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Modulation of Immunostimulatory Activity of CpG Oligonucleotides by Site-Specific Deletion of Nucleobases

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Abstract—The effect of nucleobase deletion in the 3′- or the 5′-flanking sequence to a CpG-motif on immunostimulatory activity of CpG-containing oligonucleotides was examined by cell proliferation, secretion of IL-12 and IL-6 in mouse spleen cell cultures, and by spleen enlargement in mice. Deletion of one or two nucleobases in the 3′-flanking sequence to a CpG-motif at certain positions did not affect immunostimulatory activity, while similar deletions in the 5′-flanking sequence increased immunostimulatory activity compared with the parent oligo.

Recent studies show that the mammalian immune system is activated by synthetic oligodeoxynucleotides (oligo) containing cytosine-guanine dinucleotide motifs (CpG-motif).[1–3] This activation affects a variety of cellular elements leading to proliferation of B cells, macrophages as well as production of cytokines, including 1L-6, 1L-12, γ-IFN and TNF-α.[1–3] These immunological properties of synthetic oligos containing CpG-motif (CpG–DNA) have yielded promising results as immunomodulatory agents.[1–3] The use of these agents for the treatment and prevention of diseases is currently being examined.[4,5]

While the presence of a CpG-motif is a major factor for the immunostimulatory activity of CpG–DNA, the presence of specific purine and pyrimidine nucleotides in the flanking regions also affect the immunostimulatory activity.[6,7] In addition to the base sequence, modification of the internucleoside linkage between cytosine and guanine is known to affect the immunostimulatory activity.[8] Site-specific substitution of deoxynucleosides in the flanking region of the CpG-motif with 2′-0-methyl, 2′-O-methoxyethyl, or 3′-O-methylribonucelosides is also known to affect the immunostimulatory activity.[9] Substitutions made in the vicinity of the CpG-motif caused suppression of immunostimulatory activity, while substitutions made three or four deoxynucleosides away from the CpG-motif on the 5′-side caused an increase in immunostimulatory activity.[10] Our recent results suggest that an accessible 5′-end of the CpG oligo is critical for the immunostimulatory activity.[11]

We have shown that substitution of C or G in a CpG-motif with modified pyrimidine or purine bases does affect the immunostimulatory activity of CpG oligos.[12] We have also shown that site-specific substitution of anionic internucleoside linkage with non-ionic methylphosphonate linkage also affects the immunostimulatory activity of CpG oligos.[13] In continuation of our studies to dissect the structure–activity relationships of CpG oligos, for the first time, we studied the effect of deletion of nucleobases at specific sites in CpG oligos on their immunostimulatory activity. Deletion of nucleobases was achieved by incorporating 1′,2′-dideoxyribose without a nucleobase referred to here as 'd-spacer' (Fig. 1).

For the present studies, we chose two CpG oligos containing either a 'GACGTT' (oligo 1) or an 'AGCGTT' (oligo **6**) hexameric motif. Both the motifs have been shown to stimulate an immune response in mice. To understand the effect of site-specific deletion of the nucleobase, we synthesized oligos **2**–**5** and **7**–**17** (Table 1) in which one or two nucleobases were deleted in the 3′- or the 5′-flanking sequence to the hexameric motif by incorporating 'd-spacer'.[14] Sequences and nucleobase deletion sites in each oligo are shown in Table 1. In oligos **2** and **7**, a C or G nucleobase of the CpG-motif was deleted, respectively. In oligos **3**, **4**, and **8**–**11** a nucleobase was deleted in the 5′-flanking region to

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the CpG-motif at different positions. Similarly, in oligos **5**, **12**, and **13** a nucleobase was deleted in the 3′-flanking region at an increasing distance from the CpG-motif. Oligos **14**–**17** contained two nucleobase deletions at the positions shown (Table 1).

The immunostimulatory activity of oligos listed in Table 1 was studied in mouse spleen cell cultures as assessed by the extent of cell proliferation. BALB/c mouse spleen lymphocytes were cultured with oligos at concentrations of 0.1, 1.0, 3.0, and 10.0 μg/mL for 48 h and cell proliferation was determined by ^{3}H-uridine incorporation.[8] Oligos **1** and **6** used in this study are the parent CpG oligos that did not have nucleobase deletions and had shown proliferation indices of 14.3±0.05 (Fig. 2A) and 0.9±0.4, respectively, at 0.1 μg/mL concentration. The immunostimulatory activity of parent oligos was also evaluated in vivo by administering intraperitoneally a single dose of oligos to BALB/c mice and measuring the spleen weight at 72 h. Mice which received oligo **1** at doses of 5 and 10 mg/kg had spleen weights of 120 and 148 mg, respectively. These spleen weights of mice treated with oligo **1** are about 41 and 74% higher than the spleen weights of the control mice that were treated with PBS. Similarly, administration of oligo **6** at doses of 5 and 10 mg/kg resulted in spleen weights of 130 and 152 mg, respectively. These are about 53 and 79% higher than the control mice spleen weight. In addition, we also measured induction of IL-12 and IL-6 by oligos **1** and **6** at different concentrations in BALB/c mouse spleen cell cultures. Both the oligos induced concentration-dependent cytokine production in spleen cell cultures (Figs. 2 and 3). At a concentration of 0.1 μg/mL, oligos **1** and **6** induced 1292.3±73.1 and 1159.0±74.6 pg/mL of IL-12 secretion, and 1067.3±123.3 and 133.7±19.4 pg/mL of IL-6 secretion, respectively.

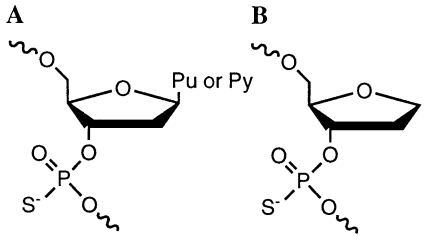

Figure 1. Structures of (A) a 2′-deoxyribonucleotide and (B) a 1′,2′-dideoxyribonucleotide ('d-spacer'; represented by **X** in sequences in Table 1). Pu and Py stand purine (A, G) and pyrimidine (C, T) nucleobases, respectively.

Table 1. Sequences of CpG-oligodeoxynucleotides showing position of d-spacer substitution

Oligo number	Sequence (5′→3′)[a]
Parent oligo	
1	CTATCTGACGTTCTCTGT
Nucleobase deleted oligos corresponding to oligo **1**	
2	CTATCTGA**X**GTTCTCTGT
3	CTATC**X**GACGTTCTCTGT
4	CTA**X**CTGACGTTCTCTGT
5	CTATCTGACGTT**X**TCTGT
Parent oligo	
6	CCTACTAGCGTTCTCATC
Nucleobase deleted oligos corresponding to oligo **6**	
7	CCTACTAGC**X**TTCTCATC
8	CCTAC**X**AGCGTTCTCATC
9	CCTA**X**TAGCGTTCTCATC
10	CCT**X**CTAGCGTTCTCATC
11	CCT**X**CTAGCCTTCTCATC
12	CCTACTAGCGTT**X**TCATC
13	CCTACTAGCGTTC**X**CATC
14	CCT**XX**TAGCGTTCTCATC
15	**XX**TACTAGCGTTCTCATC
16	CCTACTAGCGTTC**XX**ATC
17	CCT**X**CT**X**GCGTTCTCATC

[a]**X** indicates the postion of d-spacer, CpG-motif is shown as underlined.

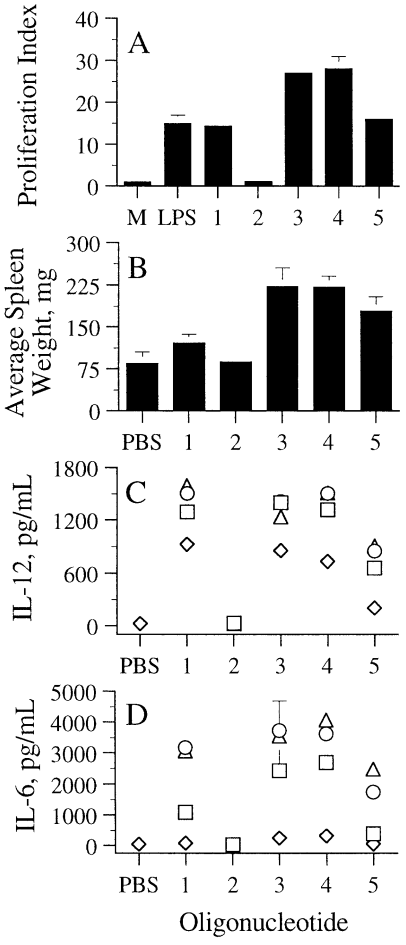

Figure 2. Immunostimulatory activity of GACGTT motif oligos, **1**–**5**, containing d-spacer. (A) Proliferation indices of oligos in BALB/c mouse spleen cell cultures at 0.1 μg/mL concentration. M and LPS represent media (control) and lypopolysaccharide, respectively. (B) Spleen enlargement in BALB/c mice following intraperitoneal administration of oligos at a dose of 5 mg/kg. Control mice received vehicle (PBS). Four animals were used for each oligo treatment. Secretion of IL-12 (C) and IL-6 (D) in BALB/c mouse spleen cell cultures at 0.03 (◇), 0.1 (□), 0.3 (○) and 1.0 (△) μg/mL concentration of oligos after 24 h incubation. Each value is an average of four replicate samples.

Deletion of C or G Nucleobase of the CpG-motif

When a C-nucleobase of CpG-motif of oligo **1** or G-nucleobase of CpG-motif of oligo **6** was deleted (oligos **2** and **7**, respectively, Table 1), no cell proliferation in cell cultures or spleen enlargement in mice was observed at any concentration studied (Figs. 2 and 3). In mouse spleen cell cultures, neither of the oligos induced IL-12 or IL-6 secretion (Figs. 2 and 3). These results suggest that nucleobases C and G of CpG-motif are essential for immunostimulatory activity of CpG oligos.

Deletion of Nucleobases in the 5′-Flanking Region to the CpG-motif

To study if deletion of a nucleobase in the 5′-flanking region of CpG-motif has any effect on immunostimulatory activity, we synthesized oligos **3** and **4** that contained a GACGTT motif (Table 1). In oligos **3** and **4**, the third and fourth nucleobases from the CpG-motif in the 5′-flanking sequence were deleted, respectively. Oligos **3** and **4**, in mice cell culture assays, showed proliferation indices of 27.1 ± 0.9 and 28.2 ± 2.8, respectively (Fig. 1A). The administration of oligos **3** and **4** to mice caused spleen enlargement which was about 85% higher in both the cases than that observed with oligo **1** administration (Fig. 2B). In mouse spleen cell cultures, oligos **3** and **4** induced a concentration dependent IL-6 and IL-12 production (Fig. 2C and D). At a concentration of 0.1 μg/mL, oligos **3** and **4** induced 1398 ± 48.9, and 1319.7 ± 84.6 pg/mL of IL-12 secretion, respectively. At the same concentration, they induced 2419.3 ± 91.7, and 2694 ± 118.9 pg/mL of IL-6 production, respectively. These results indicate that deletion of a nucleobase in the 5′-flanking region at a distance of three or four nucleotides from the CpG-motif results in increased immunostimulatory activity compared with the parent oligo.

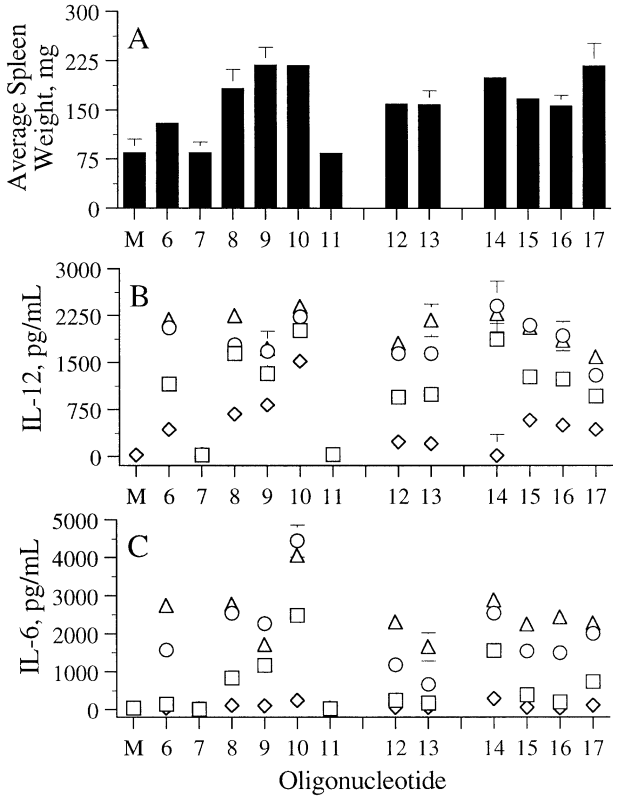

Figure 3. Immunostimulatory activity of AGCGTT motif oligos, **6**–**17**, containing d-spacer. (A) Spleen enlargement in BALB/c mice at 5 mg/kg dose (see Fig. 2 for details). Secretion of IL-12 (B) and IL-6 (C) in BALB/c mouse spleen cell cultures at 0.03 (◇), 0.1 (□), 0.3 (○) and 1.0 (△) μg/mL concentration of oligos as described in Figure 2.

To establish if deletion of nucleobases in the 5′-flanking region to the CpG-motif results in increased immunostimulatory activity is a general phenomenon, we introduced similar deletions in the second oligo (oligo **6**) that contained AGCGTT motif, resulting in the synthesis of oligos **8**–**10**. All three oligos **8**–**10** showed higher cell proliferation indices in spleen cell cultures compared with the parent oligo **6** (data not shown). Administration of oligos **8**–**10** to mice caused 42–68% increase in spleen weight compared with oligo **6** (Fig. 3A). These oligos also induced higher levels of IL-12 and IL-6 secretion in spleen cell cultures, especially at lower concentrations (0.03 and 0.1 μg/mL), compared with oligo **6**. Oligo **11**, which is similar to oligo **10** except that the CpG-motif is changed to CpC, induced no cell proliferation (data not shown) or cytokine induction in mouse spleen cell cultures or spleen enlargement in mice (Fig. 3). These results confirm that increased immunostimulatory activity is not the result of deletion of nucleobase but requires the presence of a CpG-motif.

Deletion of Nucleobases in the 3′-Flanking Region to the CpG-motif

To study the effect of deletion of the nucleobase in the 3′-flanking region to the CpG-motif, we synthesized oligo **5**, in which third nucleobase from the CpG-motif was deleted. Oligo **5** showed no loss of immunostimulatory activity as determined by lymphocyte proliferation in cell cultures (Fig. 2A) and spleen enlargement in mice (Fig. 2B), suggesting that deletion of a nucleobase in the 3′-flanking region at a distance of about three nucleotides from the CpG-motif had less impact on immunostimulatory activity. However, lower levels of IL-12 and IL-6 (Fig. 2C and D) were observed in spleen cell cultures when treated with oligo **5** than when treated with oligo **1**, suggesting that oligo **5** induced cytokines that were not assayed, which could be responsible for the observed lymphocyte proliferation and splenomegaly.

To confirm these results, oligos **12** and **13** were synthesized in which a nucleobase was deleted three or four nucleosides away from the CpG-motif in the 3′-flanking sequence, respectively. Oligos **12** and **13** caused about 22% higher increase in spleen weight in mice (Fig. 3A) and induced similar levels of IL-6 and IL-12 secretion in spleen cell cultures compared with oligo **6** (Fig. 3B and C). These data indicate that the deletion of a nucleobase at a distance of about three or four nucleosides away from the CpG-motif in the 3′-flanking sequence does not affect immunostimulatory activity.

Deletion of Two Nucleobases in the 5′- or the 3′-Flanking Region to the CpG-motif

After observing that deletion of one nucleobase in the flanking region to the CpG-motif increases immunostimulatory activity, we further asked what will be the impact of deletion of two nucleobases on immunostimulatory activity. We synthesized oligos **14** and **15** containing deletion of two nucleobases at adjacent positions, three nucleosides away from the CpG-motif in the 5′-flanking sequence, and oligo **16** containing two contiguous deletions of nucleobases in the 3′-flanking region. Oligo **14** showed a proliferation index of 2.8 ± 4.4 compared with oligo **6**, which had a proliferation index of 0.9 ± 0.4. Administration of oligos **14** and **15** to mice caused 53 and 29% increases, respectively, in spleen weight compared with control oligo **6** (Fig. 3A). Deletion of two nucleobases in the 3′-flanking region (oligo **16**) caused only a 20% increase in spleen weight and similar levels of IL-12 and IL-6 secretion (Fig. 3B and C) compared with oligo **6**, suggesting that deletion of nucleobases in the 3′-flanking region had less of an impact on immunostimulatory activity compared with deletions made in the 5′-flanking region.

We further asked how immunostimulatory activity would be affected if deletions of two nucleobases were made at two or three nucleosides apart, rather than contiguous in the 5′-flanking region to the CpG-motif. Oligo **17**, in which second and fifth nucleobases were deleted, produced a 68% increase in spleen weight compared with oligo **6**. Oligo **17** caused similar spleen enlargement as oligo **10**, which has one nucleobase deletion, suggesting that deletions of nucleobase at a gap of more than three nucleosides from the CpG-motif is primarily responsible for the increased immunostimulatory activity.

In conclusion, the present results suggest that deletion of a nucleobase in the 5′-flanking region at a distance of three or more nucleosides from CpG-motif increases immunostimulatory activity, and similar deletions in the 3′-flanking sequence does not affect immunostimulatory activity compared with unmodified parent oligo. Deletion of two nucleobases in the 5′- or the 3′-flanking region to the CpG-motif showed results similar to deletion of one nucleobase. Increased immunostimulatory activity was not only due to deletion of nucleobases; in addition, the presence of a CpG-motif was required. An increase in immunostimulatory activity observed with deletion of nucleobases seems to be a general phenomenon if the deletion is introduced at an appropriate position.

Our previous studies showed that substitution of deoxynucleosides with a 2′-substituted ribonucleoside or 3′-*O*-methylribonucleoside in the 5′-flanking region to the CpG-motif significantly increases immunostimulatory activity.[9,10] In addition, the substitution of an anionic internucleosidic linkage with a non-ionic linkage at about three or more internucleoside linkages away from the CpG-motif in the 5′-flanking sequence increases immunostimulatory activity.[13] Taken together, these results suggest that a nucleobase and a sugar ring, except an internucleoside phosphate linkage, beyond the fourth or fifth nucleoside from the CpG-motif on either side of the sequence play an insignificant role in the recognition and/or binding of the CpG oligos to the receptor/protein in the immunostimulatory pathway. In fact, as shown in the present study, the deletion of a nucleobase on the 5′-side to the CpG-motif increases immunostimulatory activity.

The changes in the secretion of cytokine levels observed further suggest that the modifications incorporated in CpG oligos alter recognition and/or binding affinity to receptors leading to different cytokine secretion profiles compared with the unmodified parent oligo. The distinct immunostimulatory properties of these modified novel CpG oligos may allow design of immunotherapeutic agents to induce cytokines of interest to treat specific diseases. In addition, the modifications introduced would also contribute to improved bioavailablity of CpG oligos in vivo.

References and Notes

1. Tokunaga, T.; Yamamoto, T.; Yamamoto, S. *Jpn. J. Infect. Dis.* **1999**, *52*, 1.
2. Gurunathan, S.; Klinman, D. M.; Seder, R. A. *Annu. Rev. Immunol.* **2000**, *18*, 927.
3. Pisetsky, D. S. *Curr. Top. Microbiol. Immunol.* **2000**, *247*, 143.
4. Davis, H. L.; Suparto, I. I.; Weeratna, R. R.; Jumintarto Iskandriati, D. D.; Chamzah, S. S.; Mairuf, A. A.; Nente, C. C.; Pawitri, D. D.; Krieg, A. M.; Heriyanto Smits, W.; Sajuthi, D. D. *Vaccine* **2000**, *18*, 1920.
5. Tighe, H.; Takabayashi, K.; Schwartz, D.; Marsden, R.; Beck, L.; Corbeil, J.; Richman, D. D.; Eiden, J. J.; Spiegelberg, H. L.; Raz, E. *Eur. J. Immunol.* **2000**, *30*, 1939.
6. Krieg, A. M.; Yi, A. K.; Matson, S.; Waldschmidt, T. J.; Bisshop, G. A.; Teasdale, R.; Koretzky, G. A.; Klinman, D. M. *Nature* **1995**, *374*, 546.
7. Pisetsky, D. S. *Immunol. Res.* **1999**, *19*, 35.
8. Zhao, Q.; Temsamani, J.; Idarola, P.; Jiang, Z.; Agrawal, S. *Biochem. Pharmacol.* **1996**, *51*, 173.
9. Zhao, Q.; Yu, D.; Agrawal, S. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 3453.
10. Zhao, Q.; Yu, D.; Agrawal, S. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1051.
11. Yu, D.; Zhao, Q.; Kandimalla, E. R.; Agrawal, S. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2585.
12. Kandimalla, E. R.; Yu, D.; Zhao, Q.; Agrawal, S. *Bioorg. Med. Chem.* **2001**, *9*, 807.
13. Yu, D.; Kandimalla, E. R.; Zhao, Q.; Cong, Y; Agrawal, S. *Bioorg. Med. Chem.* **2001**, in press.
14. Oligonucleotides were synthesized using β-cyanoethyl-phosphoramidite chemistry on PerSeptive Biosystem's 8900 Expedite DNA synthesizer on 1–2 mmol scale. Phosphoramidites of dA, dG, dC and T were obtained from PerSeptive Biosystems, and 1′,2′-dideoxyribonucleoside (d-spacer) phosphoramidite was purchased from Glen Research. Beaucage reagent was used as an oxidant to obtain phosphorothioate backbone modification. After the synthesis, oligos were deprotected as required, purified by HPLC, and dialyzed against distilled water. Then the PS-oligos were lyophilized, redissolved in distilled water and the concentrations were

determined by measuring the UV absorbance at 260 nm. PS-oligos were characterized by CGE, and MALDI-TOF mass spectrometry (Bruker Proflex III MALDI-TOF mass spectrometer with 337 nm N2 laser). Molecular weights observed and (calculated) for each oligo are **1**, 5702 (5703.6); **2**, 5596 (5594.4); **3**, 5596 (5594.4); **4**, 5581 (5579.4); **5**, 5596 (5594.4); **6**, 5660 (5657.6); **7**, 5510 (5508.4); **8**, 5535 (5533.4); **9**, 5551 (5548.4); **10**, 5526 (5524.4); **12**, 5551 (5548.4); **13**, 5535 (5533.4); **14**, 5417 (5415.4); and **15**, 5440 (5439.2). Others were not determined.