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Immunostimulatory Activity of CpG Containing Phosphorothioate Oligodeoxynucleotide is Modulated by Modification of a Single Deoxynucleoside

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Abstract—Phosphorothioate oligodeoxynucleotides (PS-oligos) containing the CpG motif have immunostimulatory properties. Our earlier study had shown that the immunostimulatory activity of PS-oligos containing the CpG motif can be modulated by incorporation of 2′-*O*-methylribonucleosides (Zhao, Q.; Yu, D.; Agrawal, S. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 3453). Here we show that the immunostimulatory activity of a PS-oligo containing a CpG motif can be modulated by substitution of a single deoxynucleoside at specific sites with either 2′-*O*-methylribonucleoside or 3′-*O*-methylribonucleoside in the flanking region to CpG motif. Furthermore, substitution of deoxynucleosides with 2′-*O*-methoxyethoxyribonucleosides also results in modulating immunostimulatory activity of PS-oligos.

Phosphorothioate oligodeoxynucleotides containing unmethylated CpG dinucleosides (CpG motif) are immune stimulatory, including inducing B-cell proliferation and cytokine production.[1–5] The immunostimulatory activity of a PS-oligo containing a CpG motif depends on the position of the CpG motif and its flanking sequence.[1–5] The mechanisms of how the cells are activated by a CpG-containing PS-oligo are not yet well established,[6,7] but internalization of PS-oligo into cells is required for the immune stimulation.[8] Preclinical studies have indicated that PS-oligos containing CpG motifs have therapeutic potential as vaccine adjuvants and for the immunotherapy of cancer and infectious and allergic diseases.[9–13] Certain modifications of the CpG motif in a given PS-oligo, e.g., substitution of cytosine in the CpG motif with 5-methylcytosine,[1,4] modification of the internucleotide linkage between C and G of CpG motifs,[4] and substitution of CpG with 2′-*O*-methylribonucleoside (2′-OMe)[4] have been shown to minimize the immunostimulatory responses. PS-oligos containing modified CpG motifs exert minimal side effects in mice.[14]

In a recent study, we observed that the substitution of two contiguous deoxynucleosides with 2′-OMe residues immediately upstream (at the 5′-end) of the CpG motif suppressed the immunostimulatory activity of the PS-oligo.[15] Conversely, substitution of two deoxynucleosides of PS-oligos with 2′-OMe further upstream of the CpG motif resulted in increased immunostimulatory activity. Increases in immunostimulatory activity of PS-oligos were also observed when substitutions of deoxynucleosides with 2′-OMe were made at a point toward the 3′-end of CpG motifs. The results obtained were intriguing and raised a number of other questions. For example, what minimal number of deoxynucleoside substitutions with 2′-OMe in a CpG-containing PS-oligo confers the ability to modulate immunostimulatory activity? Is it specific to incorporation of 2′-OMe or do other 2′-*O*-alkylribonucleosides have the same effect? Furthermore, what is the effect of substitution of deoxynucleoside at a specific site with 3′-*O*-methyribonucleoside (3′-OMe), which would also result in 2′–5′-linkage, on immunostimulatory activity.

To address these questions, we have employed a PS-oligo containing one CpG motif (oligo **1**), which has been shown to be immunostimulatory,[1] and synthesized oligos **2**–**14** in which substitution of one or two deoxynucleosides was carried out with modified nucleosides at specific sites as shown in Table 1.[16] Oligos **1**–**14** (Table 1) were evaluated for their immunostimulatory activity in a mouse spleen lymphocyte proliferation assay.[4] The comparative data presented in Figure 1 is of 0.1 μg/mL concentration.

In oligos **2**, **3**, and **4**, one deoxynucleoside at the 5′-end of the CpG motif of oligo **1** was substituted with 2′-OMe, leaving no nucleoside, one, and two deoxynucleosides, respectively, between the substitution and the CpG

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Table 1. Oligodeoxynucleotide phosphorothioates and site of modification

Oligo No.	Sequence & Modification (5'-3')
1	TCCATGACGTTCCTGATGC
2	TCCATG(A)CGTTCCTGATGC
3	TCCAT(G)ACGTTCCTGATGC
4	TCCA(U)GACGTTCCTGATGC
5	TCCATGACG(U)TCCTGATGC
6	TCCATGACGT(U)CCTGATGC
7	TCCATGACGTT(C)CTGATGC
8	TCCATG[A]CGTTCCTGATGC
9	TCCA[U]GACGTTCCTGATGC
10	TCC[A]TGACGTTCCTGATGC
11	TCCATGACG[U]TCCTGATGC
12	TCCATGACGTTCCTGAUGC
13	TCCATGACGTTCCUGAUGC
14	TCCATGACGTTCCUGAUGC

Normal face

Boxed nucleosides

Circled nucleosides

Underlined nucleosides

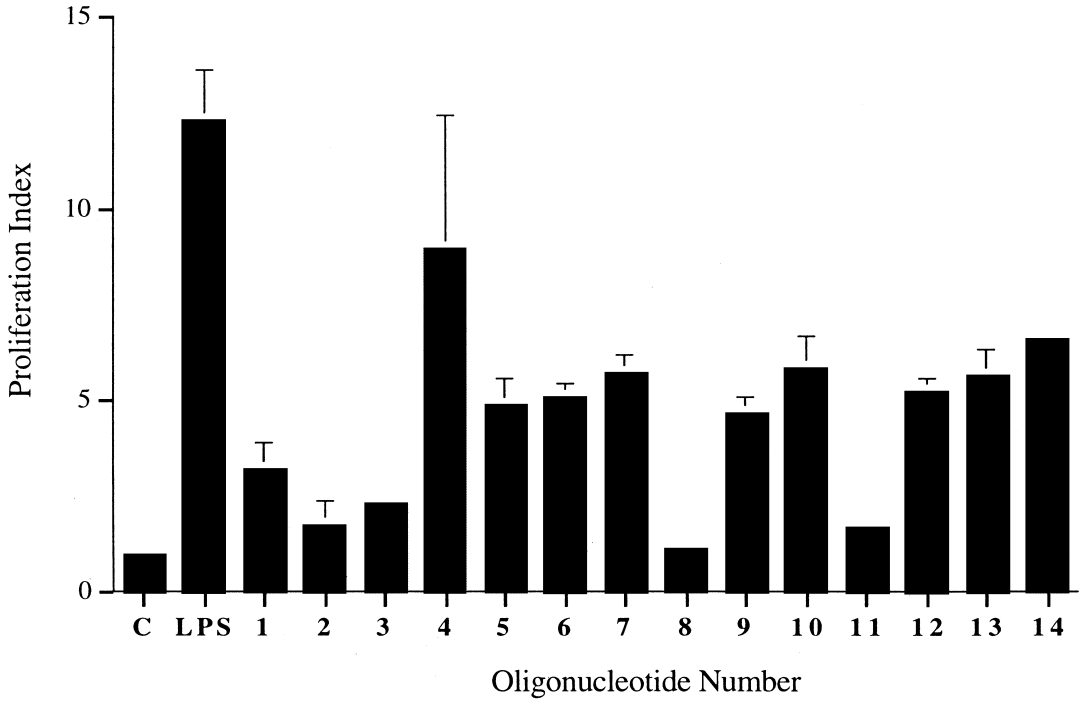

Figure 1. Proliferation of murine spleen lymphocytes cultured with oligonucleotides. Spleen lymphocytes of female CD-1 mice (4–5 weeks) were cultured in the presence of various oligonucleotides (oligos **1**–**14**) at a concentration of 0.1 μg/mL using the same experimental conditions as reported earlier.[4] Cells cultured with medium alone served as a negative control (C) and cells cultured with 10 μg/mL LPS (lipopolysaccharides) served as a positive control (LPS). After 44 h, cells were pulsed with 1μCi/well of ^{3}H-uridine for 4 h. Cells were harvested using automated cell harvester and the radioactivities were transferred to filters and counted. The assays were performed in triplicate.

motif. In oligos **5**, **6** and **7**, one deoxynucleoside on the 3′-end of the CpG motif of oligo **1** was substituted with 2′-OMe leaving no nucleoside, one, and two deoxynucleosides, respectively, between the substitution and the CpG motif. As shown in Figure 1, the proliferation index of oligo **1** was 3.13 ± 0.72. The proliferation indices of oligos **2** and **3**, which have 2′-OMe residues close to the CpG motif at the 5′-end, were 1.68 ± 0.63 and 2.24 ± 0.03, respectively. The proliferation indices of oligos **2** and **3** indicate that substitution of either deoxynucleoside close to the CpG results in suppression of immunostimulatory activity. The proliferation index of oligo **4** was 8.9 ± 3.4, which is an increase of over 180% compared with that of oligo **1**. Substitution of one deoxynucleoside with 2′-OMe in the 3′-flanking region either immediately next to the CpG motif (oligo **5**) or at a distance (oligos **6** and **7**) resulted in an increase in the immunostimulatory activity. The proliferation indices of oligos **5**, **6** and **7** were 4.8 ± 0.72, 5.02 ± 0.34, and 5.64 ± 0.53, respectively, which is an increase of 55 to 80% compared with that of oligo **1** ($p < 0.05$). These results are in agreement with our previous observation that the substitution of deoxynucleosides immediately next to the CpG motif on the 5′-end suppresses immunostimulatory activity and further upstream or downstream increases the immunostimulatory activity. It is important to note that substitution of a single deoxynucleoside with 2′-OMe affects the immunostimulatory activity of CpG containing PS-oligo.

Our next goal was to understand if the modulation of the immunostimulatory activity observed in oligo **1** with 2′-OMe substitution was a function of 2′-*O*-methylribonucleosides. We incorporated 3′-OMe at selected positions in oligo **1** and synthesized oligos **8**–**11**. Incorporation of 3′-OMe in oligos **8**–**11** led not only to incorporation of the 3′-OMe group, but also to incorporation of 2′–5′-phosphorothioate linkages at the site of substitution. While the effects on the immunostimulatory activity of oligos **8**–**11** were in general the same as with oligos **2**–**7**, notable changes were observed. The substitution of one deoxynucleoside with 3′-OMe immediately upstream to the CpG motif (oligo **8**) resulted in almost complete suppression of the proliferation index (Fig. 1). Substitution of one deoxynucleoside with 3′-OMe in the 5′-flanking region of the CpG motif (oligos **9** and **10**) caused an increase in immunostimulatory activity. The proliferation indices of oligos **9** and **10** were 4.6 ± 0.43 and 5.7 ± 0.87, respectively, which is an increase of 50 to 80% over that of oligo **1** ($p < 0.05$). A notable difference was observed with oligo **11**, in which substitution of one deoxynucleoside with 3′-OMe immediately downstream to the CpG caused suppression of the immunostimulatory activity. The proliferation index of oligo **11** was 1.6 ± 0.19, compared with the value of 3.1 ± 0.72 observed with oligo **1** ($p < 0.05$). This is in contrast to oligo **5**, in which the same substitution was made with 2′-OMe and produced no effect on immunostimulatory activity compared to oligo **1**. The results on the immunostimulatory activity of oligo **11** indicate that alteration of the 3′–5′-linkage to 2′–5′-linkage alone or in combination with 3′-OMe group had a suppressive effect on CpG motif-mediated immunostimulatory activity.

In continuation of our studies on the effect of substitution of deoxynucleosides with 2′-OMe in CpG-containing PS-oligos we have extended our earlier observation using other 2′-*O*-alkylribonucleosides.[15] We synthesized oligos **12**–**14**, in which two deoxynucleosides were substituted with 2′-*O*-methoxyethoxyribonucleosides (2′-OMOE) in the 5′- (oligo **12**) and 3′-(oligo **13**) flanking region or in both 5′- and 3′-(oligo **14**) flanking regions. In cell culture assays, all three oligos (oligos **12**–**14**), showed an increase in immunostimulatory activity compared with oligo **1**. The proliferation indices of oligos **12**, **13**, and **14** were 5.18 ± 0.34, 5.60 ± 0.67, and 6.57 ± 0.20, respectively, which is an increase of 50 to 80% for oligos **12** ($p < 0.01$) and **13** ($p < 0.01$) and 110% for oligo **14** ($p < 0.01$) compared with the value for oligo **1**.

To confirm the results of the cell culture assay, the selected oligos were administered to mice and the degree of splenomegaly was measured as an indicator of the level of the immunostimulatory effects. The oligos studied in mice were oligos **1**, **2**, **4**, and **8**–**12**. A single dose of 5 mg/kg of oligo was administered intraperitoneally to BALB/c mice (female, 4–6 weeks old, Harlan–Sprague–Dawley Inc.). The mice were sacrificed at 72 h after administration and the spleens were harvested and weighed. The administration of oligo **1** caused a 74% increase in the spleen weight compared with the mice in the control group ($p < 0.01$) (Fig. 2). Oligo **2**, in which only one deoxynucleoside immediately at the 5′-end of the CpG motif was substituted with 2′-OMe caused only an 18% increase in spleen weight ($p < 0.05$). Oligo **4**, in which one deoxynucleoside in the 5′-flanking region upstream of the CpG motif was substituted with 2′-OMe, caused a 124% increase in spleen weight compared with mice in the control group ($p < 0.01$), indicating its increased immunostimulatory activity compared with oligo **1**.

The results of spleen enlargement in mice following administration of oligos **8**–**11** were in agreement with the results of the cell proliferation assay. Administration of oligos **8** and **11**, in which one deoxynucleoside on the 5′- or 3′- end, respectively, immediately next to the CpG motif was substituted with 3′-OMe, caused less increase in spleen weight than did oligo **1** (Fig. 2). Oligos **9** and **10**, in which 3′-OMe substitution was incorporated in the 5′-flanking region farther from the CpG motif caused 179% ($p < 0.05$) and 194% ($p < 0.01$) increases in spleen weight compared with untreated mice, whereas oligo **1** caused only a 74% increase in spleen weight. Oligo **12**, in which two deoxynucleosides were substituted in the 5′-flanking region with 2′-OMOE caused an increase of 173% compared with untreated mice ($p < 0.05$), indicating an increase in immunostimulatory activity, compared to oligo **1**.

The above results clearly indicate that the substitution of deoxynucleosides immediately 5′- or 3′-to the CpG motif with 2′-OMe or 3′-OMe significantly suppresses immunostimulatory activity. However, an increase in immunostimulatory activity is observed if substitutions are made in the flanking region at least two nucleosides away from the CpG motif. These substitutions are not limited to 2′-OMe; bulkier 2′-OMOE also caused similar

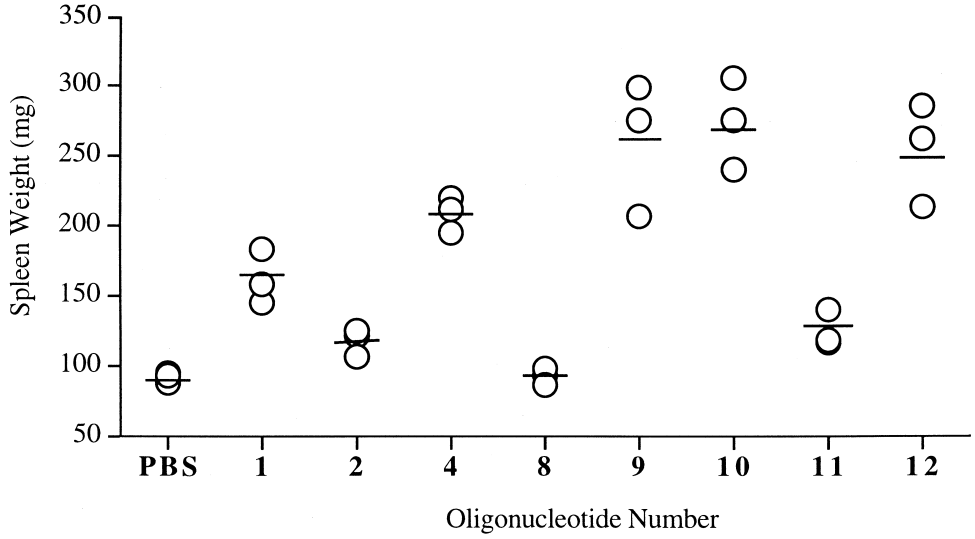

Figure 2. Spleen enlargement of mice following administration of various oligonucleotides (oligos **1**, **2**, **4**, and **8**–**12**). Female BLAB/c mice (6–8 weeks, 19–21 g) were divided into nine groups with three mice in each group. Oligonucleotides (oligos **1**, **2**, **4**, and **8**–**12**) were dissolved in sterile PBS and were administered intraperitoneally to mice at a dose of 5 mg/kg. One group of mice received sterile PBS alone to serve as a control (PBS). After 72 h, mice were sacrificed and spleens were harvested and weighed. Each circle represents the spleen weight of an individual mouse and the line represents the mean weight value of each group.

increase in immunostimulatory activity. Substitution with 3′-OMe, in which the internucleotide linkages with the preceding nucleoside were 2′–5′-linkages, in addition to the 3′-OMe group, also results in modulation of the immunostimulatory activity of CpG-containing PS-oligos. The modifications studied here provide a rational approach for modulating immunostimulatory properties of PS-oligos containing CpG motifs. These single-nucleoside chemical-modifications will have only minimal influence on the binding affinity of these oligos to RNA compared to control oligo **1**. For example, the 2′-*O*-alkyl substitution slightly increases RNA binding affinity, and the 2′,5′-linkage slightly decreases RNA binding affinity. The immunosuppresive modifications proposed in this paper impact antisense studies in two ways: (i) the sites containing CpG motifs on mRNA would no longer be limiting factors in target site selection, and (ii) the introduction of these modifications in antisense PS-oligos decreases non-antisense related effects and thereby the resulting side effects caused by CpG motifs in PS-oligos. In addition, the chemical modifications that enhance immunostimulatory activity of PS-oligos containing CpG motifs are of particular interest for the development of oligos as vaccine adjuvants and for the immunotherapy of cancer, infectious and allergic diseases. Further studies are ongoing to establish if there are any differences in the pattern and/or kinetics of cytokine induction with use of these modified PS-oligos.

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16. Oligonucleotides were synthesized on 1 μmol scale using an automated DNA synthesizer (Expedite 8909 PerSeptive Biosystems). Deoxynucleosides and 2′-*O*-methylribonucleoside phosphoramidites were obtained from PerSeptive Biosystems (Foster City, CA). 2′-*O*-methoxyethoxyribonucleoside and 3′-*O*-methylribonucleosides phosphoramidites were obtained from ChemGenes (Ashland, MA). Modified nucleosides were incorporated into the oligonucleotides at a specific site using normal coupling cycles. After the synthesis, oligonucleotides were deprotected using concentrated ammonium hydroxide and purified by reversed-phase HPLC, followed by dialysis. Purified oligonucleotides as sodium salt form were lyophilized prior to use. Purity of oligonucleotides was checked by CGE and MALDI-TOF MS (Bruker Proflex III MALDI-TOF Mass Spectrometer). Molecular weight observed and calculated (in brackets) for each oligonucleotide were as follows: oligo **1**, 6041 (6043); oligo **2**, 6075 (6073); oligo **3**, 6075 (6073); oligo **4**, 6060 (6059); oligo **5**, 6061 (6059); oligo **6**, 6060 (6059); oligo **7**, 6075 (6073); oligo **8**, 6075 (6073); oligo **9**, 6061, (6059); oligo **10**, 6075 (6073); oligo **11**, 6060 (6059); oligo **12**, 6193 (6191); oligo **13**, 6178 (6177); oligo **14**, 6327 (6327).