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Requirement of Nucleobase Proximal to CpG Dinucleotide for Immunostimulatory Activity of Synthetic CpG DNA

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Abstract—Synthetic oligodeoxyribonucleotides containing CpG dinucleotides exhibit potent immunostimulatory activity in vertebrates. Although the molecular mechanisms of recognition and interaction of CpG DNA sequences with receptors are not well understood, the current evidence suggests that the receptor shows considerable selectivity for CpG DNA sequences with different preferences in mouse (GACGTT) and human (GTCGTT) species. In our continued effort to understand the chemical and structural characteristics of CpG DNA required for the immunostimulatory activity and thereby for the recognition of receptors in the immunostimulatory pathway, we examined the requirement of nucleobases in the two adjacent nucleotide positions on the 5′- and the 3′-side to the CpG dinucleotide ($P_1P_2CGP_3P_4$) for the immunostimulatory activity. These studies, in which a natural nucleoside is substituted with an abasic nucleoside (X), suggest that a nucleobase is absolutely required in C, G, P_3, and P_4 positions for immunostimulatory activity. Surprisingly, an abasic nucleoside is permitted at either P_1 or P_2 depending on the neighboring base. It was found that 'GXCGTT' motif has an intermediate immunostimulatory activity between those of 'GACGTT' and 'GTCGTT' in the mouse cells.

Introduction

Mammalian immune system recognizes synthetic oligodeoxyribonucleotides and bacterial DNA containing CpG dinucleotides in specific sequence contexts (CpG DNA).[1–4] The immune responses to CpG DNA include stimulation of B cell proliferation, activation of macrophages, monocytes, and dendritic cells.[1–6] The activation of immune cells by CpG DNA results in the expression of several co-stimulatory molecules and secretion of a number of cytokines including IL-12, IFN-γ, IL-6 and TNF-α.[1–6] The use of CpG DNAs as antitumor, antiviral, antibacterial, and anti-asthmatic agents and as adjuvants in immunotherapy has been reported.[7–13]

Our laboratory has been studying the effects of sequence and structural changes in CpG DNA that potentiate or neutralize their immunostimulatory activity. We have shown that modifications changing the secondary structure or conformation of oligonucleotides result in altered immune recognition or activation.[14] These studies include the effects of chemical modifications in phosphate backbone,[14–16] sugar[14,17–19] and nucleobase,[20] deletion of nucleobases[21] or nucleotides,[22] and the requirement of an accessible 5′-end[23,24] for immunostimulatory activity.

While the presence of a CpG dinucleotide is essential for immunostimulatory activity, the flanking sequences also play a critical role.[1–6] Optimal immunostimulatory sequences vary from species to species. For instance, a 'GACGTT' hexameric sequence activates mouse immune system. Human immune cells, however, respond poorly to this sequence and prefer instead 'GTCGTT', 'TTCGTT', or 'AACGTT' sequence.

In general, the common immunostimulatory sequence that could elicit activity in different vertebrate species, at least in rodent and human systems, is $P_1P_2CGT(P_3)T(P_4)$, where (P_1P_2) are d(GA), d(AA), d(GT), or (TT) with variations from species to species. P_3 and P_4 are generally natural 2′-deoxypyrimidine nucleosides, preferably thymidines. Several lines of evidence suggest that any chemical modifications introduced within the CpG dinucleotide or proximal to the

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CpG dinucleotide severely impair the immunostimulatory activity of CpG DNA.[14,15,17,19–22] Recently, we have shown that deletion of one or two nucleobases distal to the CpG dinucleotide at certain positions in the 3′-flanking sequence has an insignificant effect on immunostimulatory activity, while the same deletions in the 5′-flanking sequence increased activity compared with unmodified parent CpG DNA.[21] To determine the effect on immune stimulation of deleting nucleobases at either P_1–P_4 positions, we synthesized a series of oligodeoxyribonucleotides with an abasic linker (d-spacer) at these positions and studied for immunostimulatory activity.

5′-NNNNNNP_1P_2CGP_3P_4NNNNNN-3′

1 5′-CCTACTAGCGTTCTCATC-3′
2 5′-CTATCTGACGTTCTCTGT-3′
3 5′-CTATCTGTCGTTCTCTGT-3′
4 5′-CTCACTTTCGTTCTCTGT-3′

5 5′-CTATCT**X**ACGTTCTCTGT-3′
6 5′-CTATCT**X**CCGTTCTCTGT-3′
7 5′-CTATCT**X**GCGTTCTCTGT-3′
8 5′-CTATCT**X**TCGTTCTCTGT-3′

9 5′-CTATCTA**X**CGTTCTCTGT-3′
10 5′-CTATCTC**X**CGTTCTCTGT-3′
11 5′-CTATCTG**X**CGTTCTCTGT-3′
12 5′-CTATCTT**X**CGTTCTCTGT-3′

13 5′-CCTACTAGCG**X**TCTCATC-3′
14 5′-CCTACTAGCGT**X**CTCATC-3′
15 5′-CTATCTGACG**X**TCTCTGT-3′
16 5′-CCTACTAGC**X**TTCTCATC-3′
17 5′-CTATCTGA**X**GTTCTCTGT-3′

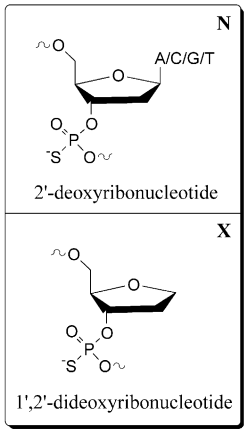

Figure 1. Structure of deoxyribonucleotide phosphorothioates (N) and 1′,2′-dideoxyribonucleotide phosphorothioate or abasic linker (**X**). A general sequence showing designation of positions 1–4 proximal to the CpG dinucleotide at the top of the sequence listing. Sequences **1–4** are parent CpG DNAs without any modifications containing different hexameric immunostimulatory sequences (underlined). The abasic linker (**X**) substitution in CpG DNAs **5–17** is shown in bold.

Results and Discussion

Here, we report immunostimulatory activities for all eight possible variations at positions P_1 and P_2 having one abasic nucleoside in combination with a natural deoxynucleotide (CpG DNAs **5–12** in Fig. 1). Abasic substitutions were also made at P_3 and P_4 positions (CpG DNAs **13–15** in Fig. 1). These were compared with sequences containing the active hexameric motifs, 'AGCGTT', 'GACGTT', 'GTCGTT', and 'TTCGTT' (CpG DNAs **1–4**, Fig. 1).

Immunostimulatory activity of CpG DNAs

CpG DNAs **1–4** were tested for their ability to induce cytokines (IL-12 and IL-6) in spleen cell cultures from two different mice, BALB/c and C57BL/6. All four CpG DNAs produced a concentration-dependent secretion of both cytokines IL-12 and IL-6. Results of mouse spleen cell cultures at 1.0 (BALB/c) and 3.0 (C57BL/6) μg/mL concentrations are shown in Table 1. As expected, mouse specific motif containing CpG DNA **2** induced highest IL-12 and IL-6 secretion in both cell cultures. In general, CpG DNAs **1**, **3** and **4** induced more IL-12 than IL-6 in BALB/c mouse spleen cell cultures. However, in C57BL/6 mouse spleen cell cultures, all four CpG DNAs induced a more IL-6 secretion than IL-12.

Oligonucleotides containing CpG dinucleotides induce splenomegaly in mice that is considered a result of immune stimulation.[25,26] To further confirm the immunostimulatory activity in vivo, CpG DNAs **1–4** were injected intraperitoneally to BALB/c mice at 5 mg/kg

Table 1. Effect of CpG DNAs on cytokine secretion in mouse spleen cell cultures and spleen enlargement in vivo

CpG DNA	BALB/c			C57BL/6	
Number	Spleen Wt. (mg)[b]	IL-12 (pg/mL)[c]	IL-6 (pg/mL)[c]	IL-12 (pg/mL)[d]	IL-6 (pg/mL)[d]
1	169±15	1515±242	1375±294	1902±147	14775±378
2	188±13	4083±158	6432±1348	1974±145	17028±1005
3	148±12	1468±254	655±145	1170±83	6157±249
4	157±5	1350±470	106±6	980±92	7576±498
5	ND	4487±771	6472±170	1613±90	12176±268
6	ND	46±22	176±45	1139±65	11944±603
7	ND	4984±1162	7876±1415	2106±123	14737±380
8	ND	2605±686	3455±651	947±97	7481±330
9	84±13	2300±357	2895±711	984±33	8143±112
10	133±23	1147±314	853±184	738±136	5441±456
11	171±10	4020±633	4409±1226	1271±57	10523±525
12	128±11	1406±282	648±37	532±28	2963±50
13	98±15	22±2	77±8	ND	ND
14	109±7	21±1	16±4	ND	ND
15	105±14	38±2	21±2	ND	ND
16[a]	ND	215±24	8±0.01	ND	ND
17[a]	88±5	20±1	19±1	ND	ND
Medium	86±6	66±12	21±2	96±12	128±17

[a]From ref 20.
[b]Average spleen weight of three or four mice after 72 h of administration of CpG DNA at a dose of 5 mg/kg; ND; not determined.
[c]At a concentration of 1.0 μg/mL of CpG DNA.
[d]At a concentration of 3.0 μg/mL of CpG DNA.

and changes in spleen weights were determined 72 h later (Table 1). Spleen weights were increased about 69, 88, 48, and 57% by CpG DNAs **1**–**4**, respectively, compared with the control mice that were treated with vehicle (PBS). These data from in vivo studies again show that CpG DNAs **1** and **2** are more immunostimulatory than CpG DNAs **3** and **4**.

Together, these results show that all four CpG DNAs induced immunostimulatory activity in BALB/c and C57BL/6 mice that is dependent on the hexameric sequence and also the mouse strain. The 'GACGTT' motif in **2** is more potent than 'AGCGTT' motif in **1** in both BALB/c and C57BL/6 mice. As expected, the two CpG DNAs **3** and **4**, containing 'GTCGTT' and 'TTCGTT' sequences, which are known to be selectively recognized by human cells, were less active in these assays. Earlier, we found that CpG DNAs **16** and **17**, having abasic linkers in place of one or other of the CpG nucleotides did not induce splenocyte proliferation or cytokine secretion (Table 1) suggesting that a nucleobase is required within the CpG dinucleotide for immunostimulatory activity.[21]

Effect of deletion of P_3 or P_4 nucleobase

Several of the potent immunostimulatory sequences contain two thymidines following the CpG dinucleotide.[4–6] To explore the need for the thymine bases in hexameric sequences 'AGCGTT' and 'GACGTT', we replaced P_3 or P_4 with an abasic linker in CpG DNAs **13**–**15** (Fig. 1). These CpG DNAs showed little or no immunostimulatory activity in cytokine secretion assays and spleen enlargement in BALB/c mice (Table 1). These results suggest that a nucleobase is required in both the positions for immunostimulatory activity.

Effect of deletion of P_1 nucleobase

After confirming that nucleobases at P_3 and P_4 positions are absolutely required for immunostimulatory activity, we examined the effect of replacing P_1 with an abasic linker. We synthesized four CpG DNAs (**5**–**8**) containing an abasic linker at P_1 followed by A, C, G, or T base in position P_2. Induction of cytokines by CpG DNAs **5**–**8** was determined in BALB/c mouse and C57BL/6 mouse spleen cell cultures (Table 1). CpG DNAs **5**, **7** and **8**, which have A, G, and T, respectively, at P_2, showed a concentration-dependent stimulation of IL-12 and IL-6 production (data not shown). CpG DNA **6**, which has cytosine at position P_2, induced IL-12 and IL-6 secretion just above background level in BALB/c spleen cell culture assays (Table 1). In C57BL/6 spleen cell cultures, CpG DNA **6** induced slightly higher levels of IL-12 and IL-6 than did CpG DNA **8** that has the 'XTCGTT' sequence (Table 1). These findings with CpG DNA **6** are consistent with the earlier reports that a CpG dinucleotide preceded by a cytosine is not a potent immunostimulator.[4] CpG DNA **5**, with hexameric sequence XACGTT, induced levels of IL-12 and IL-6 that were similar to or slightly lower than those found with the parent sequence containing 'GACGTT' (CpG DNA **2**) in BALB/c or C57BL/6 spleen cells, respectively (Figs 2 and 3). In a similar comparison, hexameric sequence 'XTCGTT' in CpG DNA **8** produced slightly higher or similar levels of both IL-12 and IL-6 in BALB/c and C57BL/6 spleen cell cultures, respectively, when compared with the parent CpG DNA **3**, with a 'GTCGTT' hexameric sequence (Figs 2 and 3).

Effect of deletion of P_2 nucleobase

Next we assessed the effect of an abasic linker at P_2 with dA, dC, dG, or T at P_1 (CpG DNAs **9**–**12**) on immunostimulation. In BALB/c mouse spleen cell cultures, all four CpG DNAs (**9**–**12**) induced a concentration-dependent IL-12 and IL-6 production (data not shown). Representative data are shown in Table 1 and Figures 4 and 5. Again, the effect varied depending on the nucleobase present at position P_1. CpG DNA **11**, with G at position P_1, induced the highest levels of cytokines. Compared with the parent CpG DNA **2**, which has a 'GACGTT' hexameric sequence, **11** induced slightly lower levels of IL-12 at 0.1 and 0.3 μg/mL concentration. However, at higher concentrations, **11** induced equal or

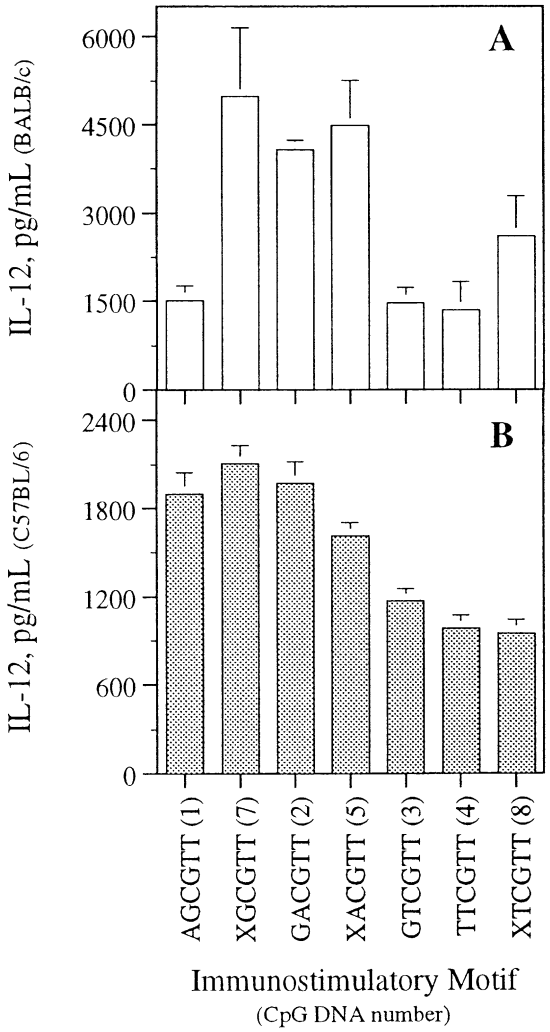

Figure 2. Comparison of effect of abasic linker (**X**) at P_1 position of CpG DNAs on secretion of IL-12 in BALB/c (A) and C57BL/6 (B) mice spleen cell cultures at 1.0 or 3.0 μg/mL concentration, respectively.

higher production of IL-12 compared with parent CpG DNA **2** (Fig. 4). Compared with parent CpG DNA **3**, which has a 'GTCGTT' hexameric sequence, CpG DNA **11** showed a potent induction of IL-12 and IL-6 production (Figs 4 and 5).

To further confirm the immunostimulatory activity in vivo, CpG DNAs **9**–**12** were intraperitoneally injected to BALB/c mice at a dose of 5 mg/kg and spleen weights were determined 72 h later. Average spleen weights are shown in Table 1. Except CpG DNA **9**, which has an 'AXCGTT' sequence, the other three CpG DNAs induced potent immunostimulatory activity compared to controls.

Spleen cells consist of different subsets of cell population than peripheral blood mononuclear cells (PBMCs). To examine, if this difference in cell population could result in different activity, we isolated PBMCs from BALB/c mouse blood and tested for the ability of P_2 substituted CpG DNAs to induce IL-12 and IL-6 secretion. The results are presented in Table 2. Again CpG DNA **11** showed greater activity compared with other three CpG DNAs containing abasic linker at P_2. CpG DNA **9** induced higher IL-12 secretion but lower level of IL-6 compared with parent CpG DNA **3**. These data further confirm the results of spleen cell culture assays.

The Toll-like receptor 9, TLR9, has been identified as the receptor that recognizes CpG DNA.[27] As yet there

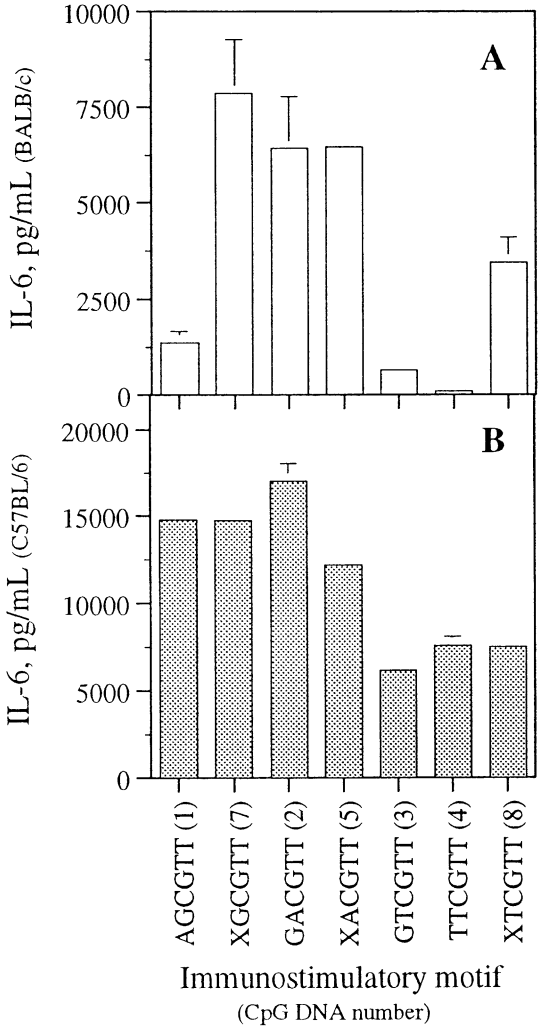

Figure 3. Comparison of effect of abasic linker (**X**) at P_1 position of CpG DNAs on secretion of IL-6 in BALB/c (A) and C57BL/6 (B) mice spleen cell cultures at 1.0 or 3.0 μg/mL concentration, respectively.

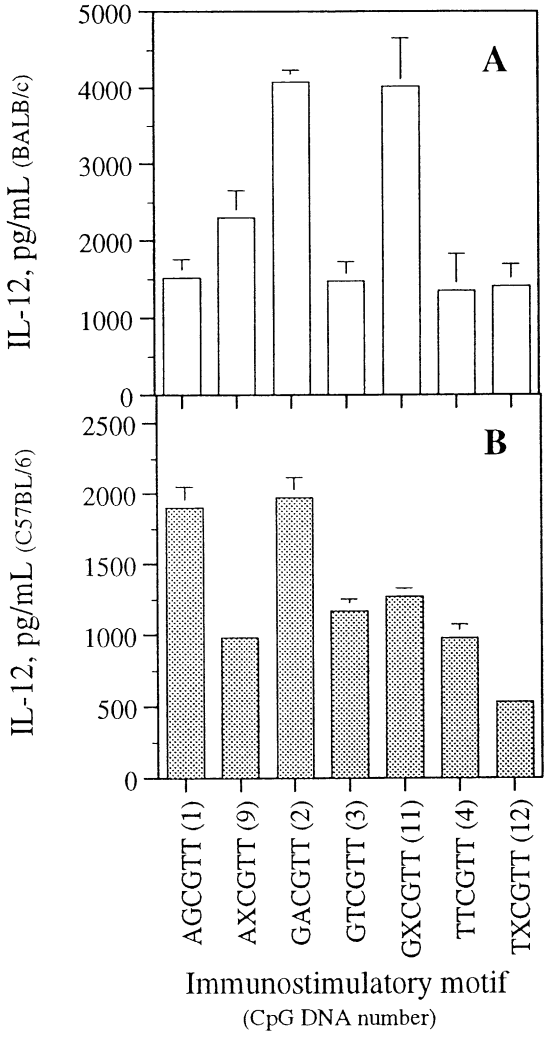

Figure 4. Effect of abasic linker (**X**) at P_2 position of CpG DNAs on secretion of IL-12 in BALB/c (A) and C57BL/6 (B) mice spleen cell cultures at 1.0 or 3.0 μg/mL concentration, respectively.

Table 2. CpG DNA induced cytokine secretion in BALB/c mouse PBMC cultures[a]

CpG DNA	IL-12 (pg/mL)[b]		IL-6 (pg/mL)[b]	
Number	3 μg/mL	10 μg/mL	3 μg/mL	10 μg/mL
2	522±26	599±40	278±28	251±8
3	86±13	140±33	53±6	190±3
9	101±4	162±3	42±2	52±6
10	23±3	47±5	27±11	44±34
11	209±40	488±23	103±25	177±13
12	27±0.3	108±12	14±2	27±6
Medium	ND		ND	

[a]See Figure 1 for sequences and position of modification.
[b]After 24 h incubation with CpG DNA; ND: not detectable.

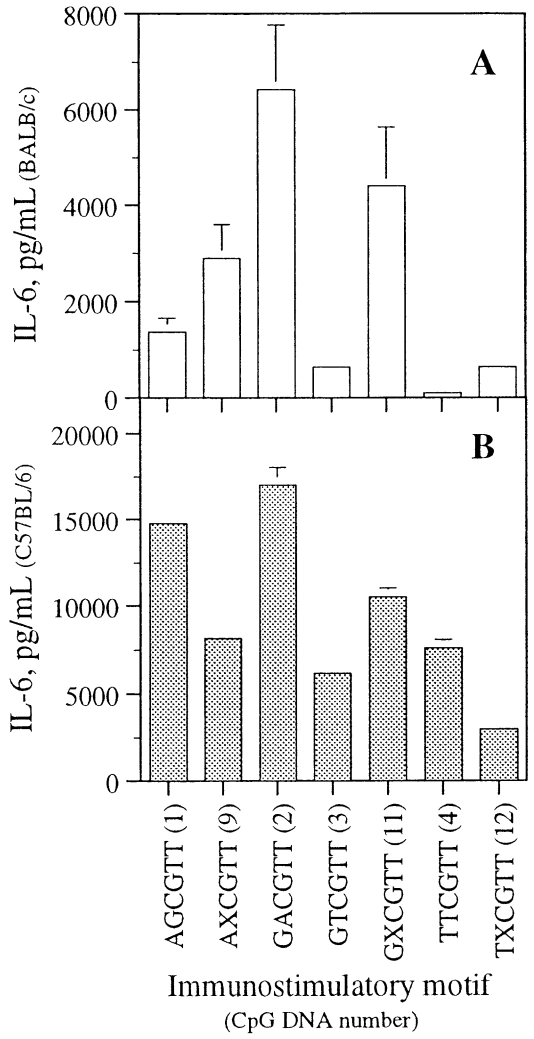

Figure 5. Effect of abasic linker (**X**) at P_2 position of CpG DNAs on secretion of IL-6 in BALB/c (A) and C57BL/6 (B) mice spleen cell cultures at 1.0 or 3.0 μg/mL concentration, respectively.

have been no reports elucidating the chemical contacts between TLR9 and CpG DNA. There is growing evidence that TLR9 proteins from immune cells of different species exhibit considerable variability in their sequence specificity.[27–31] The presence of a CpG dinucleotide, however, is absolutely required for immunostimulatory activity of CpG DNA. Deleting either cytosine or guanine from the CpG dinucleotide results in complete loss of immunostimulatory activity, suggesting that a CpG dinucleotide in certain sequence contexts is specifically recognized by the receptors. Our recent studies have identified important functional groups on cytosine and guanine that may be involved in receptor recognition.[20] The present studies also confirm that the CpG dinucleotide is required for immunostimulatory activity.

Our results of the mouse spleen cell assays indicate that the immunostimulatory activity of the same CpG DNA varied between the two mouse strains, BALB/c and C57BL/6 as had been reported with other CpG DNAs by us and others.[32,33] In general, the BALB/c strain showed higher IL-12 secretion in response to CpG DNA compared with the C57BL/6 strain. The reverse was true for IL-6 secretion. The differences in the cytokine secretion profiles induced by CpG DNAs in these two strains could be due to differences in the recognition of CpG DNAs by TLR9 receptor or the subsequent signaling events initiated by TLR9.

Taken together, the present results suggest that the bases proximal to the CpG dinucleotide, especially two thymidines at P_3 and P_4, play a critical role in immune stimulation. The substitution of either of these thymidines with the abasic linker severely impairs activity and it appears that a nucleobase is absolutely required at these positions. In contrast, the two nucleobases proximal to CpG dinucleotide on the 5′-side (P_1 and P_2) appear not to be very critical. Specifically, an abasic linker at P_1 did not significantly reduce immunostimulatory activity. An abasic linker at P_2 was tolerated but the nature of the nucleobase at P_1 had significant influence. For example, with an abasic linker at P_2, greatest immunostimulation in mouse cells required a guanosine at P_1. We are currently testing the effects of these CpG DNAs on different immune cell types obtained from different hosts including humans.

Experimental

Oligodeoxynucleotide synthesis and purification

CpG DNAs were synthesized using β-cyanoethylphosphoramidite chemistry on a PerSeptive Biosystem's 8909 Expedite DNA synthesizer on a 1 to 2 μmol scale. The phosphoramidites of dA, dG, dC and T were obtained from PerSeptive Biosystems. The 1′,2′-dideoxy-5′-DMT-3′-phosphoramidite was purchased from Glen Research. Beaucage reagent was used as an oxidant to obtain phosphorothioate backbone modifications. After the synthesis, CpG DNAs were deprotected as required, purified by HPLC and dialyzed against distilled water. Then the CpG DNAs were lyophilized and redissolved in distilled water and the concentrations were determined by measuring the UV absorbance at 260 nm. CpG DNAs were characterized by CGE and MALDI-TOF mass spectrometry (Brucker Proflex III MALDI-TOF mass spectrometer with 337 nm N2 laser) for purity and molecular mass, respectively.

Mouse splenocyte cultures

BALB/c or C57BL/6 mouse (4–8 weeks) spleen cells were cultured in RPMI complete medium as described earlier.[14] All other culture reagents were purchased from Mediatech (Gaithersburg, MD).

Isolation of mouse PBMCs

Peripheral blood mononuclear cells (PBMCs) from freshly drawn BALB/c mouse blood were isolated by Ficoll-Paque density gradient centrifugation method (Histopaque-1077, Sigma). Briefly, pooled, heparinized

blood was layered on to the Histopaque-1077 (equal volume) in a centrifuge tube and centrifuged at 400 *g* for 30 min at room temperature. The buffy coat, containing mononuclear cells, was removed carefully. The cells were washed twice with isotonic phosphate buffered saline (PBS) by centrifugation at 250 *g* for 10 min. The resulting pellet was then resuspended in RPMI 1640 medium containing L-glutamine and supplemented with 10% heat-inactivated FCS and penicillin-streptomycin (100 U/mL). Cells were cultured in 24 well plates for different time periods at 1×10^6 cells/mL/well in the presence or absence of CpG DNAs. After 24 h, supernatants were harvested and stored frozen at −70 °C until assayed for IL-12 and IL-6 by sandwich ELISA as described below.

Assays for IL-12 and IL-6 secretion in mouse spleen cell cultures

BALB/c or C57BL/6 mouse spleen cells were plated in 24-well dishes at a density of 5×10^6 cells/mL. 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 μg/mL to spleen 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 in 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 of appropriate biotinylated antibody and incubated at 25 °C for 1.5 h. Then the plates were washed extensively with PBS/0.05% Tween 20 and further incubated at 25 °C for 1.5 h after adding 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 900HDI 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 g) were divided into groups of three or four. CpG DNAs were dissolved in sterile PBS and administered intraperitoneally to mice at a dose of 5 mg/kg. After 72 h, the mice were sacrificed and their spleens harvested and weighed.

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