Design, Synthesis, and Immunostimulatory Properties of CpG DNAs Containing Alkyl-Linker Substitutions: Role of Nucleosides in the Flanking Sequences

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Bacterial and synthetic DNA containing unmethylated CpG dinucleotides activate the innate immune system and promote Th1-like immune responses. Recently, a receptor, TLR9, has been shown to recognize CpG DNA and activate immune cascade. But there have been no reports on the molecular mechanisms of recognition between CpG DNA and the receptor(s). Our earlier studies described a number of the chemical and structural characteristics of CpG dinucleotide and the sequences flanking the CpG dinucleotide that are critical for immunostimulatory activity. In the present study, we examined the effect of the presence and absence of a nucleoside in the flanking sequences by replacing one or two natural deoxyribonucleosides at various positions with one or more alkyl- (C2−C12), branched alkyl- (glyceryl or aminobutyryl-propanediol), or ethyleneglycol- (tri or hexa) linkers. The results suggest that a linker substitution at the first two nucleoside positions adjacent to the CpG dinucleotide on the 5′- or the 3′-side neutralizes the immunostimulatory activity, as determined by in vitro mouse spleen cell proliferation, cytokine secretion, and in vivo mouse spleen enlargement. The same substitutions placed about three to six nucleotides away from the CpG dinucleotide either did not affect or potentiated immunostimulatory activity compared with parent CpG-DNA without modifications. Substitution of deoxyribonucleosides with a C3 or C4 alkyl-linker was found to be optimal for potentiating immunostimulatory activity.

Introduction

The cells of the vertebrate immune system (macrophages, monocytes, dendritic cells, NK cells, and B-cells) recognize unmethylated CpG dinucleotides present in bacterial and synthetic oligodeoxynucleotides (CpG-DNA) in a sequence-specific manner and secrete a myriad of cytokines and chemokines, including IL-12, IL-6, TNF-α, and INF-γ, and up-regulate expression of costimulatory molecules.[1−4] The cytokines secreted provide nonspecific protection against infectious pathogens and strongly enhance the generation of antigen-specific immune responses.[5−9] A number of CpG-DNAs are at various stages of preclinical and clinical evaluation as antitumor, antiviral, antibacterial, and anti-asthmatic agents and as adjuvants in immunotherapy.[6−19]

The presence of a CpG dinucleotide with specific nucleotides in the flanking sequences is critical for the immunostimulatory activity of CpG-DNAs.[3,20,21] Our earlier studies have shown that sugar (ribose or 2′-O-methylribose) or backbone (nonionic methylphosphonate) modifications within a CpG dinucleotide neutralized the immunostimulatory activity.[22] The same modifications distal to the CpG dinucleotide did not neutralize the immunostimulatory activity of CpG DNAs.[23−25] Moreover, the incorporation of these modifications in the 5′-flanking sequence enhanced the immunostimulatory activity of CpG DNA.[23−25] Additionally, our recent studies suggest that an accessible 5′-end of CpG-DNA is critical for immunostimulatory activity.[26]

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We have shown that substitution of C or G within a CpG dinucleotide with certain modified pyrimidine or purine bases does not affect the immunostimulatory activity of CpG DNA.[27] We have also shown that site-specific substitution of an anionic internucleoside linkage with a nonionic methylphosphonate linkage also affects the immunostimulatory activity of CpG-DNA.[25] The deletion of a nucleobase at specific sites distal to the CpG dinucleotide enhances the immunostimulatory activity of CpG-DNA.[28] Our recent studies, in which a 3′-deoxyribonucleoside is incorporated for 2′-deoxyribonucleoside in CpG DNA, suggest that structural perturbation caused by 2′-5′-internucleoside linkage at least about 3−5 nucleotides distal to the CpG dinucleotide in the 5′-flanking sequence potentiates immunostimulatory activity.[29] Incorporation of the same modification in the 3′-flanking sequence has minimal effect on immunostimulatory activity.[29] To further understand the significance of nucleosides in the flanking sequences for immunostimulatory activity, we synthesized a series of CpG-DNAs with one or more natural nucleosides substituted with nonnucleoside linkers (Figure 1) and studied their structure-immunostimulatory activity relationships in BALB/c mouse spleen cell cultures and in vivo.

Results and Discussion

Two 18-mer DNA sequences containing either an "AGCGTT" (**10**) or "GACGTT" (**23**) hexameric motif containing a CpG dinucleotide were selected for the present studies (Table 1). Both parent CpG-DNAs **10** and **23** induced immune responses in mouse.[25,28,29] A series of modified CpG-DNAs incorporating one or more
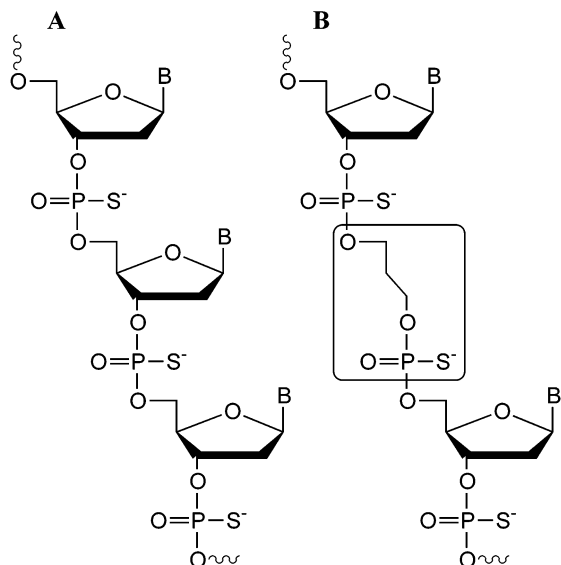
Figure 1. Representative structures of (A) a deoxyribonucleotide chain and (B) a deoxyribonucleotide chain containing a C3-linker (boxed). B stands for A, C, G, or T.

linkers (**1−9**) (Figure 2) at various nucleotide positions in parent CpG DNAs, **10**, and **23** (Table 1) were synthesized and studied for their immunostimulatory activity, as determined by spleen cell proliferation and cytokine secretion in BALB/c mouse spleen cell cultures and in vivo spleen enlargement (splenomegaly) in BALB/c mice.

Nucleosides in Certain Positions of CpG DNA Are Not Required for Immunostimulatory Activity. To study the effect of deletion of a nucleoside, a propanediol- (C3) linker, **2** (Figure 2), was incorporated in place of a 2′-deoxynucleoside at different positions in the 5′- and the 3′-flanking sequences and within the CpG dinucleotide of the parent CpG DNA **10** (CpG DNAs **11−18**; Table 1). The incorporation of propanediol-linker (**2**) allowed maintenance of appropriate distance between two adjacent phosphates similar to that found in oligodeoxyribonucleotides (Figure 1). Spleen cells obtained from BALB/c mice were cultured with CpG-DNAs at 0.1, 0.3, 1.0, and 3.0 μg/mL concentrations for 48 h, and the cell proliferation was measured by ^{3}H-uridine incorporation.[22,30] All CpG DNAs that were active showed a concentration-dependent spleen cell proliferation. The extent of spleen cell proliferation induced at 0.3 μg/mL concentration of CpG DNAs is shown in Table 2 as proliferation index (PI). The parent CpG-DNA, **10**, induced significant spleen cell proliferation (1.7 ± 0.27) compared with media (PBS) control (0.2 ± 0.06). CpG-DNA **11**, which had a C3-linker (**2**) substituted for G in CpG dinucleotide, induced spleen cell proliferation similar to that of media control. Consistent with spleen cell proliferation data, CpG DNA **10** induced cytokines IL-12 and IL-6 in a concentration-dependent manner in BALB/c mouse spleen cell cultures (Supplementary Table 1). CpG DNA **10** induced 1391 ± 179 pg/mL of IL-12 and 6644 ± 380 pg/mL of IL-6 secretion at 1.0 μg/mL concentration. CpG DNA **11** with a C3-linker in the G position of the CpG dinucleotide induced cytokine levels similar to those of background levels at 1.0 μg/mL concentration (Table 2). In in vivo experiments, parent CpG DNA **10** caused an increase of about 32% in the spleen weights of BALB/c

Table 1. Sequences of CpG DNA Showing the Position of Substitution

CpG DNA number	sequence (5′ → 3′)[a]
10	CCTACTAGCGTTCTCATC
11	CCTACTAGC**2**TTCTCATC
12	CCTACT**2**GCGTTCTCATC
13	CCTA**2**TAGCGTTCTCATC
14	CCT**22**TAGCGTTCTCATC
15	**22**TAGTAGCTTCTCATC
16	CCTACTAGCGT**2**CTCATC
17	CCTACTAGCGTTC**2**CATC
18	CCTACTAGCGTTC**22**ATC
19	CCT**6**CTAGCGTTCTCATC
20	CCTACTAGCGTTC**6**CATC
21	CCT**7**CTAGCGTTCTCATC
22	CCTACTAGCGTTC**7**CATC
23	CTATCTGACGTTCTCTGT
24	CTAT**1**TGACGTTCTCTGT
25	CTA**1**CTGACGTTCTCTGT
26	CTATCTG**2**CGTTCTCTGT
27	CTATC**2**GACGTTCTCTGT
28	CTA**2**CTGACGTTCTCTGT
29	**22222**TGACGTTCTCTGT
30	**2222**TGACGTTCTCTGT
31	**222**TGACGTTCTCTGT
32	**22**TGACGTTCTCTGT
33	**2**TGACGTTCTCTGT
34	CTAT**3**TGACGTTCTCTGT
35	CTA**3**CTGACGTTCTCTGT
36	CTA**33**TGACGTTCTCTGT
37	**33**TGACGTTCTCTGT
38	CTAT**4**TGACGTTCTCTGT
39	CTA**4**CTGACGTTCTCTGT
40	CTA**44**TGACGTTCTCTGT
41	**44**TGACGTTCTCTGT
42	CTAT**5**TGACGTTCTCTGT
43	CTA**5**CTGACGTTCTCTGT
44	CTA**55**TGACGTTCTCTGT
45	**55**TGACGTTCTCTGT
46	CTA**6**CTGACGTTCTCTGT
47	CTATCTGACGTTC**6**CTGT
48	CTA**7**CTGACGTTCTCTGT
49	CTATCTGACGTTC**7**CTGT
50	CTATCTG**8**CGTTCTCTGT
51	CTATCT**8**ACGTTCTCTGT
52	CTATC**8**GACGTTCTCTGT
53	CTAT**8**TGACGTTCTCTGT
54	CTA**8**CTGACGTTCTCTGT
55	CTATCTGACG**8**TCTCTGT
56	CTATCTGACGT**8**CTCTGT
57	CTATCTGACGTT**8**TCTGT
58	CTATCTGACGTTC**8**CTGT
59	CTATCTG**9**CGTTCTCTGT
60	CTATCT**9**ACGTTCTCTGT
61	CTA**9**CTGACGTTCTCTGT
62	CTATCTGACGT**9**CTCTGT
63	CTATCTGACGTTC**9**CTGT

[a] See Figure 2 for the chemical structures of substitutions **1–9**. All CpG DNAs are phosphorothioate backbone modified.

mice at a dose of 5 mg/kg compared with control mice injected with vehicle (PBS) (Table 2). The administration of CpG DNA **11** caused an increase of about 5% in spleen weights of BALB/c mice at the same dose. Together, these in vitro and in vivo studies suggest that the presence of a CpG dinucleotide is required for spleen cell proliferation, cytokine secretion, and spleen enlargement.

The other CpG DNAs **12−18** were also studied for their immunostimulatory activity, and the results are presented in Table 2 (for concentration-dependent cytokine data see Supplementary Table 1). These data suggest that linker substitutions within two nucleotides away from the CpG dinucleotide either in the 5′- (**12**) or the 3′-flanking sequence (**16**) resulted in a signifi-
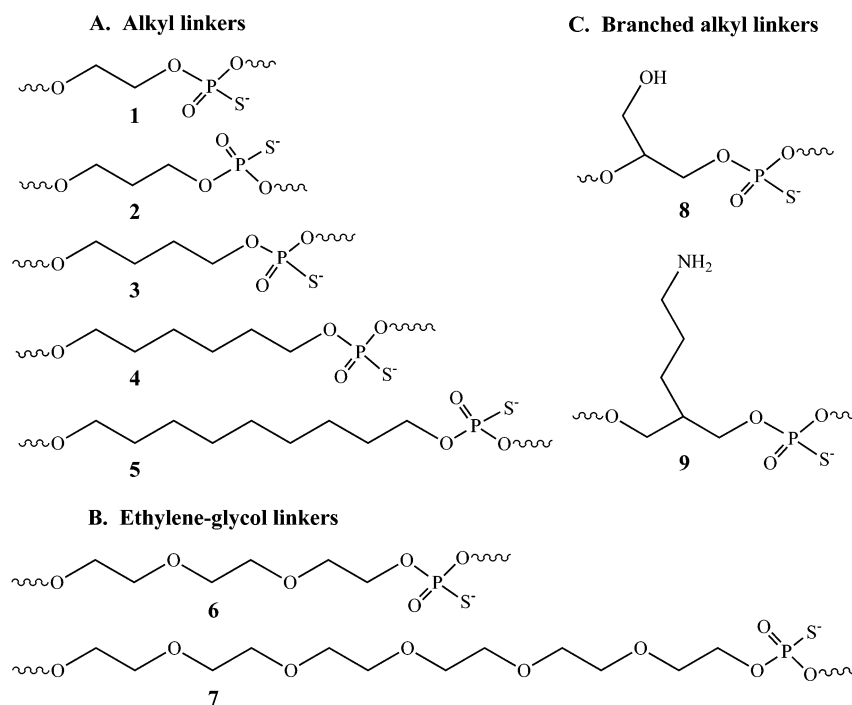
Figure 2. Chemical structures of non-nucleoside linkers used in the current study.

Table 2. Immunostimulatory Activity of CpG DNAs in BALB/c Mouse Spleen Cell Cultures and Splenomegaly in BALB/c Mice

CpG DNA number[a]	spleen cell proliferation (PI)[b]	cytokine, pg/mL[c]		spleen weight (mg)[d]
		IL-12	IL-6	
10	1.7 ± 0.27	1391 ± 179	6644 ± 380	130 ± 8
11	0.3 ± 0.02	119 ± 9	73 ± 10	105 ± 15
12	1.2 ± 0.1	491 ± 38	5435 ± 236	159 ± 18
13	2.1 ± 0.1	1709 ± 189	16105 ± 1767	211 ± 10
14	1.8 ± 0.1	2356 ± 191	15575 ± 814	230 ± 12
15	1.4 ± 0.1	1090 ± 50	6198 ± 682	203 ± 18
16	0.3 ± 0.02	157 ± 29	123 ± 62	90 ± 13
17	1.6 ± 0.2	1170 ± 59	5500 ± 673	170 ± 17
18	2.1 ± 0.3	2205 ± 182	10405 ± 1889	170 ± 12
M/V	0.2 ± 0.06	85 ± 23	59 ± 16	106 ± 17
LPS	2.6 ± 0.8	−	−	−

[a] See Table 1 for sequence, nature, and position of modification incorporated. [b] At 0.3 μg/mL concentration of CpG DNA. [c] Cytokine secretion in 24 h cell cultures at 1.0 μg/mL concentration of CpG DNA and [d]at 5 mg/kg dose, spleen weights measured after 48 h, and each value is an average of three or four mice.

cantly lower immunostimulatory activity. As the linker is moved away from the CpG dinucleotide (**13** and **17**), an increased immunostimulatory activity was observed, as determined in splenomegaly assays (Table 2). However, in spleen cell culture assays, substitution in the 5′-flanking sequence (**13**) caused an increase in spleen cell proliferation and IL-12 and IL-6 secretion (Table 2). CpG DNA **17**, which had the same modification substituted in the 3′-flanking sequence, caused a spleen cell proliferation and IL-12 and IL-6 secretion similar to that of the parent CpG DNA **10** (Table 2). The results of CpG DNAs **14**, **15**, and **18** suggested that substitution of two linkers did not result in an additive effect on immunostimulatory activity. More importantly, either single- or double-linker substitutions away from the CpG dinucleotide did not neutralize the immunostimulatory activity (Table 2). These results suggest that a nucleobase and a sugar ring are not required in the flanking sequences at least four to seven nucleotides away from the CpG dinucleotide for immunostimulatory activity. The data in Table 2 suggest that certain components in the CpG DNA are not required for its

recognition by the receptors. In fact, the deletion of nucleobases as well as sugar rings at these nucleoside positions facilitates better recognition and/or interaction of modified CpG DNA by receptors and thereby potentiates immunostimulatory activity.

To examine if maintenance of proper interphosphate distance is required for immunostimulatory activity, ethyleneglycol-linkers (**6**, **7**) were incorporated in CpG DNA (**19−22**), and these modified CpG DNAs were examined for their immunostimulatory activity. The data presented in Table 3 (also see Supplementary Table 2 for concentration-dependent cytokine data) suggest that the substitution of either a tri- (**6**) or hexa- (**7**) ethyleneglycol-linker did not have a detrimental effect on the immunostimulatory activity of CpG DNA. However, substitution in the 5′-flanking sequence resulted in potentiation of the immunostimulatory activity compared with substitutions in the 3′-flanking sequence. Taken together, the results of in vitro and in vivo studies suggest that the substitution in the 3′-flanking sequence has a minimal effect on immunostimulatory activity compared with substitutions in the 5′-flanking sequence.

Table 3. Immunostimulatory Activity of CpG DNAs in BALB/c Mouse Spleen Cell Cultures and Splenomegaly in BALB/c Mice

CpG DNA number[a]	Cytokine, pg/mL[b]		spleen weight (mg)[c]
	IL-12	IL-6	
10	1391 ± 179	6644 ± 380	130 ± 8
19	2071 ± 44	15640 ± 887	275 ± 13
20	1189 ± 119	3921 ± 268	155 ± 9
21	1518 ± 148	14520 ± 825	263 ± 19
22	1151 ± 386	4046 ± 721	164 ± 22
M/V	85 ± 23	59 ± 16	106 ± 17

[a] See Table 1 for sequence, nature, and position of modification incorporated. [b] Cytokine secretion in 24 h cell cultures at 1.0 μg/mL concentration of CpG DNA and [c]at 5 mg/kg dose, and each value is an average of three or four mice.

Table 4. Splenomegaly Induced by CpG DNA Containing a C3-Alkyl-Linker in BALB/c Mice

CpG DNA number[a]	spleen weight (mg)[b]
23	121 ± 16
26	120 ± 14
27	228 ± 19
28	248 ± 20
vehicle	102 ± 8

[a] See Table 1 for sequences and position of modification. [b] Average spleen weight for four mice at a dose of 5 mg/kg.

Effect of C2−C9-Linker Substitutions in CpG DNA. To examine the effect of the length of an alkyl-linker on immunostimulatory activity, linkers of different carbon chain lengths, C2 to C9 (**1**−**5**), were incorporated in parent CpG DNA **23**, which had a "GACGTT" hexameric sequence, and these compounds were tested for their immunostimulatory activity. Since the preceding experiments had shown that incorporation of a C3-linker in the 5′-flanking sequence had a greater effect than it did in the other positions, we incorporated linkers **1**−**5** only in the 5′-flanking sequence to the CpG dinucleotide as shown in Table 1 to determine the optimal linker for potentiation of immunostimulatory activity.

The data from the splenomegaly assay for CpG DNAs **26**−**28** containing a C3-linker (**2**) at different nucleotide positions in the 5′-flanking sequence to the CpG dinucleotide are shown in Table 4. The parent CpG DNA **23** at a dose of 5 mg/kg caused an increase of about 21% in spleen weight compared with vehicle (PBS) control. At the same dose, CpG DNA **26**, which had a C3-linker adjacent to CpG dinucleotide in the 5′-flanking sequence, caused spleen enlargement similar to that caused by the parent CpG DNA. In contrast, a C3-linker placed two or five nucleotides away from CpG dinucleotide in the 5′-flanking sequence (CpG DNAs **27** and **28**) caused extensive splenomegaly (Table 4). These results are in agreement with the results observed with CpG DNAs **12** and **13**.

To evaluate the optimal linker size for potentiation of immunostimulatory activity, we measured IL-12 and IL-6 secretion induced by modified CpG DNAs in BALB/c mouse spleen cell cultures. All CpG DNAs induced concentration-dependent IL-12 and IL-6 secretion. Figure 3 shows data obtained at 1 μg/mL concentration of selected CpG DNAs, **25**, **28**, **35**, **39**, and **43**, which had a linker at the fifth nucleotide position in the 5′-flanking sequence to the CpG dinucleotide compared with the parent CpG DNA. The CpG DNAs, which contained C2- (**1**), C3- (**2**), and C4-linkers (**3**), induced
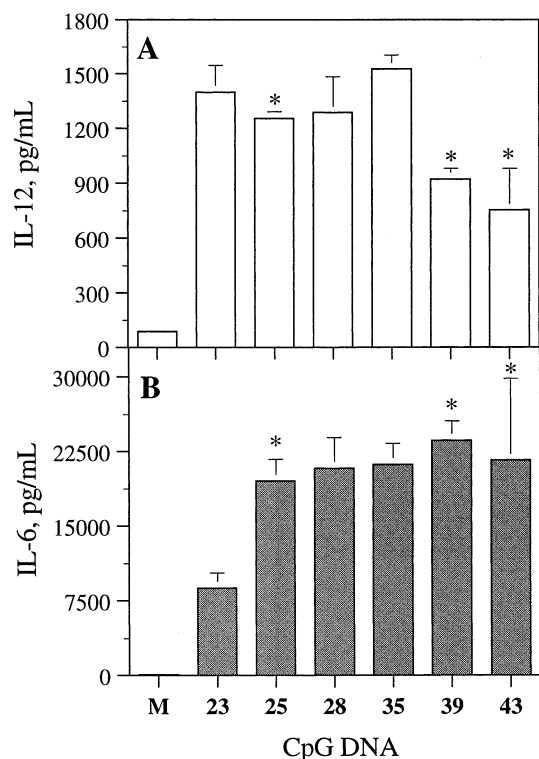

Figure 3. Cytokine (IL-12 and IL-6) secretion induced by CpG DNAs with a "GACGTT" hexameric motif containing a C2- (**25**), C3- (**28**), C4- (**35**) C6- (**39**), or C9-linker (**43**). M stands for media control. BALB/c mice spleen cell cultures were incubated with 1.0 μg/mL concentration of CpG DNAs for 24 h, and the levels of cytokines IL-12 (A) and IL-6 (B) secreted were measured as described in the Experimental Section. Each value is an average of three replicate samples. * indicates $p < 0.05$.

secretion of IL-12 production similar to that of the parent CpG DNA **23**. The CpG DNA that contained C6 and C9-linkers (**4** and **5**) at the fifth nucleotide position from CpG dinucleotide in the 5′-flanking sequence induced lower levels of IL-12 secretion than did the parent CpG DNA (Figure 3A), suggesting that substitution of linkers longer than a C4-linker results in the induction of lower levels of IL-12. All five CpG DNAs, which had linkers, induced 2−3 times higher IL-6 secretion than did the parent CpG DNA. The presence of a linker in these CpG DNAs showed a significant effect on the induction of IL-6 compared with CpG DNAs that did not have a linker. However, we did not observe length-dependent linker effect on IL-6 secretion.

Effect on Immunostimulatory Activity of CpG DNA Containing Ethylenegylcol-Linkers. We synthesized CpG DNAs **46** and **47**, in which a triethyleneglycol-linker (**6**) is incorporated at the fifth nucleotide position in the 5′- and at the fourth nucleotide position in the 3′-flanking sequences to the CpG dinucleotide, respectively. Similarly, CpG DNAs **48** and **49** contained a hexaethyleneglycol-linker (**7**) in the 5′- or the 3′-flanking sequence to the CpG dinucleotide, respectively. All four modified CpG DNAs (**46**−**49**) were tested in BALB/c mouse spleen cell cultures for cytokine induction (IL-12, IL-6, and IL-10) in comparison with parent CpG DNA **23**. All CpG DNAs induced concentration-dependent cytokine production over the concentration range tested (0.03−10.0 μg/mL) (Supplementary Table 3). The levels of cytokines induced at 0.3 μg/mL con-

Table 5. Cytokine Secretion Induced by CpG DNAs Containing an Ethyleneglycol-Linker in BALB/c Mice Spleen Cell Cultures

CpG DNA number[a]	cytokine, pg/mL[b]		
	IL-12	IL-6	IL-10
23	1887 ± 233	2130 ± 221	86 ± 14
46	2106 ± 143	2362 ± 166	78 ± 21
47	1888 ± 259	1082 ± 25	47 ± 14
48	2066 ± 153	2507 ± 66	73 ± 17
49	1318 ± 162	476 ± 13	25 ± 5
Medium	84 ± 13	33 ± 6	2 ± 1

[a] See Table 1 for sequence, nature, and position of modification incorporated. [b] Cytokine secretion in 48 h cell cultures at a concentration of 0.3 µg/mL of CpG DNA.

Table 6. Spleen Cell Proliferation Induced by CpG DNA Containing an Aminobutyryl Propanediol-Linker in BALB/c Mice Spleen Cell Cultures and Splenomegaly in BALB/c Mice

CpG DNA number[a]	spleen cell proliferation (PI)[b]	spleen weight (mg)[c]
23	3.7 ± 0.8	121 ± 16
59	2.5 ± 0.6	107 ± 11
60	9.2 ± 0.7	169 ± 16
61	8.8 ± 0.4	220 ± 8
63	7.8 ± 0.04	177 ± 12
M/V	1.2 ± 0.3	102 ± 8
LPS	2.8 ± 0.5	ND

[a] See Table 1 for sequences and position of modification. [b] At 0.1 µg/mL concentration in 48 h cell cultures. [c] Average spleen weight for four mice at a dose of 5 mg/kg; ND stands for not determined.

centration of CpG DNAs **46**–**49** are shown in Table 5. CpG DNAs **46** and **48**, which had an ethyleneglycol-linker in the 5′-flanking sequence, induced higher levels of IL-12 (2106 ± 143 and 2066 ± 153 pg/mL) and IL-6 (2362 ± 166 and 2507 ± 66 pg/mL) secretion than did parent CpG DNA **23** (Table 5). At the same concentration, **46** and **48** induced slightly lower levels of IL-10 secretion than did the parent CpG DNA (Table 5). CpG DNA **47**, which had a shorter ethyleneglycol-linker (**6**) in the 3′-flanking sequence, induced IL-12 secretion similar to that of the parent CpG DNA, but significantly lower levels of IL-6 and IL-10 (Table 5). CpG DNA **49**, which had a longer ethyleneglycol-linker (**7**), induced significantly lower levels of all three cytokines tested compared with the parent CpG DNA (Table 5).

Though triethyleneglycol-linker (**6**) had a chain length similar to that of C9-linker (**5**), the CpG DNA containing triethyleneglycol-linker had better immunostimulatory activity than did CpG DNA containing C9-linker, as determined by induction of cytokine secretion in spleen cell cultures. These results suggest that the lower immunostimulatory activity observed with CpG DNA containing longer alkyl-linkers (**4** and **5**) may not be related to their increased length but to their hydrophobic characteristics. This observation prompted us to examine substitution of branched alkyl-linkers containing hydrophilic functional groups on immunostimulatory activity.

Effect on Immunostimulatory Activity of CpG DNA Containing Branched Alkyl-Linkers. Two branched alkyl-linkers containing a hydroxyl (**8**) or an amine (**9**) functional group were incorporated in parent CpG DNA **23**, and the effects on immunostimulatory activity of the resulting modified CpG DNAs (**50**–**63**, Table 1) were examined. The data obtained with CpG DNAs **59**–**63**, containing amino-linker **9** at different nucleotide positions, in BALB/c mouse spleen cell cultures (proliferation) and in vivo (splenomegaly) are shown in Table 6.

Parent CpG DNA **23** showed a proliferation index of 3.7 ± 0.8 at a concentration of 0.1 µg/mL. At the same concentration, modified CpG DNAs **60**–**63** containing amino-linker **9** at different positions caused higher spleen cell proliferation than did the parent CpG DNA (Table 6). As observed with other linkers, when the substitution was placed adjacent to CpG dinucleotide (**59**), a lower proliferation index was noted compared with parent CpG DNA (Table 6), further confirming that the placement of a linker substitution adjacent to CpG dinucleotide has a detrimental effect on immunostimulatory activity. In general, substitution of an amino-

linker for 2′-deoxyribonucleoside in the 5′-flanking sequence (**60** and **61**) resulted in higher spleen cell proliferation than found with the substitution in the 3′-flanking sequence (**62** and **63**). Similar results were observed in the splenomegaly assay (Table 6), confirming the results observed in spleen cell cultures. Modified CpG DNAs containing glycerol-linker (**8**) showed immunostimulatory activity similar to or slightly higher than that observed with modified CpG DNA containing amino-linker (**9**) (data not shown).

To compare the immunostimulatory effects of CpG DNA containing linkers **8** and **9**, we selected CpG DNAs **54** and **61**, which had substitution in the 5′-flanking sequence and assayed their ability to induce cytokines IL-12 and IL-6 secretion in BALB/c mouse spleen cell cultures. Both CpG DNAs **54** and **61** induced concentration-dependent cytokine secretion. Figure 4 shows the levels of IL-12 and IL-6 induced by **54** and **61** in mouse spleen cell cultures at 0.3 µg/mL concentration compared with parent CpG DNA **23**. Both CpG DNAs induced higher levels of IL-12 and IL-6 than did parent CpG DNA **23**. CpG DNA containing glycerol-linker (**8**) induced slightly higher levels of cytokines (especially IL-12) than did CpG DNA containing amino-linker (**9**) (Figure 4). These results further confirm that the linkers containing hydrophilic groups are more favorable for the immunostimulatory activity of CpG DNA.

Effect on Immunostimulatory Activity of CpG DNA Containing Multiple Linker Substitutions. We examined two different aspects of multiple linker substitutions in CpG DNA. In one set of experiments, we kept the length of nucleotide sequence to 13-mer and incorporated one to five C3-linker (**2**) substitutions at the 5′-end (**29**–**33**). These modified CpG DNAs permitted us to study the effect of an increase in the length of linkers without causing solubility problems. In the second set of experiments, we incorporated two of the same linker substitutions (**3**, **4**, or **5**) in adjacent positions in the 5′-flanking sequence to the CpG dinucleotide to study if there would be any additive effect on immunostimulatory activity.

Modified CpG DNAs were studied for their ability to induce cytokine production in BALB/c mouse spleen cell cultures in comparison with parent CpG DNA **23**. All CpG DNAs induced concentration-dependent cytokine production. The data obtained at 1.0 µg/mL concentration of CpG DNAs is shown in Table 7. In this assay, parent CpG DNA **23** induced 967 ± 28 pg/mL of IL-12, 1593 ± 94 pg/mL of IL-6, and 14 ± 6 pg/mL of IL-10 secretion at 1.0 µg/mL of concentration. The data pre-
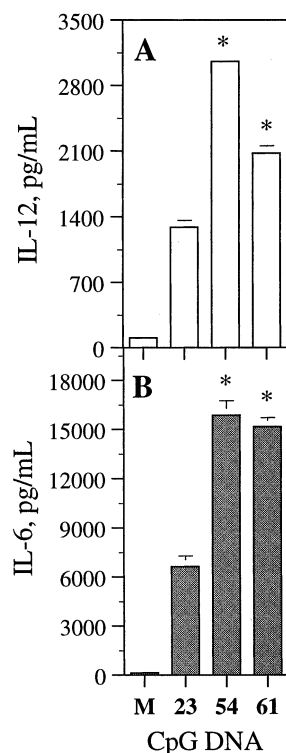
Figure 4. Cytokine (IL-12 and IL-6) secretion induced by CpG DNAs with a "GACGTT" hexameric motif containing a glyceryl- (**54**) or amino-propanediol-linker (**61**). M stands for media control. BALB/c mice spleen cell cultures were incubated with 1.0 μg/mL concentration of CpG DNAs for 24 h, and the levels of cytokines IL-12 (A) and IL-6 (B) secreted were measured as described in the Experimental Section. Each value is an average of three replicate samples. * indicates $p < 0.05$.

Figure 5. Cytokine (IL-12 and IL-6) secretion induced by CpG DNAs with a "GACGTT" hexameric motif containing two C4- (**36**, **37**) C6- (**40**, **41**), or C9-linker (**44**, **45**). M stands for media control. BALB/c mice spleen cell cultures were incubated with 1.0 μg/mL concentration of CpG DNAs for 24 h, and the levels of IL-12 (A) and IL-6 (B) secreted were measured as described in the Experimental Section. Each value is an average of three replicate samples. * indicates $p < 0.05$.

Table 7. Cytokine Secretion Induced by CpG DNA Containing Multiple C3-Linkers in BALB/c Mouse Spleen Cell Cultures

CpG DNA number[a]	cytokine (pg/mL)[b]		
	IL-12	IL-6	IL-10
23	967 ± 28	1593 ± 94	14 ± 6
29	1424 ± 325	3293 ± 478	36 ± 6
30	2336 ± 344	2409 ± 134	28 ± 1
31	1886 ± 142	2194 ± 184	36 ± 2
32	744 ± 164	3359 ± 706	45 ± 1
33	633 ± 255	3309 ± 337	58 ± 19
medium	126 ± 18	67 ± 8	5 ± 1

[a] See Table 1 for sequences, nature, and position of modification.
[b] At a concentration of 1.0 μg/mL in 24 h cell culture.

sented in Table 7 suggest that as the number of linker substitutions decreased, IL-12 induction decreased. However, the induction of lower levels of IL-12 secretion by CpG DNAs **32** and **33** could be the result of the shorter length of CpG DNAs. Our studies with unmodified CpG DNA shorter than 15-nucleotides showed insignificant immunostimulatory activity (data not shown). Neither length nor the number of linker substitutions has a smaller effect on IL-6 secretion. Though IL-10 secretion increased with linker substitutions, the overall IL-10 secretion by these CpG DNAs was minimal.

CpG DNAs containing two linker substitutions (linker **3**−**36**; linker **4**−**40**; linker **5**−**44**) at the fourth and fifth positions in the 5′-flanking sequences to the CpG dinucleotide and the corresponding 5′-truncated versions **37**, **41**, and **45**, respectively, were tested for their ability to induce cytokine secretion in BALB/c mouse
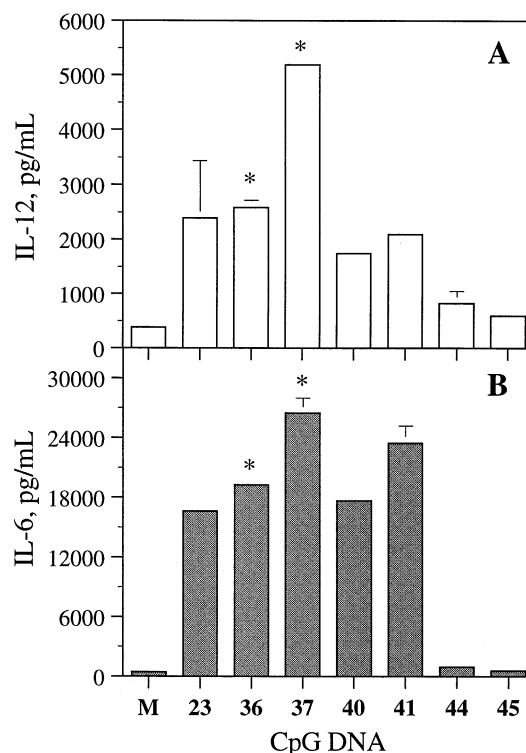
spleen cell cultures. The levels of IL-12 and IL-6 secreted at 1.0 μg/mL concentration are shown in Figure 5. The results presented in Figure 5 suggest that the immunostimulatory activity is dependent on the nature of the linker incorporated. The substitution of the fourth and fifth nucleosides with C4-linker **3** (CpG DNA **36**) had an insignificant effect on cytokine secretion compared with parent CpG DNA **23**, suggesting that the nucleobase and sugar ring at these positions are not required for receptor recognition and/or binding. The deletion of the nucleotides beyond the linker substitutions (CpG DNA **37**) caused higher IL-12 and IL-6 secretion than that found with CpG DNAs **23** and **36**. As expected, the substitution of two C6-linkers (**4**) resulted in IL-12 secretion lower than and IL-6 secretion similar to that induced by parent CpG DNA **23**. The 5′-truncated CpG DNA **41** induced higher cytokine secretion than did CpG DNA **40**. The CpG DNAs **44** and **45**, which had two C9-linkers (**5**), induced insignificant cytokine secretion, confirming the results obtained with monosubstituted CpG DNA containing the same linker as described above.

A number of first-generation CpG DNAs are currently being evaluated in clinical trials as monotherapies and in combination with vaccines against cancers and as adjuvants with vaccines and allergens for infectious diseases and allergies.[5,6,8,31,32] The current results indicate that the immunostimulatory activity of CpG DNAs can be modulated by incorporation of alkyl-linkers at appropriate positions in a CpG DNA molecule. These studies suggest that nucleotides in certain posi-

tions, especially in the 5′-flanking sequence to the CpG dinucleotide, are not significantly involved in recognition and/or interaction with the receptors. In fact, the deletion of a nucleoside at about three to six nucleotides away from the CpG dinucleotide in the 5′-flanking sequence potentiated immunostimulatory activity. The same modifications incorporated in the 3′-flanking sequence did not neutralize or increase the immunostimulatory activity compared with the parent CpG DNA, suggesting that perhaps only phosphate backbone, but not the sugar and nucleobase moieties, of the nucleotide portion involves in binding. In contrast, the CpG dinucleotide and the first two nucleotides adjacent to the CpG dinucleotide on both the 3′- and 5′-side play a critical role in immunostimulatory activity. Certain other modifications, however, are tolerated in the adjacent nucleotide positions to the CpG dinucleotide, as reported earlier.[24−26,28]

In another aspect, the linkers used in the study can be viewed as tethers that are joining two DNA fragments together. The lower immunostimulatory activity observed with shorter CpG DNAs (**33**) can be attained by attaching a short DNA fragment through the use of an appropriate tether (**28**). The results presented here with linker substitutions at the 5′-end of CpG DNA (**29**–**33**) suggest that these substitutions do not block the 5′-accessibility of CpG DNA for receptor recognition, in contrast to 5′-5′-linked CpG DNA.[26]

The CpG DNAs that contained C2-, C3-, and C4-linkers had similar immunostimulatory effects, suggesting that the length of the alkyl linker has an insignificant influence on recognition and/or binding of the modified CpG DNAs to the receptor(s). Similarly, tri- and hexa-ethyleneglycol-linkers also potentiated immunostimulatory activity when they were incorporated in the 5′-flanking sequence. However, the lower immunostimulatory activity observed with longer carbon linkers, such as C6 and C9, compared with shorter alkyl-linkers and long ethyleneglycol-linkers suggests that modifications that introduce increased hydrophobicity have adverse effect on immunostimulatory activity. It is important to note that within a CpG dinucleotide, a hydrophobic methyl substitution at the 5-position of cytosine neutralizes CpG-related immune stimulation[22] and a hydrophilic hydroxyl substitution at the same position retains immune stimulation.[27]

These studies suggest that while the presence of a CpG dinucleotide is critical for the observed immunostimulatory activity, modifications introduced in the 3′-flanking sequence have minimal effects on immunostimulatory activity. The introduction of nonnatural chemical modifications in oligonucleotides could contribute to increased nuclease stability and/or cellular uptake of oligonucleotides, which could result in altered immunostimulatory activity. In general, the 3′-exonucleases are mostly responsible for degradation of oligonucleotides in vivo. A number of studies have reported an increase in the in vivo stability of oligonucletides with appropriate chemical modifications incorporated at the 3′-end of the oligonucleotide.[33−36] In the present study, the alkyl-linker modifications incorporated toward the 3′-end should have imparted a relatively higher stability against nucleases than those incorporated toward the 5′-end; therefore, the former

should have had a relatively greater immunostimulatory activity. In contrast, the results presented here show that CpG DNAs that have alkyl-linker substitutions in the 5′-flanking sequence (at least three to six nucleotides away from the CpG dinucleotide) had greater immunostimulatory activity than did the CpG DNAs that had substitutions in the 3′-flanking sequence, These results suggest that the observed increase in immunostimulatory activity was not the result of increased nuclease stability, but the result of the structural modifications introduced in the CpG DNA.

In addition, modified CpG DNAs containing different alkyl-linkers may have slightly different cellular uptake properties, depending on the nature of the linker incorporated (for example alkyl-linkers vs ethyleneglycol-linkers). CpG DNAs that contained the same kind of linker at different nucleotide positions should have possessed similar cellular uptake properties (for example, a C3-linker). We observed in the experiments reported here, however, that the immunostimulatory activity varied depending on the position of substitution within the CpG DNA sequence. These results suggest that the observed differences in the immunostimulatory activity could be the result of change in the required sequence (modifications incorporated within and adjacent to the CpG dinucleotide) and/or structural changes caused because of the modifications introduced (substitutions incorporated in the 5′- and 3′-flanking sequences), but not as a result of different cellular uptake properties.

In conclusion, the substitution of certain nucleotides with alkyl-linkers in the 5′-flanking sequence to the CpG dinucleotide potentiated immunostimulatory activity. Interestingly, the same substitution in the 3′-flanking sequence did not affect immunostimulatory activity compared with parent CpG DNA. While a C3-linker optimally potentiated immunostimulatory activity, longer ethyleneglycol- and branched alkyl-linkers also showed increased immunostimulatory activity. The linker substitutions in the 5′-flanking sequence increased IL-6 secretion several fold over that seen with unmodified CpG DNA. The ability of modified CpG DNAs to induce IL-12 secretion similar to that of parent CpG DNA is sufficient to skew the resulting immune response toward Th1 phenotype; the secretion of higher levels of IL-6 could be of an advantage for maturation of B-cells and rapid onset of T-cell specific humoral immune responses, when they are used as adjuvants with vaccines, allergens, and mAbs. It is not clear whether the differences observed in the immunostimulatory activity of the modified CpG DNAs resulted from altered recognition/binding events with the receptor(s) or initiation of different upstream signaling and transcriptional events compared with the parent CpG DNA. The ongoing studies of modified CpG DNA with specific immune-cell lineages should help us to understand the molecular mechanisms of interactions in detail and enable us to further fine-tune the incorporation of modifications, eventually allowing the broad application of CpG DNAs as immunological tools and therapeutic agents.

Experimental Section

CpG DNA Synthesis and Purification. CpG DNA were synthesized using β-cyanoethylphosphoramidite chemistry on a PerSeptive Biosystem's 8909 Expedite DNA synthesizer on

1−2 μmol scale. The phosphoramidites of dA, dG, dC, and T were obtained from PE Biosystems. The DMT-protected phosphoramides of different linkers used in the study were obtained from Glen Research or ChemGenes. Beaucage's reagent was used as an oxidant to obtain phosphorothioate backbone modification.[37] 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 DNAs 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 molecular weights of representative CpG DNAs as determined by MS are (calculated; found): **10** (5658; 5660), **11** (5466; 5468), **12** (5482; 5484), **13** (5506; 5508), **14** (5331; 5333), **15** (5355; 5357), **16** (5491; 5493), **17** (5491; 5493), **18** (5340; 5342), **19** (5557; 5550), **21** (5589; 5584), **23** (5704; 5702), **24** (5539; 5626), **26** (5528; 5530), **27** (5537; 5539), **28** (5537; 5539), **34** (5567; 5565), **39** (5580; 5578), **43** (5622; 5620), **46** (5612; 5610), **48** (5744; 5740), **53** (5569; 5568), and **62** (5609; 5606). The purity of full length CpG DNAs ranged from 89−95%, as determined by CGE and the rest being n-1 and n-2. All CpG DNAs were synthesized and purified under identical conditions to minimize endotoxin contamination. In addition, CpG DNAs were tested for endotoxin contamination by Limulus assay (Bio-Whittaker) and endotoxin levels were <0.075 EU/mL.

Mouse Lymphocyte Proliferation Assay. Lymphocytes obtained from BALB/c mouse (4−8 weeks) spleens were cultured in RPMI complete medium as described earlier.[22,30] The cells were plated in 96-well dishes at a density of 10^6 cells/mL in a final volume of 100 μL. The CpG DNA, LPS (lipopolysaccharide, a positive control) (10 μg/mL), or medium was added to the cell culture in 10 μL of TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) at final concentrations of 0.1, 0.3, 1.0, and 3.0 μg/mL. The cells were then incubated at 37 °C. After 44 h, 1 μCi ^{3}H-uridine (Amersham) was added to the culture in 20 μL of RPMI medium, and the cells were pulse-labeled for another 4 h. The cells were harvested by automatic cell harvester, and the filters were counted by a scintillation counter. The experiments were performed two or three times for each CpG DNA in triplicate at each concentration. The averages were calculated, normalized, and presented as proliferation index.

Assays for IL-12, IL-6, and IL-10 Secretion in Mouse Spleen Cell Cultures. The secretion of IL-12, IL-6, and IL-10 in BALB/c mouse spleen cell cultures was measured by sandwich ELISA. The required reagents, including cytokine antibodies and cytokine standards, were purchased form PharMingen. ELISA plates (Costar) were incubated with appropriate antibodies at 5 μg/mL in PBSN buffer (PBS/0.05% sodium azide, pH 9.6) overnight at 4 °C and then blocked with PBS/10% FBS at 37 °C for 30 min. Cell culture supernatants and cytokine standards were appropriately diluted with PBS/10% FBS, added to the plates in triplicate, and incubated at 25 °C for 2 h. Plates were overlaid with 1 μg/mL appropriate biotinylated antibody and 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 Chromogenic reagent chromatin (Kirkegaard and Perry), and the color change was measured on a Ceres 900 HDI Spectrophotometer (Bio-Tek Instruments). The levels of IL-12, IL-6, and IL-10 in the cell culture supernatants were calculated from the standard curve constructed under the same experimental conditions for IL-12, IL-6, and IL-10, respectively.

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

Statistical Analysis. All results are presented as mean ± SD. The unpaired Student's t-test was used to determine the statistical significance of representative CpG DNAs in cytokine assays for any difference in immunostimulatory activity between the modified and parent CpG DNAs (Statpak, v 4.1).

Supporting Information Available: Tables of immunostimulatory activity and cytokine secretion. This material is available free of charge via the Internet at http://pubs.acs.org.

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