

Conjugation of Ligands at the 5'-End of CpG DNA Affects Immunostimulatory Activity

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Bacterial DNA and synthetic oligonucleotides containing unmethylated CpG dinucleotides (CpG DNA) activate the vertebrate immune system and promote Th1-like immune responses. Several CpG DNAs are currently being tested in clinical trials as either alone or in combination with vaccines, antibodies, and allergens separately or as conjugates for a number of disease indications including cancers, allergies, and asthma. In this paper, we show that conjugation of an oligonucleotide and a CpG DNA through their 5'-ends (5'-5'-linked DNA) significantly reduces the immunostimulatory activity of the CpG DNA. In addition, we found that the reduction in immunostimulatory activity of 5'-5'-linked CpG DNA depends on the size of the oligonucleotide conjugated to CpG DNA. Conjugation of a smaller group or molecule, such as a phosphorothioate group, at the 5'-end of CpG DNA has an insignificant effect on immunostimulatory activity. However, conjugation of a mononucleotide, tetra- or longer oligonucleotide or a fluorescein molecule to the 5'-end of a CpG DNA (5'-5'-linked DNA) significantly suppresses the immunostimulatory activity of CpG DNA. Surprisingly, conjugation of an oligonucleotide or a ligand through the 3'-end of CpG DNA (3'-3'-linked DNA) has an insignificant effect on immunostimulatory activity. Studies of cellular uptake and activation of transcription factor NF- κ B in J774 cells using fluorescein-conjugated CpG DNAs suggest that the differences in the immune stimulation of 5'- and 3'-end-conjugated CpG DNAs is not as a result of differences in their cellular uptake properties. These results suggest that for optimal immunostimulatory activity, ligands should not be attached at the 5'-end of the CpG DNA.

INTRODUCTION

The immune system of vertebrates recognizes unmethylated CpG dinucleotides present in bacterial DNA and synthetic oligonucleotides (CpG DNA) in a sequence-selective manner. As a result of activation by CpG DNA, immune system cells secrete a myriad of cytokines, including IL-12, IL-6, TNF- α , and INF- γ , and up-regulate several costimulatory molecules (1–4). The cytokines and costimulatory molecules secreted provide nonspecific protection against infectious pathogens and strongly enhance the generation of antigen-specific B-cell responses (5–7). A number of CpG DNAs are at various stages of preclinical and clinical evaluation as antitumor, antiviral, antibacterial, and antiinflammatory agents and as adjuvants in immunotherapy (5–7).

A receptor, TLR9, recognizes CpG dinucleotides present in certain specific sequence contexts in CpG DNA and initiates signaling pathways that lead to the activation of NF- κ B and the subsequent up-regulation of cytokines and costimulatory molecules (8). Alternately, CpG DNA activates DNA protein kinase, which phosphorylates I κ B-kinaseB, leading to the activation of NF- κ B, which leads to the production of cytokines (9). It is not clear whether the two pathways are activated sequentially or in parallel leading to a common function of activating the NF- κ B pathway. The growing evidence suggests that TLR9 exhibits considerable sequence selectivity for CpG DNA (10, 11). In addition, the receptor recognizes CpG-dinucleotides with a strict bias for the chemical and

conformational nature of the unmethylated d(CpG) dinucleotide. However, it can accommodate a range of flanking sequences (3, 12) that may vary from species to species (13, 14).

Our laboratory has been studying the effects of sequence and structural changes in the flanking sequences that potentiate or neutralize the immunostimulatory activity of CpG DNA in an attempt to understand the molecular recognition events between the receptors and CpG DNA. Our studies suggest that incorporation of certain modified bases in place of C or G in a CpG dinucleotide does not affect immunostimulatory activity (15). However, the receptor recognizes only 2'-deoxyribonucleotides with a negatively charged internucleotide linkage between C and G of the CpG-dinucleotide (16). Further, a number of different modifications at the 2'-position of the sugar and phosphate backbone are tolerated about three or more nucleotides away from the CpG-dinucleotide in both the 5'- and 3'-flanking sequences (17–19). In fact, the incorporation of modifications in the 5'-flanking sequence usually potentiates the immunostimulatory activity of CpG DNA, while the incorporation of the same in the 3'-flanking sequence has minimal effect on the immunostimulatory activity. We have also shown that the presence of a nucleobase on nucleosides is not required for the immunostimulatory activity in certain positions in the 5'- or the 3'-flanking sequence to the CpG-dinucleotide (20). Although the incorporation of 2'-5'-deoxyribonucleosides within or adjacent to CpG dinucleotide is detrimental for immunostimulatory activity, the incorporation of the same in the flanking sequences is well tolerated and in certain positions they even potentiate the immunostimulatory activity (21).

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In recent years, the conjugation of antigens and vaccines to CpG DNA has been reported (22–26). The use of such CpG DNA conjugates induces more efficient antigen/vaccine-specific immune responses than does CpG DNA and antigen/vaccine combination treatments (25, 26). Our recent studies suggest that an accessible 5'-end of the CpG DNA is critical for the immunostimulatory activity (27). To confirm this observation, establish the size of the ligand conjugated at the 5'-end that could block immunostimulatory activity, and examine if cellular uptake is effected by 3'- or 5'-conjugation that could lead to different immunostimulatory activity, we carried out the study described herein. In this study, CpG-DNA was conjugated to different lengths or sizes of nucleotides or ligands through their 5'- or 3'-ends, and the impact on immunostimulatory activity was studied. These studies define the most appropriate site for conjugation on CpG DNA with large molecules such as antigens/vaccines for optimal therapeutic uses.

EXPERIMENTAL PROCEDURES

CpG DNA Synthesis and Purification. CpG DNA and their conjugates were synthesized using β -cyanoethyl phosphoramidite chemistry on a PerSeptive Biosystem's 8909 Expedite DNA synthesizer on 1 to 2 μ mol scale as described earlier (27–30). The phosphoramidites of dA, dG, dC, and T were obtained from PE Biosystems. The 5'-phosphoramidites of dA, dG, dC, and T were obtained from Glen Research Corporation. The DMT-protected fluorescein phosphoramidite and DMT-protected CPG-bound fluorescein were obtained from Glen Research. Beaucage's reagent was used as an oxidant to obtain phosphorothioate backbone modification (31). After the synthesis, CpG DNAs were deprotected using standard protocols, purified by HPLC, and dialyzed against USP quality sterile water for irrigation (Braun). The CpG DNA conjugates were lyophilized and dissolved again in distilled water, and the concentrations were determined by measuring the UV absorbance at 260 nm. All the CpG DNAs synthesized were characterized by CGE and MALDI-TOF mass spectrometry (Brucker Proflex III MALDI-TOF mass spectrometer with 337 nm N_2 laser) for purity and molecular mass, respectively. The purity of full-length CpG DNA conjugates ranged from 89 to 95% and the rest being n-1 and n-2 as determined by CGE and/or denaturing PAGE.

Cell Culture Conditions and Reagents. BALB/c mouse (4–8 weeks) spleen cells were cultured in RPMI complete medium as described earlier (16, 32). Murine macrophage-like cells, J774 (American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FCS and antibiotics (100 IU/mL of penicillin G/streptomycin). All other culture reagents were purchased from Mediatech (Gaithersburg, MD).

ELISAs for IL-12 and IL-6. BALB/c mouse spleen or J774 cells were plated in 24-well dishes at a density of 5×10^6 or 1×10^6 cells/mL, respectively. The CpG DNA dissolved in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) was added to a final concentration of 0.03, 0.1, 0.3, 1.0, 3.0, or 10.0 μ g/mL to mouse spleen cell cultures and 1.0, 3.0, or 10.0 μ g/mL to J774 cell cultures. The cells were then incubated at 37 °C for 24 h, and the supernatants were collected for ELISA assays. The experiments were performed two or three times for each CpG DNA in triplicate for each concentration.

The secretion of IL-12 and IL-6 was measured by sandwich ELISA. The required reagents, including cy-

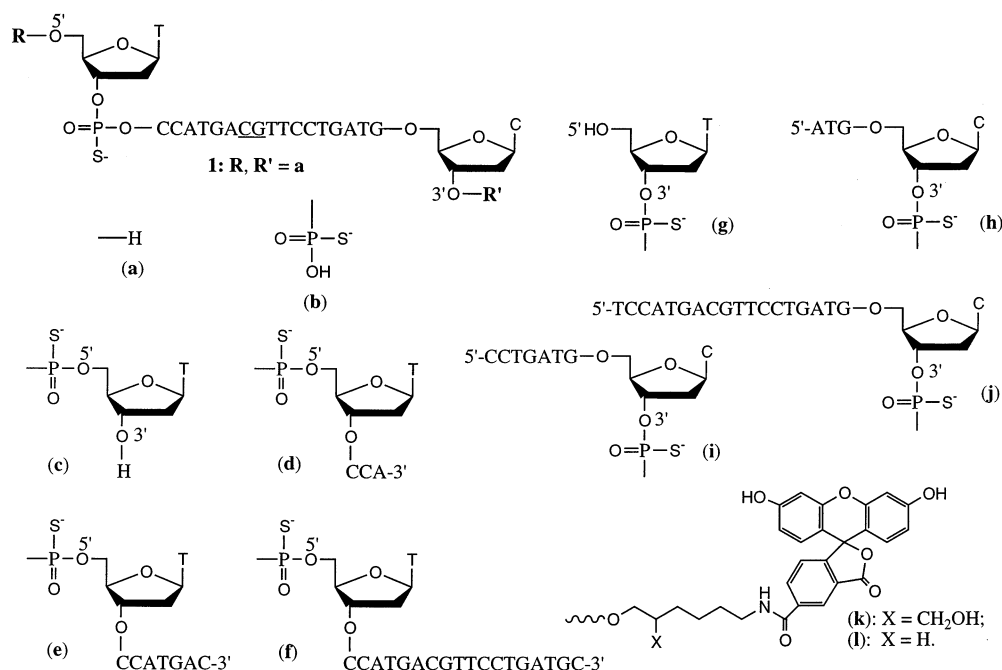
tokine antibodies and standards, were purchased from PharMingen. ELISA plates (Costar) were incubated with appropriate antibodies at 5 μ g/mL in PBSN buffer (PBS/0.05% sodium azide, pH 9.6) overnight at 4 °C and then blocked with PBS/1% BSA at 37 °C for 30 min. Cell culture supernatants and cytokine standards were appropriately diluted with PBS/1% BSA, added to the plates in triplicate and incubated at 25 °C for 2 h. Plates were washed, 1 μ g/mL of appropriate biotinylated antibody was added, and the plates were incubated at 25 °C for 1.5 h. The plates were washed extensively with PBS/0.05% Tween 20 and then further incubated at 25 °C for 1.5 h after the addition of streptavidine-conjugated peroxidase (Sigma). The plates were developed with Sure Blue (Kirkegaard and Perry) chromogenic reagent, and the reaction was terminated by adding Stop Solution (Kirkegaard and Perry). The color change was measured on a Ceres 900 HDI Spectrophotometer (Bio-Tek Instruments) at 450 nm. The levels of IL-12 and IL-6 in the cell culture supernatants were calculated from the standard curve constructed under the same experimental conditions for IL-12 and IL-6, respectively.

Mouse Splenomegaly Assay. Female BALB/c mice (4–6 weeks, 19–21 gm) were divided into different groups with three or four mice in each group. CpG DNAs were dissolved in sterile PBS and administered subcutaneously to mice at a dose of 5 mg/kg. After 72 h, mice were sacrificed, and the spleens were harvested and weighed.

Cellular Uptake. J774 cells were plated at a density of 1×10^5 cells/well in an eight-well chamber slide the day before addition of CpG DNAs. The medium was changed 1 h before the experiment, fluorescein-labeled CpG DNAs were added to a concentration of 10 μ g/mL, and the cells were incubated for 1 h at 37 °C. At the end of the incubation period, medium was aspirated. The cells were washed thoroughly with ice-cold phosphate-buffered saline (PBS), fixed with cold 95% ethanol and 5% acetic acid for 10 min, washed, and finally mounted in an antifade medium (Vector Laboratories, Burlingame, CA) with DAPI (for nuclear staining). The slides were then examined, and photomicrographs were taken under a Zeiss Axiophot microscope. The fluorescein and DAPI images were merged using the Spot Imaging software.

Preparation of J774 Cell Nuclear Extracts and EMSA. For NF- κ B activation studies, J774 cells were plated at a density of 5×10^6 cells/well in six-well plates, and nuclear extracts were prepared as described earlier (33). Briefly, after stimulation of J774 cells for 1 h with CpG DNAs at a concentration of 10 μ g/mL, cells were washed three times in ice-cold PBS, harvested, and suspended in 0.24 mL of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and protease inhibitor cocktail) (Boehringer-Mannheim) and allowed to swell on ice for 15 min. Then 16 μ L of 10% Nonidet-P40 was added, and the tubes were vortexed vigorously for 10 s. Nuclei were separated from cytosol by centrifugation at 500 g for 5 min and were lysed in 60 μ L of buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA and protease inhibitor cocktail). After 30 min at 4 °C, lysates were separated by centrifugation (14000g, 10 min) and supernatants containing nuclear proteins were transferred to new vials. Protein concentrations were determined (34), and the samples were either used immediately or stored frozen at -70 °C.

The oligonucleotide probe (5'-AGTTGAGGGGACTTTC-CCAGGC-3') containing the κ B binding motif was end-labeled with [γ - ^{32} P]-ATP using T4 polynucleotide kinase

Chart 1. Sequence of Immunostimulatory CpG DNA 1 and the Chemical Structures of Ligands (b–l) Conjugated at Either the 5'-(R) or the 3'-terminal (R') of CpG DNA.**Table 1. Phosphorothioate CpG DNA Sequences and Modifications Used in the Study^a**

CpG DNA no.	sequence	length	5'-end	3'-end
1	5'-TCCATGACGTTCTGATGC-3'	19-mer	1	1
2	5'-TCCATGACGTTCTGATGC-3'-b	19-mer	1	blocked
3	5'-TCCATGACGTTCTGATGC-3'-3'-c-5'	20-mer	2	blocked
4	5'-TCCATGACGTTCTGATGC-3'-3'-d-5'	23-mer	2	blocked
5	5'-TCCATGACGTTCTGATGC-3'-3'-e-5'	27-mer	2	blocked
6	5'-TCCATGACGTTCTGATGC-3'-3'-f-5'	38-mer	2	blocked
7	b-5'-TCCATGACGTTCTGATGC-3'	19-mer	blocked	1
8	3'-g-5'-5'-TCCATGACGTTCTGATGC-3'	20-mer	blocked	2
9	3'-h-5'-5'-TCCATGACGTTCTGATGC-3'	23-mer	blocked	2
10	3'-i-5'-5'-TCCATGACGTTCTGATGC-3'	27-mer	blocked	2
11	3'-j-5'-5'-TCCATGACGTTCTGATGC-3'	38-mer	blocked	2
12	5'-TCCATGACGTTCTGATGC-3'-k	19-mer	1	blocked
13	l-5'-TCCATGACGTTCTGATGC-3'	19-mer	blocked	1

^a See Chart 1 for chemical structures **b–l**; 5'-CG-3' dinucleotide is shown underlined.

(Invitrogen) and purified over two successive G-50 mini columns (Amersham Pharmacia Biotech). Reaction mixtures (25 μL) containing 10 μg of nuclear protein, 5 mM Tris (pH 7.5), 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 4% glycerol (v/v), and 0.08 mg/mL salmon sperm DNA were preincubated on ice for 15 min. After preincubation, 1×10^6 cpm of the probe was added and the binding reaction was allowed to proceed for 20 min at room temperature. DNA–protein complexes were then resolved in a 6% native polyacrylamide gel in TBE buffer (22.5 mM Tris, 22.5 mM boric acid and 0.5 mM EDTA, pH 8.3) at 140 V for 2–3 h. Gels were dried and exposed to Kodak Biomax MR film at -70°C . Films were scanned, and the images were processed using Adobe imaging software.

RESULTS AND DISCUSSION

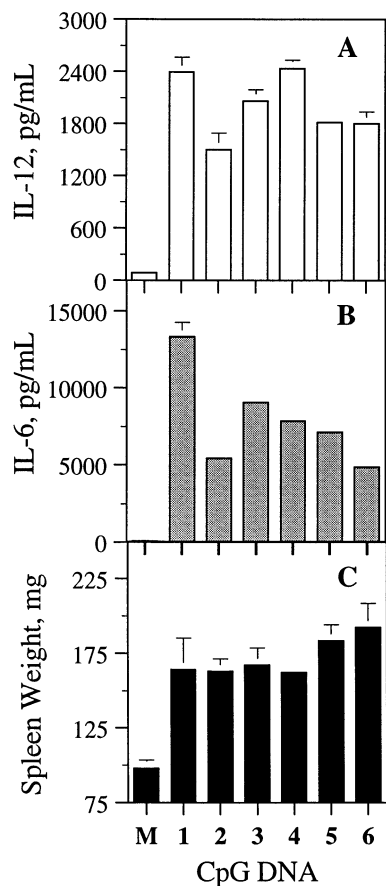
We chose an oligonucleotide sequence (Chart 1, Table 1) containing a CpG dinucleotide (**1**), which showed immunostimulatory activity in earlier studies (27). We synthesized a number of analogues of CpG DNA sequence **1** conjugating a phosphorothioate group (**b**), mono- (**c**, **g**), 4- (**d**, **h**), 8- (**e**, **i**), or 19-mer (**g**, **j**) oligonucleotide, or a fluorescein (**l** or **k**) either at the 5'-(R) or the 3'-end (R') (see Chart 1 for structures and Table 1 for sequences).

CpG DNA conjugates **2–6** and **12**, in which a ligand conjugated to CpG DNA **1** through its 3'-hydroxyl group, are shown in Table 1. The 3'-end of these molecules is blocked as a result of conjugation at the 3'-end and CpG DNAs **3–6** have two 5'-ends. We also synthesized CpG DNAs **7–11** and **13**, in which a ligand conjugated to parent CpG DNA **1** through its 5'-hydroxyl group (Table 1). The 5'-end of these molecules is blocked by conjugation and, therefore, CpG DNAs **8–11** have two 3'-ends.

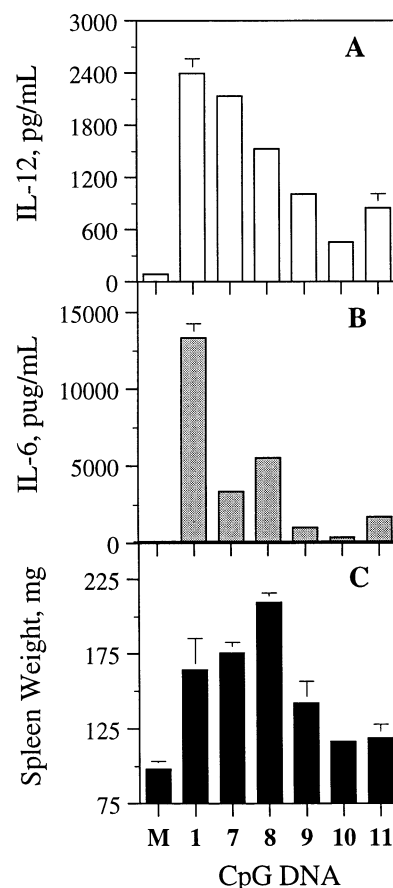
Phosphorothioate and Nucleotide Conjugation at the 3'-End of CpG DNA. In BALB/c mouse spleen cell cultures, all CpG DNAs induced a concentration-dependent IL-12 and IL-6 secretion (Table 2). At a concentration of 1.0 $\mu\text{g}/\text{mL}$, parent CpG DNA **1** induced 2391 ± 175 and 13320 ± 960 pg/mL of IL-12 and IL-6 secretion, respectively (Figure 2A and B). The secretion of IL-12 and IL-6, compared with media (PBS) control, in mouse spleen cell cultures suggest that CpG DNA **1** induced immunostimulatory activity as has been reported earlier (27). At the same concentration of 1.0 $\mu\text{g}/\text{mL}$, CpG DNA **2**, which has a phosphorothioate group conjugated to the 3'-end of the parent CpG DNA **1**, induced lower levels of IL-12 and IL-6 (1499 ± 191 and 5420 ± 370 pg/mL, respectively) secretion compared with CpG DNA **1**. CpG DNAs **3–6**, which have a mono-, 4-, 8- or 19-mer oligo-

Table 2. Induction of IL-12 and IL-6 Secretion by CpG DNA-conjugates in BALB/c Mice Spleen Cell Cultures

CpG DNA no. ^a	IL-12 (pg/mL) $\pm$ SD					IL-6 (pg/mL) $\pm$ SD				
	0.1 μ g/mL	0.3 μ g/mL	1.0 μ g/mL	3.0 μ g/mL	10.0 μ g/mL	0.1 μ g/mL	0.3 μ g/mL	1.0 μ g/mL	3.0 μ g/mL	10.0 μ g/mL
1	991 $\pm$ 121	1820 $\pm$ 224	2391 $\pm$ 175	3507 $\pm$ 127	2615 $\pm$ 279	652 $\pm$ 48	2858 $\pm$ 180	13320 $\pm$ 960	18625 $\pm$ 1504	17229 $\pm$ 1750
2	526 $\pm$ 32	2100 $\pm$ 175	1499 $\pm$ 191	3019 $\pm$ 35	3489 $\pm$ 162	1387 $\pm$ 152	1426 $\pm$ 124	5420 $\pm$ 370	19096 $\pm$ 484	19381 $\pm$ 2313
3	1030 $\pm$ 11	1348 $\pm$ 102	2060 $\pm$ 130	3330 $\pm$ 130	3582 $\pm$ 259	923 $\pm$ 22	2542 $\pm$ 81	9054 $\pm$ 120	14114 $\pm$ 179	13693 $\pm$ 264
4	1119 $\pm$ 159	1726 $\pm$ 207	2434 $\pm$ 100	2966 $\pm$ 204	3215 $\pm$ 464	870 $\pm$ 146	1905 $\pm$ 56	7841 $\pm$ 350	17146 $\pm$ 1246	15713 $\pm$ 693
5	1175 $\pm$ 68	2246 $\pm$ 124	1812 $\pm$ 75	2388 $\pm$ 320	2545 $\pm$ 202	1152 $\pm$ 238	3499 $\pm$ 116	7142 $\pm$ 467	14064 $\pm$ 167	13566 $\pm$ 477
6	1087 $\pm$ 121	1705 $\pm$ 163	1797 $\pm$ 141	2522 $\pm$ 195	3054 $\pm$ 103	1039 $\pm$ 105	2043 $\pm$ 157	4848 $\pm$ 288	15527 $\pm$ 224	21021 $\pm$ 1427
7	1173 $\pm$ 107	2170 $\pm$ 155	2132 $\pm$ 58	2812 $\pm$ 203	3689 $\pm$ 94	807 $\pm$ 0.5	927 $\pm$ 0.5	3344 $\pm$ 0.5	10233 $\pm$ 0.5	9213 $\pm$ 0.5
8	866 $\pm$ 51	1564 $\pm$ 63	1525 $\pm$ 63	2666 $\pm$ 97	4030 $\pm$ 165	750 $\pm$ 63	1643 $\pm$ 30	5559 $\pm$ 415	11549 $\pm$ 251	11060 $\pm$ 651
9	227 $\pm$ 3	495 $\pm$ 96	1007 $\pm$ 68	897 $\pm$ 15	1355 $\pm$ 97	302 $\pm$ 18	374 $\pm$ 22	1000 $\pm$ 68	9106 $\pm$ 271	13077 $\pm$ 381
10	139 $\pm$ 18	211 $\pm$ 12	452 $\pm$ 22	458 $\pm$ 29	1178 $\pm$ 237	220 $\pm$ 23	235 $\pm$ 18	383 $\pm$ 35	1706 $\pm$ 33	11530 $\pm$ 254
11	181 $\pm$ 85	282 $\pm$ 105	846 $\pm$ 165	2082 $\pm$ 185	3185 $\pm$ 63	467 $\pm$ 122	437 $\pm$ 85	1697 $\pm$ 283	9781 $\pm$ 13	11213 $\pm$ 294
medium	86 $\pm$ 6					60 $\pm$ 12				

^a See Table 1 for sequences.**Figure 1.** Secretion of IL-12 (A) and IL-6 (B) in BALB/c mouse spleen cell cultures in the presence of CpG DNAs **1–6** at a concentration of 1.0 μ g/mL. Assays were carried out as described in Experimental Section. M stands for media/vehicle (PBS) control. (C) Spleen enlargement in BALB/c mice following subcutaneous administration of CpG DNAs **1–6** at a dose of 5 mg/kg. Control mice received vehicle (PBS). Three or four mice were used for each CpG DNA treatment, and the averages were calculated.

nucleotides conjugated through their 3'-ends to the 3'-end of parent CpG DNA **1**, induced 2060 ± 130 , 2434 ± 100 , 1812 ± 75 , and 1797 ± 141 pg/mL of IL-12 (Figure 2A), and 9054 ± 120 , 7841 ± 350 , 7142 ± 467 , and 4848 ± 288 pg/mL of IL-6 (Figure 2B), respectively, at the same concentration. These in vitro cytokine secretion studies suggest that the conjugation of a ligand at the 3'-end of a CpG DNA, in general, results in a similar or lower levels of IL-12 secretion and lower levels of IL-6 compared with parent CpG DNA. It is important to note that CpG DNA **6** contains two CpG dinucleotides as opposed to one in CpG DNAs **1–5**. In addition, the length

**Figure 2.** Induction of IL-12 (A) and IL-6 (B) secretion in BALB/c mouse spleen cell cultures in the presence of CpG DNA **1** and **7–11** at a concentration of 1.0 μ g/mL. Assays were carried out as described in Experimental Section. M stands for media (PBS) control. (C) Spleen enlargement in BALB/c mice following subcutaneous administration of CpG DNA **1** and **8–11** at a dose of 5 mg/kg. See Figure 1 legend for other experimental details.

of CpG DNA **6** is twice that of **1**, and **6** contains two 5'-ends compared with CpG DNA **1**, which has a 3'- and a 5'-end. The results presented here, however, suggest that the number of CpG dinucleotides and the length of CpG DNA do not affect immunostimulatory activity.

CpG DNAs **1** and **3–6** were studied for their ability to induce splenomegaly (increase in spleen weight) in BALB/c mice at a single dose of 5 mg/kg administered subcutaneously. The parent CpG DNA **1** caused a 67.5% increase in spleen weight compared with control mice injected with vehicle (PBS) (Figure 1C). At the same dose, CpG DNAs **3–6** caused 70.4, 64.3, 86.7, and 96.2% increase in spleen weight, respectively. These results

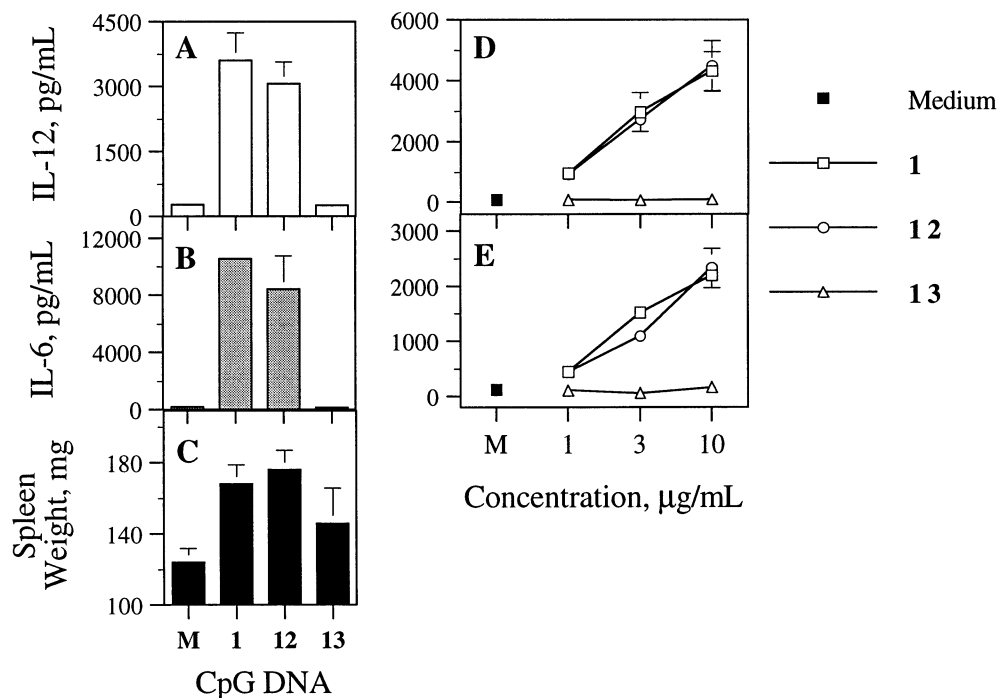


Figure 3. Induction of IL-12 (A) and IL-6 (B) secretion in BALB/c mouse spleen cell cultures in the presence of CpG DNAs **1**, **12**, and **13** at $1.0 \mu\text{g/mL}$ concentration. Assays were carried out as described in Experimental Section. M stands for media (PBS) control. (C) Spleen enlargement in BALB/c mice following subcutaneous administration of CpG DNAs **1**, **12**, **13** at a dose of 5 mg/kg . Control mice received vehicle (PBS). Three or four mice were used for each CpG DNA treatment, and the averages were calculated. Induction of IL-12 (D) and IL-6 (E) secretion in J774 cell cultures in the presence of CpG DNAs **1**, **12**, and **13** at different concentration. Assays were carried out as described in Experimental Section. M stands for media (PBS) control.

suggest that 3'-conjugation (CpG DNAs **3–6**) does not interfere with immunostimulatory activity compared with parent CpG DNA. In fact, CpG DNAs **5** and **6** showed a greater spleen weight increase than did parent CpG DNA **1** (Figure 1C).

Phosphate and Nucleotide Conjugation at the 5'-End of CpG DNA. CpG DNAs, **7–11**, which have either phosphate or nucleotide(s) conjugated at the 5'-end of the parent CpG DNA, were also tested for induction of cytokine secretion in BALB/c mouse spleen cell cultures. All CpG DNAs induced concentration-dependent IL-12 and IL-6 secretion (Table 2). The induction of IL-12 and IL-6 secretion by CpG DNAs **7–11** at a concentration of $1.0 \mu\text{g/mL}$ is shown in Figure 2A and 2B, respectively. CpG DNA **7**, which has a phosphorothioate group conjugated at its 5'-end, induced 2132 ± 58 and $3344 \pm 0.5 \text{ pg/mL}$ of IL-12 and IL-6 secretion, respectively, at $1.0 \mu\text{g/mL}$ concentration. At the same concentration, CpG DNA **8**, which has a mononucleotide conjugated through its 5'-end to the 5'-end of **1**, induced 1525 ± 45 and $5559 \pm 415 \text{ pg/mL}$ of IL-12 and IL-6 secretion, respectively. CpG DNAs **9–11**, which have a 4-, 8- or 19-mer oligonucleotide conjugated through their 5'-ends to CpG DNA **1**, induced 1007 ± 68 , 452 ± 22 , and $846 \pm 165 \text{ pg/mL}$ of IL-12, respectively, at $1.0 \mu\text{g/mL}$ concentration (Figure 2A). At the same concentration, CpG DNAs **9–11** induced 1000 ± 68 , 383 ± 35 , and $1697 \pm 283 \text{ pg/mL}$ of IL-6 secretion, respectively (Figure 2B). These *in vitro* results suggest that a phosphorothioate group conjugated at the 5'-end of CpG DNA has an insignificant effect on IL-12 secretion. However, the conjugation of nucleotides (1–19-mers) to CpG DNA through 5'-end (5'-5'-conjugation) resulted in significantly lower IL-12 and IL-6 secretion.

CpG DNAs **8–11** were further examined *in vivo* for their ability to increase spleen weight at a dose of 5 mg/kg as described above. CpG DNAs **8–11** showed 113.3,

44.8, 18.7, and 20.9% increase in spleen weight, respectively, compared with control mice injected with vehicle (PBS). CpG DNAs **9–11**, which have 5'-5'-conjugation produced a less spleen enlargement than did parent CpG DNA (67.5%) or the CpG DNA that contained 3'-3'-conjugations (**3–6**) (64.3–96.2%). Note that CpG DNA **11** contains two CpG dinucleotides as does CpG DNA **6**. Both CpG DNAs **6** and **11** are of the same length, but **6**, which has two free 5'-ends, caused a greater increase in spleen weight and slightly lower IL-12 secretion compared with that of CpG DNA **1**. In contrast, CpG DNA **11**, which has the 5'-ends blocked, caused a slight increase in spleen weight in BALB/c mice and induced significantly lower levels of IL-12 and IL-6 secretion in spleen cell cultures.

Fluorescein Conjugation at the 3'- or 5'-End of CpG DNA. To examine the effect of conjugation of a nonnucleoside ligand on the immunostimulatory activity of CpG DNA, we conjugated fluorescein (Chart 1, **k/l**) either at the 3'- (**12**) or the 5'-end (**13**) of CpG DNA **1**. Both conjugates (**12** and **13**) were studied in BALB/c mouse spleen cell cultures for IL-12 and IL-6 secretion and in BALB/c mice for splenomegaly. In addition, conjugates **12** and **13** were used to study the effect of 3'- and 5'-end conjugation on cellular uptake, activation of transcription factor NF- κ B, and subsequent induction of cytokine production in the murine macrophage-like cell line J774 in comparison with parent CpG DNA.

In BALB/c mouse spleen cell cultures, both CpG DNA conjugates **12** and **13** induced a concentration-dependent IL-12 and IL-6 secretion. Figure 3A and B shows the levels of IL-12 and IL-6 secreted at $1.0 \mu\text{g/mL}$ concentration of **12** and **13** compared with **1**. CpG DNA **12**, which has a fluorescein moiety conjugated at its 3'-end, induced $3059 \pm 512 \text{ pg/mL}$ of IL-12 and $8422 \pm 2327 \text{ pg/mL}$ of IL-6 secretion at $1.0 \mu\text{g/mL}$ concentration. At the same concentration, the parent CpG DNA induced 3610 ± 637

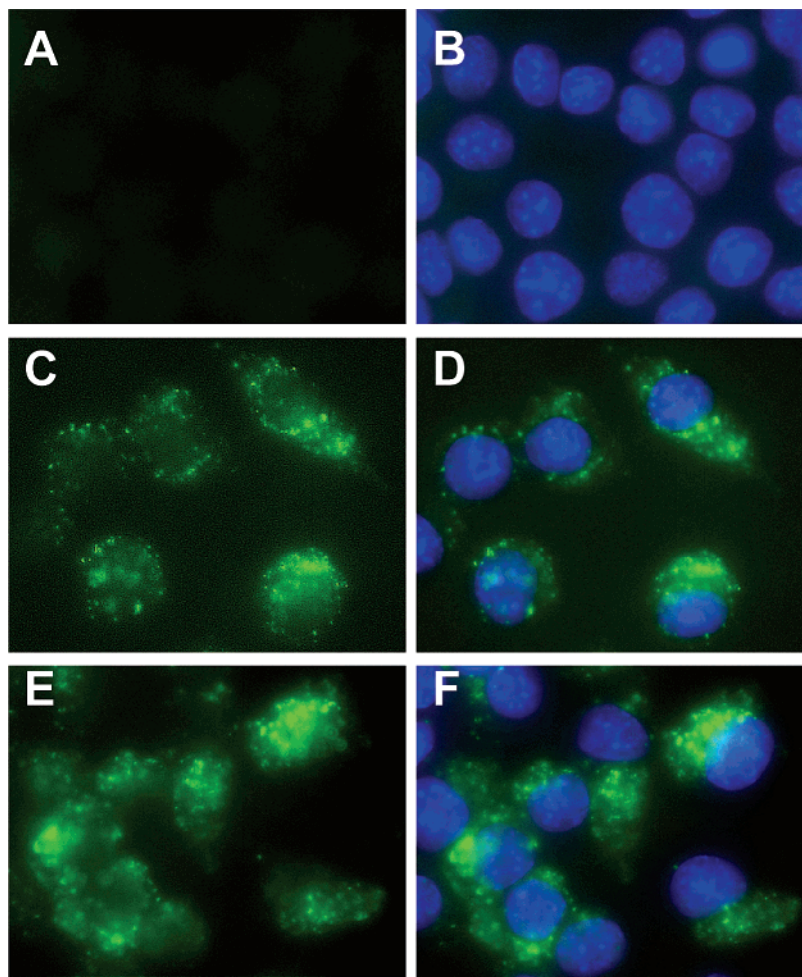


Figure 4. Cellular uptake of fluorescein-conjugated CpG DNAs **12** and **13** by J774 cells. J774 cells were incubated in medium alone (Panels A and B) or with CpG DNAs **12** (Panels C and D) and **13** (Panels E and F) and processed as described in Experimental Section. Panels A, C, and E are images taken in the fluorescein (green) channel. Panels B, D, and F are images A, C, and E superimposed with images taken in DAPI (blue, for nuclear staining) channel. Note the intense green fluorescein cytoplasmic staining in Panels C–F, indicating similar uptake of CpG DNAs **12** and **13**. Cells incubated in medium alone (A and B) are devoid of green color.

and 10540 ± 502 pg/mL of IL-12 and IL-6 secretion, respectively. In in vivo splenomegaly assay in BALB/c mice, CpG DNAs **1** and **12** caused spleen weight increases of 168 and 176 mg, respectively, compared with mice treated with vehicle (124 mg) (Figure 3C). These results suggest that conjugation of a fluorescein moiety at the 3'-end of CpG DNA does not affect (splenomegaly) or slightly reduces (cytokine secretion) immunostimulatory activity compared with the parent CpG DNA. In contrast, CpG DNA **13**, which has a fluorescein group conjugated at its 5'-end, induced 262 ± 20 pg/mL IL-12 secretion in BALB/c mice spleen cell cultures at a concentration of $1.0 \mu\text{g/mL}$ (Figure 3A). The same CpG DNA induced 168 ± 33 pg/mL of IL-6 secretion at the same concentration (Figure 3B). In BALB/c mice, CpG DNA **13** caused a spleen enlargement of 146 mg (Figure 3C). These results suggest that the conjugation of ligands at the 5'-end of a CpG DNA abrogates immunostimulatory activity significantly compared with the parent CpG DNA.

CpG DNA conjugates **12** and **13** were also examined for their ability to induce cytokine secretion in J774 cell cultures in comparison with parent CpG DNA **1**. Parent CpG DNA and its 3'-fluorescein conjugate **12** induced concentration-dependent IL-12 and IL-6 secretion (Figure 3D and E). The data presented in Figure 3D and 4E obtained from J774 cell cultures complement the results observed in murine spleen cells, which are predominantly

B lymphocytes, and confirm the results obtained in BALB/c mouse spleen cell cultures.

Does Conjugation of Ligand at the 3'- or 5'-End of CpG DNA Affect Cellular Uptake? Conjugation of ligands to CpG DNA could also affect the cellular uptake and thereby immunostimulatory activity. Since CpG DNA conjugate **12** induced similar levels of cytokine secretion to that of parent CpG DNA and **13** did not, we examined whether these differences arise from different cellular uptake properties of the two conjugates in J774 cells. Figure 4 shows the uptake of CpG DNAs **12** and **13** after 1 h of incubation in J774 cell cultures. Both CpG DNAs are taken up by J774 cells and show a similar green (FITC) punctate cytoplasmic staining pattern (Figure 4, panels C–F). In some cells at this time, the nuclei also showed positive staining, as was observed with 5'-fluorescein-labeled CpG or antisense DNAs in other studies (35, 36). Control cells that were treated with medium alone showed DAPI (blue) but no fluorescein staining (panels A and B). These results suggest that both 3'- and 5'-fluorescein conjugates are taken up by J774 cells to a similar extent and the differences observed in the immunostimulatory activities of CpG DNAs **12** and **13** are not as a result of differences in their cellular uptake but result from differential recognition of these molecules by receptors in the immunostimulatory pathway.

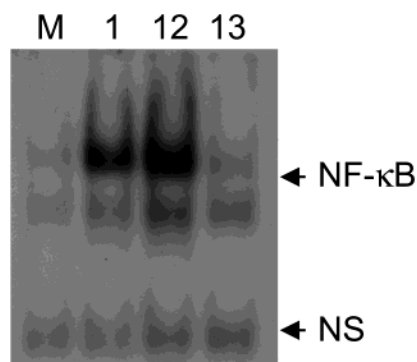


Figure 5. Effect of 3'- or 5'-fluorescein-conjugated CpG DNAs on NF- κ B activation in J774 cells. J774 cells were stimulated with CpG DNAs **1**, **12**, and **13**, nuclear extracts were prepared and analyzed on a native polyacrylamide gel as described in Experimental Section. M and NS stand for medium and nonspecific band, respectively. NF- κ B band is shown with an arrow.

Effect of 5'- or 3'-Conjugation on Activation of Transcription Factor NF- κ B in J774 Cells. CpG DNA stimulation has been shown to activate a number of signaling pathways, including the NF- κ B pathway, which plays a critical role in up-regulation of cytokine gene expression (37). To study the effect of 3'- and 5'-conjugation of ligands to CpG DNA on the role of NF- κ B activation, J774 cells were stimulated with CpG DNAs **1**, **12**, and **13** and the nuclear extracts were examined for NF- κ B activation by EMSA. The results are presented in Figure 5. The presence of complexes that correspond to NF- κ B in lanes labeled 1 and 12 suggest that parent CpG DNA **1** and its 3'-fluorescein conjugate, **12** activated transcription factor NF- κ B. In contrast, the band intensities in the lane labeled 13 were similar to that observed with control cells incubated with medium (lane M), suggesting that 5'-fluorescein-conjugated CpG DNA **13** failed to activate NF- κ B. The results of NF- κ B activation studies confirm and extend the results observed in cytokine secretion studies in J774 and BALB/c mouse spleen cell cultures.

Degradation of oligonucleotides occurs primarily by 3'-exonucleases present in vivo, which digest the oligonucleotides from their 3'-ends (38–40). Conjugation of a variety of chemical groups or ligands to the 3'-end of oligonucleotides was known to increase metabolic stability against nucleases (41–44). In the present study, the conjugation at the 3'-end could contribute to higher nuclease stability and thereby immunostimulatory activity. The splenomegaly data observed with CpG DNAs **3–6** and **12** especially support this hypothesis. It is surprising, however, to note that CpG DNAs that have a ligand conjugated at their 5'-ends (**8–11** and **13**) show lower immunostimulatory activity despite having equal, if not higher, nuclease stability compared with their parent CpG DNA **1**. In the present study, a phosphorothioate group conjugated at the 5'-end (**7**) failed to block the immunostimulatory activity (except lowering IL-6 secretion). Conjugation of a mononucleotide through 5'-5'-linkage (**8**) did not affect significantly immunostimulatory activity as determined by both in vitro and in vivo studies. Blocking the 5'-end of a CpG DNA with groups such as fluorescein or a tetranucleotide or larger oligonucleotides significantly reduced or blocked immunostimulatory activity compared with the parent CpG DNA. These results obtained with CpG DNAs **8–11** and **13** suggest that the differences in immunostimulatory activity of 3'-3'- and 5'-5'-linked DNAs do not arise from differences in nuclease stability or their cellular uptake

but from the accessibility to the 5'-end. In addition, the present results suggest that the size of ligand attached at the 5'-end determines the accessibility of the 5'-end of CpG DNA to the receptors in the immunostimulatory pathway and thereby the immunostimulatory activity. This study is focused on determining the size of ligand that blocks 5'-accessibility of CpG DNA but with insignificant affect on cellular uptake of conjugates. We are currently studying conjugation of other ligands with high lipophilic or cationic nature, including peptide ligands, that could influence physicochemical characteristics of the conjugates to understand the 5'-accessibility of CpG DNA for immunostimulatory activity.

In addition to conjugation of ligands, we also have shown in the past that the addition of guanine-rich sequences at the 5'- or 3'-end of a CpG DNA would effect its biological activity (45, 46). The presence of G-rich sequences (four or more contiguous Gs) in oligonucleotides leads to the formation of secondary structures involving the G-rich sequences. The unusual tetrameric and other higher-order structures formed at the end of the oligonucleotide could also interfere with CpG DNA recognition and thereby pertinent activation of immunostimulatory pathways, as is the case with 5'-conjugated CpG DNAs in the present study. As has been reported earlier, now there is growing evidence that CpG DNAs containing G-rich sequences at their 3'-end are more immunostimulatory than those that contain the same sequences at their 5'-end (47). However, the difference in immunostimulatory activity has been presumed in terms of their differential cellular uptake only, the present results strongly suggest that it is not related to differences in cellular uptake but related to their ability to activate immune pathways through an accessible 5'-end of CpG DNA.

CpG DNA evokes host defense mechanisms such as the activation of macrophages/monocytes, B, and NK cells and induction of Th1 cell differentiation. A number of studies have shown improved responsiveness to antigens when the antigens are used together with CpG DNAs compared with antigen alone. Several studies have shown that the direct conjugation of CpG DNA with antigens increased the effectiveness of antigens severalfold compared with the use of antigen and CpG DNA mixtures because of the optimal presentation of antigen to APC by CpG DNA when used as conjugates. In general, ligands are routinely conjugated to oligonucleotides at their 5'-ends because of convenience of attaching required functional groups at the 5'-end in solid-phase synthesis (48). However, the present studies suggest that the immunostimulatory activity of CpG DNA conjugates is dependent on whether the ligand conjugated at the 5'-end or the 3'-end and the size of the ligand conjugated.

Taken together, the current results suggest that an accessible 5'-end of CpG DNA is required for its immunostimulatory activity and smaller groups such as a phosphorothioate or a mononucleotide do not effectively block the accessibility of the 5'-end of CpG DNA to receptors or factors involved in the immunostimulatory pathway. However, the conjugation of molecules as large as fluorescein or larger at the 5'-end of CpG DNA could abrogate immunostimulatory activity. These results have a direct impact on the studies of immunostimulatory activity of CpG DNA–antigen/vaccine/monoclonal antibody (mAb) conjugates. The conjugation of large molecules such as vaccines or mAbs at the 5'-end of a CpG DNA could lead to suboptimal immunostimulatory activity of CpG DNA. The conjugation of functional ligands at the 3'-end of CpG DNA not only contributes to

increased nuclease stability but also increased immunostimulatory potency of CpG DNA in vivo.

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