

Patterns of Murine Cytokine Expression Induced by CpG DNA.

In vivo or in whole spleen cells, no significant increase in the protein levels of the following interleukins: IL-2, IL-3, IL-4, IL-5, IL-10 was detected within the first six hours (Kliman, D. M. et al., (1996) Proc. Natl. Acad. Sci. USA 93:2879-2883). However, the level of TNF-α, is increased within 30 minutes and the level of IL-6 increased strikingly within 2 hours in the serum of mice injected with CpG ODN. Increased expression of IL-12 and interferon gamma (INF-γ) mRNA by spleen cells was also detected within the first two hours.
**TABLE 5**

<table>
<thead>
<tr>
<th>ODN</th>
<th>Sequence (5'-3')</th>
<th>IL-6</th>
<th>TNF-α</th>
<th>IFN-γ</th>
<th>GM-CSF</th>
<th>IL-12</th>
</tr>
</thead>
<tbody>
<tr>
<td>512</td>
<td>TCCATGCTCGCTCTGATGCT</td>
<td>500</td>
<td>140</td>
<td>15.6</td>
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<td>16</td>
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<td>15.6</td>
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<tr>
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<td>400</td>
<td>40</td>
<td>85</td>
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<tr>
<td>1619</td>
<td>T................</td>
<td>275</td>
<td>450</td>
<td>200</td>
<td>80</td>
<td>&gt;500</td>
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<td>A...............T</td>
<td>300</td>
<td>60</td>
<td>15.6</td>
<td>15.6</td>
<td>62</td>
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<td></td>
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<td></td>
</tr>
<tr>
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<td>AA...............T</td>
<td>625</td>
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<td>15.6</td>
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<td>60</td>
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<td>300</td>
<td>70</td>
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<td>0</td>
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<tr>
<td>1708</td>
<td>CA...............TG</td>
<td>270</td>
<td>10</td>
<td>17</td>
<td>0</td>
<td>0</td>
</tr>
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<td><strong>NO:</strong> 47</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Dots indicate identity; CpG dinucleotides are underlined.

*measured by ELISA using QuantiKine kits from R & D Systems (pg/ml). Cells were cultured in 10% autologous serum with the indicated oligodeoxynucleotides (12 μg/ml) for 4 hr in the case of TNF-α or 24 hr for the other cytokines before supernatant harvest and assay. 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 (GTCGTTC) was the best inducer of TNF-α and IFN-γ secretion, and was closely followed by a nearly identical motif in oligonucleotide 1634 (GTCGTTC) (Table 5). The motifs in oligodeoxynucleotides 1637 and 1614 (GC-CGTT and GACGTT) 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 palindrome forming sequences GACGTC and CACGGT respectively).

The cells responding to the DNA appear to be monocytes, since the cytokine secretion is abolished by treatment of the cells with L-leucyl-L-leucine methyl ester (L-LME), 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, and data not shown). The cells surviving L-LME 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 all 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).


**TABLE 6**

CpG DNA induces cytokine secretion by human PBMC

<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 (50 μg/ml)</td>
<td>850</td>
<td>11,000</td>
<td>400</td>
<td>750</td>
</tr>
<tr>
<td>EC DNA (0.5 μg/ml)</td>
<td>50</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>10,000</td>
<td>15.6</td>
<td>0</td>
</tr>
<tr>
<td>EC DNA (50 μg/ml) + L-LME ²</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>EC DNA (10 μg/ml) + L-LME ²</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>

*Levels 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. Cells were pretreated for 15 min with L-lescine-L-lescine methyl ester (L-LME) to determine whether the cytokine production under these conditions was from monocytes (or other L-LME-sensitive cells). EC DNA was methylated using 2U/μg DNA of CpG methylase (New England Biolabs) according to the manufacturer’s directions, and methylation confirmed by digestion with Hpa-II and Msp-1. 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.

ND = not done

The loss of cytokine production in the PBMC treated with L-LME 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).

**TABLE 7**

CpG DNA induces cytokine expression in purified human macrophages

<table>
<thead>
<tr>
<th>Cells alone</th>
<th>IL-6 (pg/ml)</th>
<th>GM-CSF (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CT DNA (50 μg/ml)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EC DNA (50 μg/ml)</td>
<td>2000</td>
<td>15.6</td>
<td>1000</td>
</tr>
</tbody>
</table>

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

The kinetic 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 lgM (Table 2), whether the CpG motifs independently induce lgM and IL-6 production or whether the lgM production is dependent on prior IL-6 secretion was examined. The addition of neutralizing anti-IL-6 antibodies inhibited in vitro lgM 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 upregulated 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) (Pottratz, S. T. et al., 17β-estradiol) inhibits expression of human interleukin-6-promoter-reporter constructs by a receptor-dependent mechanism. J. Clin. Invest. 93:944), 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 nucleotide linkages at the 5' and 3' ends were tested. Based on previous studies of nuclease degradation of ODN, it was determined that at least two phosphorothioate linkages at the 5' end of ODN were required to provide optimal protection of the ODN from degradation by intracellular exo- and endo-nucleases. 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 thymidine 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 3' 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. At all three concentrations tested in these experiments, the S-ODN was less stimulatory than the optimal chimeric compounds.

Dependence of CSG-Mediated Lymphocyte Activation on the Type of Backbone Modification.

Phosphorothioate modified ODN (S-ODN) are far more nucleoside 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 nucleoside resistance of the former. To determine the role of ODN nucleoside resistance in immune stimulation by CpG ODN, the stimulatory effects of chimeric ODN in which the 5' and 3' ends were rendered nuclease resistant with either methylphosphonate (MP—), methylphosphorothioate (MPS—), phosphorothioate (S—), or phosphorothidithioate (S—S) internucleotide linkages were tested (Example 10). These studies showed that despite their nucleoside 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—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 in 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 S substitutions could result from any or all of the following effects: nucleosome resistance, increased cellular uptake, increased protein binding, and altered intracellular localization. However, nucleosome 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 well 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 no 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></td>
<td>Effector/Target</td>
<td>Effector/Target</td>
</tr>
<tr>
<td></td>
<td>30:1</td>
<td>100:1</td>
</tr>
<tr>
<td>None</td>
<td>-1.1</td>
<td>-1.4</td>
</tr>
<tr>
<td></td>
<td>16.1</td>
<td>24.5</td>
</tr>
<tr>
<td>3Dd</td>
<td>17.1</td>
<td>27.0</td>
</tr>
<tr>
<td>intra-CpG ODN</td>
<td>-1.0</td>
<td>-1.7</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 PBMC, but vertebrate DNA did not (Table 9). To determine whether the stimulatory activity of bacterial 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 AACGT1) from those ODN without palindromes (such as 1613 or 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’ CpTp dinucleotide and two 3’ pyrimidines. Kinetic experiments demonstrated that NK activity peaked around 18 hrs. after addition of the ODN. The data indicates 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-γβ (Example 11).

**TABLE 9**

<table>
<thead>
<tr>
<th>DNA or Cytokine Added</th>
<th>Mouse Cells</th>
<th>Human Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. 1 None</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>IL-2</td>
<td>16.68</td>
<td>15.82</td>
</tr>
<tr>
<td>E. coli DNA</td>
<td>7.23</td>
<td>5.05</td>
</tr>
<tr>
<td>Calf thymus DNA</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Expt. 2 None</td>
<td>0.00</td>
<td>3.28</td>
</tr>
<tr>
<td>1585 gggGTCAGCGTTGAggggG (SEQ ID NO:12)</td>
<td>7.38</td>
<td>17.96</td>
</tr>
<tr>
<td>1629 .........gtc ........... (SEQ ID NO:50)</td>
<td>0.00</td>
<td>4.4</td>
</tr>
<tr>
<td>Expt. 3 None</td>
<td>0.00</td>
<td>5.22</td>
</tr>
<tr>
<td>1613 GCTAGCAGCTTGTG    (SEQ ID NO:51)</td>
<td>0.02</td>
<td>HD</td>
</tr>
<tr>
<td>1769 .........z .......... (SEQ ID NO:52)</td>
<td>0.02</td>
<td>HD</td>
</tr>
</tbody>
</table>
TABLE 9-continued

<table>
<thead>
<tr>
<th>DNA or Cytokine Added</th>
<th>Mouse Cells Human Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1619 TCATGTCTTTCTGGGATCT</td>
<td>3.35</td>
</tr>
<tr>
<td>1765 ........Z............</td>
<td>0.11</td>
</tr>
</tbody>
</table>

CpG dinucleotides in ODN sequences are indicated by underlying; Z indicates cytosine.
Lower case letters indicate nuclease resistant phosphorothioate modified internucleotide linkages which, in titration experiments, were more than 20 times as potent as non-modified ODN, depending on the flanking bases. Poly G ends (g) were used in some ODN, because they significantly increase the level of ODN uptake.

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.

Identification of B Cell and Monocytel NK Cell-Specific Oligonucleotides

As shown in FIG. 6, CpG DNA can directly activate highly purified B cells and monocytes. There are 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 10).

TABLE 10

<table>
<thead>
<tr>
<th>ODN Sequence</th>
<th>B cell activation</th>
<th>NK activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1668 TCATGAGCTTTCTGGGATCT</td>
<td>42,649</td>
<td>2.52</td>
</tr>
<tr>
<td>(SEQ ID NO:54)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1758 TCTCCAGCCTGGGACCAT</td>
<td>1,747</td>
<td>6.66</td>
</tr>
<tr>
<td>(SEQ ID NO:55)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NONE</td>
<td>367</td>
<td>0.00</td>
</tr>
</tbody>
</table>

CpG dinucleotides are underlined; oligonucleotides were synthesized with phosphorothioate modified backbones to improve their nuclease resistance.

Teleological Basis of Immunostimulatory, Nucleic Acids

Vertebrate DNA is highly methylation 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 5, 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,
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 autoreactive 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 characterized by the production of anti-DNA antibodies; ii) induced by drugs which inhibit DNA methyltransferase (Comacchia, E. J. et al., *J. Clin. Invest.* 92:838 (1993)); and iii) associated with reduced DNA methylation (Richardson, B. L. et al., *Arth. Rheum.* 35:647 (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 increase of the role of CpG DNA in the sepsis syndrome is described in Cowdery, J. et al., (1996) *The Journal of Immunology* 156:4570-4575.

Proposed Mechanisms of Action

Unlike antigens that trigger B cells through their surface Ig receptor, CpG-ODN did not induce any detectable Ca\(^{2+}\) flux, changes in protein tyrosine phosphorylation, or IP\(_3\) 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 NF\(\kappa\)B 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 NF\(\kappa\)B 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 NF\(\kappa\)B activation, PDTC and glitoxin, completely block the lymphocyte stimulation by CpG DNA as measured by B cell proliferation or monocyte cell cytokine secretion, suggesting that NF\(\kappa\)B activation is required for both cell types.

There are several possible mechanisms through which NF\(\kappa\)B can be activated. These include through activation of various protein kinases, or through the generation of reactive oxygen species. No evidence for protein kinase activity 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 dihydroorhodamine 123 as described in Royall, J. A., and Ischiropoulos, H. (*Archives of Biochemistry and Biophysics* 302:498-495 (1993)). Moreover, inhibitors of the generation of these reactive oxygen species completely block the induction of NF\(\kappa\)B and the later induction of cell proliferation and cytokine secretion by CpG DNA.

Working 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 bafilomycin, which work through different mechanisms. FIG. 8A shows the results from a flow cytometry study using mouse B cells with the dihydroorhodamine 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 bafilomycin also appear to block the activation of NF\(\kappa\)B by CpG DNA as well as the subsequent proliferation and induction of cytokine secretion.

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, we used electrophoretic mobility shift assays (EMSA) 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 NF\(\kappa\)B binding site was added. This suggests that an NF\(\kappa\)B 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 NF\(\kappa\)B was strongly activated. These data therefore do not provide proof that NF\(\kappa\)B 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 NF\(\kappa\)B 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 NFκB 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 NFκB activation when CD40 is cross-linked. Examples of such TRAF proteins include TRAF-2 and TRAF-5.

Method for Making Immunosimulatory 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 β-cyanomethyl phosphorimidate method (S. L. Beaucage and M. H. Caruthers, 1981 TET. Let. 22:1859); nuclease H-phosphonate method (Gar-egg et al., 1986 TET. Let. 27:4051-4054; Froehler et al., 1986 Nucl. Acid. Res. 14: 5399-5407; Garg et al., 1986 TET. Let. 27: 4055-4058; Gaffney 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, exonuclease or endonucleases.

For use in vivo, nucleic acids are preferably relatively resistant to degradation (e.g. via endo- and exo-nuclease). 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 alkylphosphor-esters (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 Immunosimulatory 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, monocyte cells or NK cells) obtained from a subject having an immune system deficiency ex vivo and activated lymphocytes can then be reimplanted in the subject.

As reported herein, in response to unmethylated CpG containing nucleic acid molecules, an increased number of splenic cells secrete IL-6, IL-12, INF-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 monocyctic cells.

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, it 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 costimulatory 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, along 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 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 autoimmune diseases 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 INF-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., TCCATGACGGTTCCGTAGCCTT; SEQ ID NO:10), but not a control oligonucleotide (TCCATGACGGTTCCGTAGTCTT; SEQ ID NO:11) 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 oral and transdermal (e.g., via a patch). Examples of other routes of administration include injection (subcutaneous, intravenous, parenteral, intrapertioneal, intrathecal, etc.). The injection can be made 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 is 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 language “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 that 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 or 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 wk old specific pathogen free DBA/2 or BxSB 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 M (Cedarlane Laboratories, Hornby, Ontario, Canada) (“B cells”). B cells contained fewer than 1% CD4+ or CD8+ cells. 8x10^6 B cells were dispensed 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 hr at 37°C, cells pulsed with 1 μCi of [3H] uridine, 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 20μM for 48 hr. Data, reported in Table 1, represent the stimulation index compared to cells 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 oligodeoxynucleotides. 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-Thy1, anti-CD4, and anti-CD8 and complement by the method of Leibson et al., J. Exp. Med. 154:1681 (1981). Resting B cells (<0.2% 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 μM ODN or 20 μg/ml LPS for 48 hr. The number of B cells actively secreting IgM was maximal at this time point, as determined by El. Isot assay (Klinman, D. M. et al. J. Immunol. 144:506 (1990)). In that assay, B cells were incubated for 6 hrs on anti-Ig coated microtiter plates. The Ig they produced (>99% IgM) was detected using phosphatase-labelled anti-Ig (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 increases 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 a) Micrococcus lysodeikticus; b) NZB/N mouse spleen; and c) NFS/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 ELISA spot assay.

DBA/2 B cells were cultured with either no additive, 50 µg/ml LPS or the ODN 1; 1a; 4; or 4a at 50 µM. Cells were cultured and harvested at 4, 8, 24 and 48 hours. BXXXB 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 3838 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

10x10⁶ 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 (Ballas, Z. K. et al. (1993) J. Immunol. 150:17). Effector cells were added at various concentrations to 10⁵ ⁵¹Cr-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 counted 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 are 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 weighed and injected IP with 0.25 ml of sterile PBS or the indicated phosphorothioate ODN dissolved in PBS. Twenty four hours later, spleen cells were harvested, washed, and stained for flow cytometry using phycoerythrin conjugated 6H2 to gate on B cells in conjunction with biotin conjugated anti Ly-6A/E or anti-Iaα (Pharmingen, San Diego, Calif.) or anti-Blk1 (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 3D6b 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 (5x10⁵ /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 3D6b before addition of anti-IgM (1 µM). Cells were cultured for a further 24 hr. before a 4 hr. pulse with 2 µCi/well ³H thymidine. In this experiment, cells with no ODN or anti-IgM gave 90 x 10⁶ cpm of ³H thymidine incorporation by addition of anti-IgM. The phosphodiester ODN shown in Table 1 gave similar protection, though with 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 µg CpG or control phosphorothioate ODN. At various time points after injection, the mice were bled. Two mice were studied for each test 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 hr.

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 CH21.1.X 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 DNAase I (2 U/µg of DNA) at 37° C. for 2 hr in 1xSSC with 5 mM MgCl₂. To methylate the cytosine in CpG dinucleotides in E. coli DNA, E. coli DNA was treated with CpG methylase (M. Ssai; 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/ml 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 a 5% CO₂ humidified 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 cases, cells were treated with CpG O—ODN along with various concentrations (1-10 µg/ml) of neutralizing rat IgG3 antibody against murine IL-6 (hybridoma MP5-20F3) or control rat
IgG1 mAb to *E. coli* β-galactosidase (hybridoma GL13; ATCC, Rockville, Md.) (20) for 5 days. At the end of incubation, culture supernatant 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), CpG or non-CpG SODN (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 and RNA was prepared from those organs using RNAzol B (Tel-Test, Friendswood, Tex.) according to the manufacturer’s protocol.

ELISA. Flat-bottomed Immulon 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 NaHCO3, 35 mM NaHCO3) overnight at 4°C. The plates were then washed with TBPS (0.5 mM MgCl2, 0.625 M KCl, 1.47 mM KH2PO4, 0.14 M NaCl, 6.6 mM K2HPO4, 0.5% Tween 20) and blocked with 10% FCS in TPBS for 2 hr at room temperature and then washed again. Culture supernatants, mouse sera, recombinant mouse IL-6 (Pharmingen, 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 IgM (Sigma, St. Louis, Mo.) were added and incubated for 45 min. At room temperature following washes with TPBS. Horseradish peroxidase (HRP) conjugated avidin (Bio-rad Laboratories, Hercules, Calif.) at 1:4000 dilution in 10% FCS (100 µl/well) was added and incubated at room temperature for 30 min. The plates were washed and developed with o-phenylenediamine dihydrochloride (OPD; Sigma, St. Louis Mo.) 0.05 M phosphate-citrate buffer, pH 5.0, for 30 min. The reaction was stopped with 0.7 M H2SO4, and plates were read on a microplate reader (Cambridge Technology, Watertown, Mass.) at 490-600 nm. The results are shown in FIGS. 1 and 2.

RT-PCR. A sense primer, an antisense primer, and an internal oligonucleotide probe for IL-6 were synthesized using published sequences (Montgomery, R. A. and M. S. Dallman (1991), Analysis of cytokine gene expression during fetal thymic ontogeny using the polymerase chain reaction (*J. Immunol.*) 147(554)). cDNA synthesis and IL-6 PCR was done essentially as described by Montgomery and Dallman (Montgomery, R. A. and M. S. Dallman, (1991), Analysis of cytokine gene expression during fetal thymic ontogeny using the polymerase chain reaction (*J. Immunol.*) 147(554)) using RT-PCR reagents from Perkin-Elmer Corp. (Hayward, Calif.). Samples were analyzed after 30 cycles of amplification by gel electrophoresis followed by blot unlabeling analysis (Stoye, J. P. et al., 1991) DNA hybridization in dried gels with fragmented probes: an improvement over blotting techniques, Techniques 3(123). Briefly, the gel was hybridized at room temperature for 30 min: in denaturation buffer (0.05 M NaOH, 1.5 M NaCl) followed by incubation for 30 min in renaturation buffer (1.5 M NaCl, 1 M Tris, pH 8) and a 30 min wash in double distilled water. The gel was dried and prehybridized at 47°C for 2 hr: hybridization buffer (5×SSPE, 0.1% SDS) containing 10 µg/ml denatured salmon sperm DNA. The gel was hybridized with 2×106 cpm/ml [32P]ATP end-labeled internal oligonucleotide probe for IL-6 (5GATTCATCAAGTTCCCAC3) SEQ ID NO:56) overnight at 4°C, washed 4 times (2xSSC, 0.2% SDS) at room temperature and autoradiographed. The results are shown in FIG. 3.

Cell Proliferation assay. DNA2/2 mice spleen B cells (5×10^4 cells/100 µl/well) were treated with media, CpG or non-CpG SODN (0.5 µM) or O (ODN) (20 µM) for 24 hr at 37°C. Cells were pulsed for the last four hr. with either [3H]Thymidine or [3H]Uridine (1 µCi/well). Amounts of [3H] incorporated were measured using Liquid Scintillation Analyzer (Packard Instrument Co., Downers Grove, Ill.).

Transfections and CAT assays. WEHI-231 cells (10^7 cells) were electroporated with 20 µg of control or human IL-6 promoter-CAT construct (kindly provided by S. Manolagas, Univ. of Arkansas) (Potratz, S. T. et al., 1994) 17β-estradiol inhibits expression of human interleukin-6 promoter-reporter construct by a receptor-dependent mechanism. *J. Clin. Invest.* 93:944) at 250 mV and 960 µl. Cells were stimulated with various concentrations of CpG or non-CpG ODN after electroporation. Chloramphenicol acetyltransferase (CAT) activity was measured by a solution assay (Seed, B. and J. Y. Sheen (1988) A single phase-extraction assay for chloramphenicol acetyl transferase activity. *Gene* 76:271) 16 hr: after transfection. The results are presented in FIG. 5.

Example 10

Oligodeoxynucleotide Modifications Determine the Magnitude of B Cell Stimulation by CpG Motifs

ODN were synthesized on an Applied Biosystems Inc. (Foster City, Calif.) model 380A, or 394 DNA synthesizer using standard procedures (Bencze and Caruthers (1981) Deoxynucleoside phosphoramidites—A new class of key intermediates for deoxynucleoside synthesis. Tetrahedron Letters 22, 1859-1862.). Phosphodiester ODN 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 thioate containing ODN were deprotected by treatment with concentrated ammonia at 55°C. For 12 hours. The ODN were purified by gel exclusion chromatography and lyophilized to dryness prior to use. Phosphorothioate linkages were introduced by using deoxynucleoside S-(b-benzoylmercaptoethyl) pyro lidino thiophosphoramidites (Wiesler, W. T. et al., (1993) In Methods in Molecular Biology: Protocols for Oligonucleotides and Analogs Synthesis and Properties, Agrawal, S., (ed.), Humana Press, 191-206.). Dithioate containing ODN 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 methylphosphonothioates or methylphosphonates as well as phosphodiester at any desired internucleotide linkage, two different synthetic cycles were used. The major synthetic differences in the two cycles are the coupling reagent where diallylammoniummethylsulfoxide phosphines are used and the oxidation reagents in the case of methylphosphonothioates. In order to synthesize either derivative, the condensation time has been increased for the diallylammoniummethylsulfoxide phosphines due to the slower kinetics of coupling (Jager and Engels, (1984) Synthesis of deoxynucleoside methylphosphonates via a phosphonamide approach. Tetrahedron Letters 24, 1437-1440). After the coupling step has been completed, the methylphosphonodiester is treated with the sulfurizing reagent (5% elemental sulfur, 100 millimolar N,N-dimethylaminopropyline in carbon disulfide/pyridine/triethylamine), four consecutive times for 450 seconds each to produce methylphosphonothioates. To produce meth-
ylphosphonate 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; S—O—S—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 methylphosphonate modified. The ODN sequences studied (with CpG dinucleotides indicated by underlining) include:

3' (5'-GAGAAGCTTGAACGCTTTCCAGT-3'), (SEQ ID NO:14)
3' (5'-TCTTGGGCGGAAGGCAGCCT-3'), (SEQ ID NO:22)
5' (5'-GGGTTATTCGACTGCTGCCC-5'), (SEQ ID NO:57)
5' (5'-CCTAGCTTATGGCAGCCCAGCT-5'), (SEQ ID NO:58)

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 B6.SJL mice obtained from The Jackson Laboratory (Bar Harbor, Me), and maintained under specific pathogen-free conditions were used as a source of lymphocytes at 5-10 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 56°C for experiments using only modified ODN), 1.5 μM 1-glutamine, 50 μM 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin for 24 hr or 48 hr as indicated. 1 μCi of 3H thymidine or thymidine (as indicated) was added to each well, and the cells harvested after an additional 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 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 1PS level in ODN was less than 12.5 ng/mg and E. coli and calf thymus DNA contained less than 2.5 ng of 1PS/mg of DNA by Limulus assay.

Virus-free, 4-6 week old, DBA/2, C57BL/6 (B6) and congenitally athymic BALB/c mice were obtained through contract with 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 5x10^9/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 hr. All cultures were harvested at 18 hr. and the cells were used as effectors in a standard 4 hr. 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. With indicated, neutralizing antibodies against IL-4 (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 addition, 10 μg of each of the 4 MAAB (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 C56BL/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. TCCATGACGTTCGACGT; SEQ ID NO:10) or did not (i.e. control, TCCATGACGTTCGACGT; SEQ ID NO:11). Soluble SEA (10 μl in 25 μl of saline) was administered by intraperitoneal injection 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 C5I 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 only 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 a 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 INF-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 96 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.

Equivalents

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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OTHER INFORMATION: Synthetic oligonucleotide

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The invention claimed is:

1. A method for promoting a Th1 immune response in a subject, the method comprising: administering to a subject a first dose of an immunostimulatory nucleic acid; and administering to the subject a second dose of an immunostimulatory nucleic acid, wherein the immunostimulatory nucleic acid comprises a nucleotide sequence comprising 5'-CG-3' having the formula \( X_1X_2CGX_3X_4 \), wherein \( C \) is unmethylated and \( X_1, X_2, X_3 \) and \( X_4 \) are nucleotides and wherein the immunostimulatory nucleic acid is between 8 and 100 nucleotides in length, and includes more than one CpG dinucleotide.

2. The method of claim 1, wherein the second dose is administered about 7 days after the first dose.

3. The method of claim 1, wherein the subject is a human.

4. The method of claim 1, wherein \( X_1X_2 \) is GT and \( X_3X_4 \) is TT.
It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title Page, Item (60) should read:


In the Specification:

Please amend the Related Applications section in col. 1, line 4 to read:

Related Applications

This application is a continuation of U.S. Patent Application serial number 09/818,918 filed March 27, 2001, currently pending, which is divisional of U.S. Patent Application serial number 08/738,652, filed October 30, 1996, now issued as U.S. Patent 6,207,646 B1, which is a continuation-in-part of U.S. Patent Application serial number 08/386,063, filed February 7, 1995, now issued as U.S. Patent No. 6,194,388 B1, which is a continuation-in-part of U.S. Patent Application serial number 08/276,358, filed July 15, 1994, now abandoned.

Column 1, line 46, “Pharmaceutical” should be “Pharmaceutical”

Column 2, line 59, “Initogenic” should be “mitogenic”

Column 3, line 34, “fNF” should be “INF”

Signed and Sealed this
Twenty-eighth Day of December, 2010

[Signature]
David J. Kappos
Director of the United States Patent and Trademark Office
Column 9, line 25, “INF-γ” should be “IFN-γ”

Column 9, line 39, “Canisfamiliaris” should be “Canis familiaris”

Column 9, line 39, “Dermatophagoidesfarinae” should be “Dermatophagoides farinae”

Column 9, line 42, “Cryptomeriajaponica” should be “Cryptomeria japonica”

Column 9, line 57, “Paopratensis” should be “Poa pratensis”

Column 11, line 55, “INF-γ” should be “IFN-γ”

Column 13, line 32, “CvG” should be “CpG”

Column 14, line 36, “Id” should be “1D”

Column 23, line 43, “Quanikine” should be “Quantikine”

Column 23, line 46, “THF-α” should be “TNF-α”

Column 23, line 48, “oligodeoxynucle-” should be “oligodeoxynucle-”

Column 23, line 57, “INF-γ” should be “IFN-γ”

Column 26, line 37, “CSG-Mediated” should be “CpG-Mediated”

Column 29, line 22, “Monocytel NK” should be “monocyte/NK”

Column 33, line 16, “Garegg” should be “Garegg”

Column 34, line 3, “INF-γ” should be “IFN-γ”

Column 35, line 11, “INF-γ” should be “IFN-γ”

Column 40, line 44, “pyrro lidino” should be “pyrrolidino”

Column 40, line 45, “thiophosphoramid ites” should be “thiophosphoramidites”

Column 42, line 59, “CsCl” should be “CsCl”

Column 43, line 23, “INF-γ” should be “IFN-γ”

Column 61, Claim 1, “A method for of promoting” should be “A method for promoting”