Pergamon

Bioorganic & Medicinal Chemistry 9 (2001) 807–813

BIOORGANIC & MEDICINAL CHEMISTRY

Effect of Chemical Modifications of Cytosine and Guanine in a CpG-Motif of Oligonucleotides: Structure–Immunostimulatory Activity Relationships

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Received 10 November 2000; accepted 10 November 2000

Abstract—Oligodeoxynucleotides containing unmethylated CpG-motifs stimulate the innate immune system, including inducing B-cell proliferation and cytokine production. However, the mechanism of immunostimulation by CpG-oligonucleotides and the precise structural requirements and specific functional groups of cytosine and guanine necessary for recognition of and interaction with protein/receptor factors that are responsible for immune stimulation have not been elucidated. We sought to understand the critical role of each functional group of the cytosine and guanine moieties in a CpG-motif in inducing immunostimulatory activity. To this end, we examined structure–immunostimulatory activity relationships of phosphorothioate oligodeoxynucleotides (PS-oligos) containing YpG- and CpR-motifs (Y and R stand for pyrimidine and purine analogues, respectively). The PS-oligos containing a YpG-motif in which the natural deoxycytidine was replaced with deoxy-5-hydroxycytidine or deoxy-N4-ethylcytidine showed immunostimulatory activity. Substitution of deoxycytidine with a deoxy-5-methylisocytidine, deoxyuridine, or deoxy-P-base-nucleoside in the YpG-motif completely abolished the immunostimulatory activity, similar to the results observed with deoxy-5-methylcytidine. In the case of PS-oligos containing a CpR-motif, 7-deazaguanine substitution for natural guanine showed immunostimulatory activity similar to that of a parent PS-oligo. These studies suggest that the 2-keto, 3-imino and 4-amino groups of cytosine, and the 1-imino, 2-amino and 6-keto groups of guanine in a CpG-motif are important for the immunostimulatory activity of CpG-PS-oligos. The absence of N7 on guanine of the CpG-motif does not affect immunostimulatory activity significantly. These studies suggest that it is possible to develop YpG- and CpR-motifs as an alternative to CpG-motifs in PS-oligos for immunostimulatory studies.

Introduction

Bacterial DNA containing unmethylated CpG-dinucleotide motifs cause mitogenic effects, including stimulation of lymphocyte proliferation and cytokine production.[1,2] Oligodeoxynucleotides (oligos) containing CpG-motifs also stimulate the immune system[1–4] and the immunostimulatory activity of CpG-oligos is further enhanced by the presence of a phosphorothioate (PS) backbone on these oligos.[4] The immunostimulatory activity of CpG-oligos is dependent on the position of the CpG-motif and the sequences flanking the CpG-motif.[1–4] The mechanism of activation of immune stimulation by CpG-oligos is not well understood. It is believed that CpG-oligos trigger the immune cascade by binding to an intracellular receptor/protein,[3] which has not yet been characterized, which ultimately triggers stress kinase pathways, activation of NF-κB[5,6] and induction of various cytokines such as IL-6, IL-12, γ-IFN, and TNF-α.[7–11] The use of CpG-PS-oligos as antitumor, antiviral, antibacterial, and anti-inflammatory agents and as adjuvants in immunotherapy has been reported.[12–15]

In our earlier studies, to suppress the immunostimulatory activity of CpG-motif-containing antisense PS-oligos and to improve their sequence-specific antisense activity, we studied structural and chemical changes in the CpG-motif in PS-oligos.[16] These studies have shown that replacement of deoxynucleosides in a CpG-motif with 2′-*O*-methylribonucleosides suppresses immunostimulatory activity, suggesting that a rigid C3′-*endo* conformation induced by 2′-*O*-methyl modification does not allow proper recognition and/or interaction of the CpG-motif with the proteins involved in the immunostimulatory pathway.[16] It was also shown that substitution of a methyl group for an unbridged oxygen on the phosphate group

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0968-0896/01/$ - see front matter
PII: S0968-0896(00)00316-3

between C and G of a CpG-motif suppresses immunostimulatory activity, suggesting that negative charge on the phosphate group is essential for protein recognition and interaction.[16] In addition, CpG-related immunostimulatory activity of PS-oligos can also be suppressed by substituting a 5-methylcytosine for cytosine in a CpG-motif.

Now there is a growing interest in CpG-PS-oligos as therapeutic agents for cancer and inflammatory diseases, viral and bacterial infections, and as vaccine adjuvants.[5,12–15,17–19] In order to improve the immunostimulatory activity of CpG-PS-oligos and to understand the mechanism of immune stimulation, we have been studying systematic chemical changes in the backbone, sugar, and heterocyclic bases in the sequences in and around the CpG-motif in PS-oligos. We have recently shown that substitution of one or two 2′-deoxynucleosides adjacent to the CpG-motif with 2′- or 3′-*O*-methylribonucleosides on the 5′-side caused a decrease in immunostimulatory activity, while the same substitutions had insignificant effects when they were placed on the 3′-side of the CpG-motif.[20] However, the substitution of a deoxynucleoside two or three nucleosides away from the CpG-motif on the 5′-side with one or two 2′-*O*-methoxyethoxy- or 2′- or 3′-*O*-methylribonucleosides resulted in a significant increase in immunostimulatory activity.[21] In addition, we have also shown that an accessible 5′-end, but not 3′-end, was critical for immunostimulatory activity of CpG-PS-oligos.[22]

The precise structural requirements and specific functional groups of the CpG-motif necessary for the recognition of protein/receptor factor that is responsible for immune stimulation have not yet been studied in detail. In this paper, we describe the results of a systematic study in which natural cytosine or guanine in a CpG-motif was replaced with a number of pyrimidine or purine analogues. The purpose of this study was to understand which functional groups of cytosine and guanine could be involved in the recognition of and interaction with factors responsible for immune stimulation. The in vitro and in vivo studies of CpG-PS-oligos containing modified purine bases (R) suggest that the alteration of functional groups at positions 1, 2, and 6 of guanine (see Fig. 1 for structure and numbering) significantly decreased immunostimulatory activity, while the deletion of nitrogen at the 7-position (N7) had an insignificant impact. Similarly, studies with CpG-PS-oligos containing modified pyrimidine bases (Y) suggested that the alteration of functional groups at positions 2, 3, and 4 of cytosine (see Fig. 1 for structure and numbering) significantly decreased immunostimulatory activity. Substiturion of a hydrophobic methyl group at the 5-position decreased immunostimulatory activity and a hydrophilic hydroxy group at the same position did not suppress immunostimulatory activity. This is the first report of the use of chemically modified pyrimidine (Y) or purine (R) bases in place of natural cytosine or guanine, respectively, in a CpG-motif of oligos for immunomodulatory effects. In this paper, we describe the structure–immunostimulatory activity relationships of YpG- and CpR-motif-containing-PS-oligos compared with those of CpG-motif-containing-PS-oligos.

Results and Discussion

Design rationale

Figure 1 shows the chemical structures of deoxycytidine and deoxyguanosine in a CpG-motif. Cytosine has two hydrogen bond acceptor groups at positions 2 (keto-oxygen; O2) and 3 (nitrogen; N3), and a hydrogen bond donor group at position 4 (amino group; 4-NH_2). Guanine has two hydrogen bond acceptor groups at positions 6 (keto-oxygen; O6) and 7 (nitrogen; N7) and two hydrogen bond donor groups at positions 1 (imino nitrogen; N1) and 2 (amino group; 2-NH_2) that could serve as potential sites for recognizing and interacting with receptors/protein factors that are responsible for immune stimulation. In order to identify critical functional groups on cytosine and guanine in a CpG-motif for immunostimulatory activity, we have selected a number of pyrimidine (Y) (Fig. 2) and purine (R) (Fig. 3) analogues that are isostructural with natural cytosine and guanine, respectively. We synthesized a series of YpG- and CpR-PS-oligos incorporating these pyrimidine or purine analogues in place of natural cytidine (**1**) or guanosine (**8**), respectively, and studied their immunostimulatory activity in cell cultures and in vivo. The pyrimidine analogues (Y) studied included: deoxy-5-methylcytidine (**2**), deoxy-5-methylisocytidine (**3**), deoxy-5-hydroxycytidine (**4**), deoxyuridine (**5**), deoxy-N4-ethylcytidine (**6**), and deoxy-P-base-nucleoside (**7**) (Fig. 2). The modified purine nucleobases

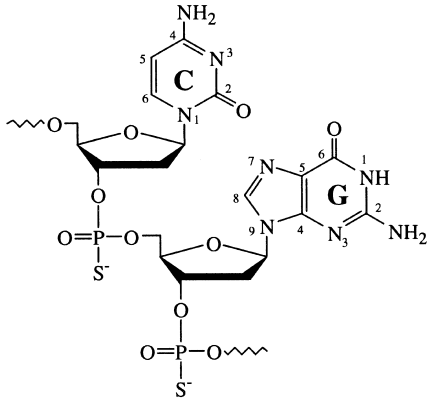

Figure 1. Chemical structure of a CpG-motif showing functional groups on cytosine and guanine that serve as hydrogen bond acceptor and donor groups.

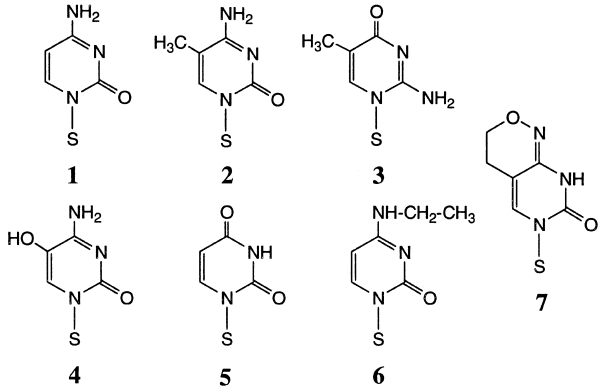

Figure 2. Chemical structures of cytosine (**1**) and its analogues (**2**–**7**) used in the study. S represents deoxyribose.

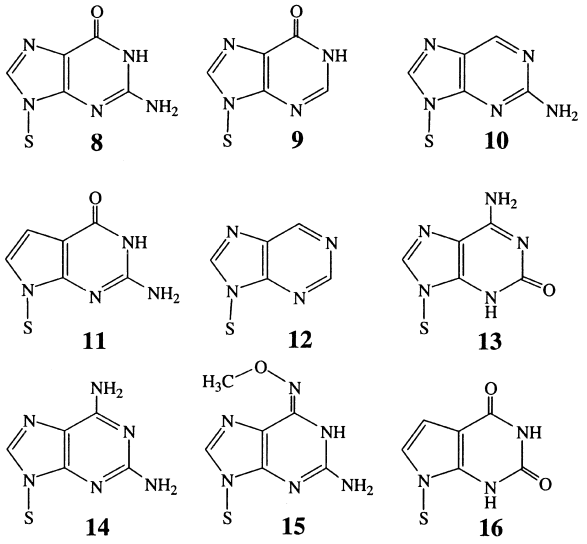

Figure 3. The structures of natural guanine (**1**) and the other purine analogues **2**–**9** used in the study. S represents 2′-deoxyribose.

(R) used in the study included inosine (**2**), 2-aminopurine (**3**), 7-deazaguanine (**4**), nebularine (**5**), isoguanine (**6**), 2-aminoadenine (**7**), K-base (**8**) and 7-deazaxanthine (**9**) (Fig. 3). These studies allowed us to assess structure–immunostimulatory activity relationships of YpG- and CpR-PS-oligos, and gain knowledge of functional groups on cytosine and guanine that could possibly be involved in recognition of and/or interaction with receptor factors in the immunostimulatory pathway. The nucleotide sequences of PS-oligos used in the study are shown in Table 1.

Lymphocyte proliferatory activity of YpG-PS-oligos

The immunostimulatory activity of YpG-PS-oligos 1–5 was studied using a BALB/c mouse lymphocyte proliferation assay. Figure 4 shows the concentration-dependent cell proliferatory activity of YpG-PS-oligos with a natural deoxycytidine (**1**) or modified cytidines, deoxy-5-hydroxycytidine (**4**) and deoxy-N4-ethylcytidine (**6**) (oligos 1–5), in mouse lymphocyte cultures. Oligo 1 had a concentration-dependent proliferatory activity. At a concentration of 3.0 μg/mL, oligo 1 had a proliferation index of 29.5±2.1. Oligo 2, in which the deoxycytidine was replaced with a deoxy-5-hydroxycytidine (**4**), also showed concentration-dependent lymphocyte proliferation. A proliferation index of 23.7±2.9 at a concentration of 3.0 μg/mL was observed for oligo 2. PS-oligo 4, which contained deoxy-N4-ethylcytidine (**6**) in the YpG-motif, also had a concentration-dependent lymphocyte proliferation activity. The proliferation index of 18.7±1.6 observed for oligo 4 at a concentration of 3 μg/mL suggests that the presence of a bulky hydrophobic substitution on the 4-amino group of cytosine slightly impedes immunostimulatory activity.

Table 1. Sequences, chemical modifications, and MALDI-TOF mass spectral analysis data of phosphorothioate oligonucleotides

Oligo no.	Sequence (5′→3′) and modification[a]	Molecular weight	
		Calculated	Observed
1	d(CTATCTGA**CG**TTCTCTGT)	5704	5705
2	d(CTATCTGA**YG**TTCTCTGT)	5720	5721
3	d(CTATCTGA**CY**TTCTCTGT)	5681	5681
4	d(CTATCTGA**YG**TTCTCTGT)	5733	5733
5	d(CTATCTGA**CY**TTCTCTGT)	5694	5693
6	d(CTATCTGA**CR**TTCTCTGT)	5704	5704
7	d(CTATCTGA**RC**TTCTCTGT)	5705	5704
8	d(TCTCCCAG**CG**TG**CG**CCAT)	5684	5685
9	d(TCTCCCAG**CR**TG**CR**CCAT)	5681	5683

[a]CpG-, YpG-, and CpR-motifs are shown in bold. **Y** represents 5-hydroxycytosine (oligos 2 and 3) or N4-ethylcytosine (oligos 4 and 5) and **R** represents 7-deazaguanine.

Oligo 3, in which deoxy-5-hydroxycytidine was placed in the deoxyguanosine position instead of the deoxycytidine position, had a proliferation index that was similar to that observed for media control (Fig. 4). Similarly, the control oligo 5 in which deoxyguanosine in the CpG-motif was substituted with cytidine analogue **6** showed cell proliferation similar to that of media control.

The other oligos, in which deoxycytidine in the YpG-motif was replaced with deoxy-5-methylcytidine (**2**), deoxy-5-methylisocytidine (**3**), deoxyuridine (**5**), or deoxy-P-base-nucleoside (**7**) showed no or insignificant cell proliferation activity in the same assay system. These results suggest that (i) cytidine analogues **4** and **6** in place of natural cytidine in a YpG-motif in PS-oligos (2 and 4, respectively) are responsible for cell proliferation activity and (ii) the presence of these analogues in place of natural guanosine in a CpG-motif does not induce cell proliferation.

Lymphocyte proliferatory activity of CpR-PS-oligos

The immunostimulatory activity of CpR-PS-oligos was also studied using a BALB/c mouse lymphocyte proliferation assay. Figure 5 shows lymphocyte proliferation activity of CpR-PS-oligos (1 and 6–9) with a natural guanine or 7-deazaguanine in lymphocyte cultures at 1.0 μg/mL concentration. All oligos showed a concentration-dependent cell proliferation activity. At a concentration of 1.0 μg/mL, oligo 1 showed a proliferation index of 19.3±0.7 in this assay. Oligo 6, in which the natural guanine was replaced with a 7-deazaguanine (**11**), had a proliferation index of 14.5±0.5 at the same concentration. At this concentration, the proliferation index observed for oligo 6 was about 75% of the proliferation index observed for oligo 1. Oligo 7, in which CpR-motif was reversed to produce a RpC-motif, at the same concentration of 1.0 μg/mL, had a proliferation index of 0.67±0.08, which was similar to that observed for media control (Fig. 5). These results suggest that (i) a CpR-motif in PS-oligo 2 that has 7-deazaguanine in the R-position is responsible for cell proliferation, and (ii) the presence of 7-deazaguanine in the cytosine position in the CpG-motif does not induce cell proliferation.

The PS-oligo that contained inosine (**9**) in place of natural guanine showed about 35% cell proliferation compared with oligo 1 (data not shown), suggesting that the absence of an amino group at position 2 impedes recognition of and interaction with the protein factors, thereby reducing immunostimulatory activity. The

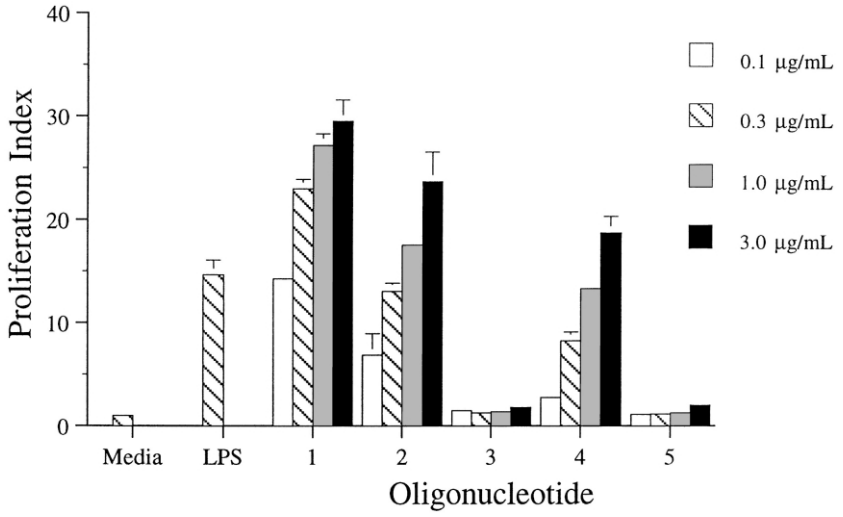

Figure 4. Lymphocyte proliferation of YpG-PS-oligos in mouse spleen lymphocyte cultures at indicated concentrations. LPS stands for lipopolysaccharide.

CpR-PS-oligos in which guanine was replaced with other purine analogues, **10** and **12–16** induced insignificant cell proliferation (8–19%) compared with parent PS-oligos containing natural guanine, suggesting that these modifications do not permit proper recognition and/or interaction with protein/receptor factor and thereby do not activate immune system.

To confirm the critical role of N7 of guanine in immune stimulation, we synthesized two CpR-PS-oligos with natural guanosines and 7-deazaguanosines in the R-position and studied their ability to induce cell proliferation (oligos 8 and 9). This sequence contained two CpG-motifs. Both PS-oligos 8 and 9 induced a concentration-dependent cell proliferation. The PS-oligo 9 that contained 7-deazaguanines in the CpR-motifs had a proliferation index of 5.6±0.6 at a concentration of 1.0 μg/mL (Fig. 5). At the same concentration, its control PS-oligo 8 with natural guanines in both the CpG-motifs had a proliferation index of 7.1±2.2. This proliferation index for oligo 9 corresponded to about 78% cell proliferation compared with its parent oligo 8, which contained natural guanines. These results further support that N7 of guanine is not required for protein recognition/interaction and that 7-deazaguanine could be used to replace natural guanine in CpG-motifs without significantly affecting CpG-related immunostimulatory activity.

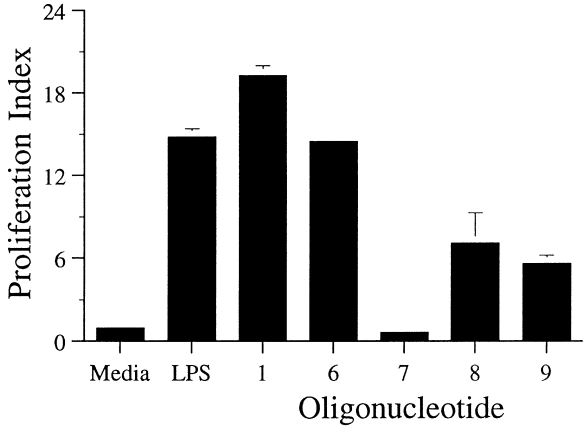

Figure 5. Lymphocyte proliferation of CpR-PS-oligos in mouse spleen lymphocyte cultures at 1.0 μg/mL concentration. LPS stands for lipopolysaccharide.

Splenomegaly in mice

To confirm the in vitro effects of CpG-PS-oligos, oligos 1, 2, 4, and 6–9 were injected intraperitoneally (ip) to BALB/c mice at a dose of 10 mg/kg and the change in spleen weight was measured as described earlier[16] as an indicator of the level of immunostimulatory activity of each PS-oligo. The changes in spleen weights as a result of treatment with YpG- and CpR-PS-oligos are presented in Figures 6 and 7, respectively.

Oligo 1, which had a natural deoxycytidine (**1**) in the YpG-motif, produced about 45% increase in spleen weight, compared with the control group of mice that received PBS. Oligo 2, which had a deoxy-5-hydroxycytidine (**4**) in place of deoxycytidine in the YpG-motif,

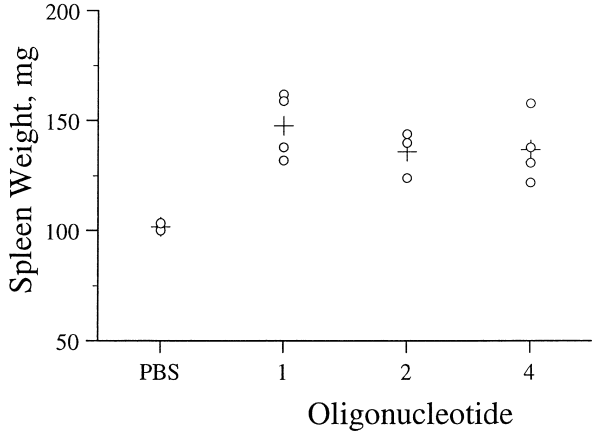

Figure 6. Spleen enlargement of mice following administration of YpG-PS-oligos. Each circle represents the spleen weight of an individual mouse and the + represents the mean spleen weight for each group.

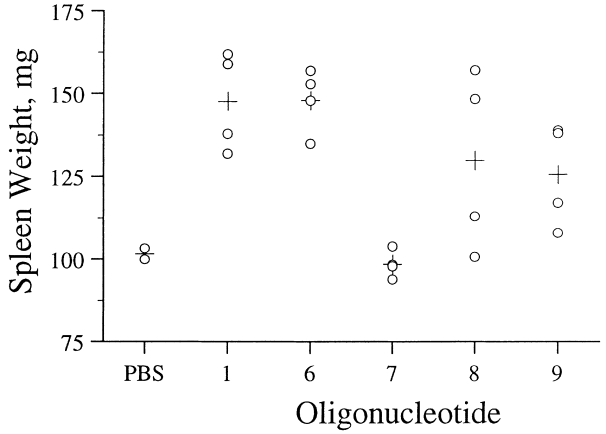

Figure 7. Spleen enlargement of mice following administration of CpR-PS-oligos. Each circle represents the spleen weight of an individual mouse and the + represents the mean spleen weight for each group.

produced about 35% increase in spleen weight at the same dose. Oligo 4, which had deoxy-N4-ethylcytidine (**6**) in place of deoxycytidine in the YpG-motif, produced about 34% increase in spleen weight at the same dose compared with the control group. These data confirm the results observed in lymphocyte proliferation assays for these oligos containing modified deoxycytidine analogues in place of natural deoxycytidine in CpG-motif.

Oligo 6, which had 7-deazaguanine in place of R in the CpR-motif, also produced about the same increase in spleen weight as that of the same dose of oligo 1, which containied natural guanine. The control oligo 7, with a 7-deazaguanine in RpC-motif, produced no change in spleen weight, suggesting that 7-deazagunine serves as a replacement for natural guanine in the CpG motif only when it is in the right position. Oligo 8, which had two CpG motifs produced about 28% increase in spleen weight, and oligo 9 with 7-deazaguanine in place of natural guanine produced a 24% increase in spleen weight. These data confirm the results observed in lymphocyte proliferation assays with oligos containing CpR-motif.

Structure–immunostimulatory activity relationships of YpG- and CpR-motif-containing-PS-oligos

Depending on the flanking sequences, CpG-motifs in oligonucleotides activate the immune system, causing lymphocyte proliferation in cell cultures and splenomegaly in mice. The presence of a methyl group at the 5-position of cytosine (deoxy-5-methylcytidine, **2**) in a CpG-motif suppresses CpG-related immunostimulatory effects of CpG-PS-oligos. Based on the results observed in our in vitro and in vivo experiments, we constructed structure–immunostimulatory activity relationships for the PS-oligos containing YpG- and CpR-motifs.

The replacement of natural deoxycytidine (**1**) in the CpG-motif with a deoxy-5-methylisocytidine (**3**) resulted in a complete loss of immunostimulatory activity, as was the case with deoxy-5-methylcytidine (**2**). This observation could be the result of switching the keto and amino groups at the 2- and 4-positions, respectively, and/or of the placement of a hydrophobic methyl group at the 5-position of cytosine. Oligo 2, containing a hydrophilic hydroxy substitution at the 5-position of cytosine (**4**) in the YpG-motif, showed immunostimulatory activity similar to that of oligo 1, which contained a natural deoxycytidine. This observation suggests that bulky hydrophilic groups are better tolerated than are hydrophobic groups at this position for immunostimulatory activity of CpG-PS-oligos. Perhaps the binding pocket of the receptor for the CpG-oligos is hydrophilic in nature and cannot accommodate a hydrophobic group at the 5-position of cytosine.

When the deoxycytidine in the CpG-motif was replaced with a deoxyuridine (**5**), in which keto groups were present at both the 2- and 4-positions, no immunostimulatory activity was observed, suggesting that a hydrogen bond donor amino group at the 4-position of cytosine is critical for immunostimulatory activity. When a large hydrophobic ethyl group was placed on the 4-amino group of cytosine in a CpG-motif, reduced lymphocyte proliferation and a slightly reduced increase in spleen weight in mice were observed. This result suggests that a bulky ethyl group at this position does not alter recognition, but slightly interfere with binding of the YpG-PS-oligo to the receptor factors responsible for immunostimulatory activity. In spite of the presence of ethyl substitution, the 4-amino group of Y-analogue **6** can participate in hydrogen bond formation with an acceptor. The YpG-PS-oligo containing the modified pyrimidine base nucleoside deoxy-P, in which the nitrogen at the 4-position was involved in ring-structure formation with the 5-position, and which could no longer serve as a hydrogen bond donor, had no lymphocyte proliferation activity in cultures. This result suggests that the hydrogen bond donor amino group at position 4 of cytosine in a CpG-motif is critical for immunostimulatory activity.

An excellent structure–activity relationship can also be correlated for CpR-motif-PS-oligos. The PS-oligo that contained inosine (**9**) in the CpR-motif, which lacks the 2-amino group, showed about 65% lower cell proliferation than did the parent oligo 1, suggesting that the functional groups at positions 1, 6, and 7 are responsible for partial recognition of and/or interaction with the protein/receptor factor. The PS-oligo that contained 2-aminopurine (**10**) in the CpR-motif, which retained hydrogen bond donor amino group at position 2, but lacked hydrogen bond donor at position 1 and hydrogen bond acceptor keto oxygen at position 6, also showed little or no cell proliferation compared with PS-oligo 1. This result suggests that functional groups at positions 1 and 6 are required for immunostimulatory activity.

The in vitro and in vivo immunostimulatory activity observed for CpR-PS-oligo 6, which contained 7-deazaguanine (**11**) in the CpR-motif, was similar to that of control PS-oligo 1, suggesting that functional groups N1, 2-NH_2, and O6, but not N7, are critical for immune stimulation. The CpR-PS-oligo that contained nebularin (**12**), which lacks a hydrogen bond acceptor keto

group at position 6, and the two hydrogen bond donor groups at positions 1 and 2, showed no activity compared with control oligo 1. This result suggests that one or more of these three functional groups at positions 1, 2, and 6 are important for immunostimulatory activity.

Substitution of natural guanine with isoguanine (**13**), in which the hydrogen bond donor amino group at position 2 and the hydrogen bond acceptor keto group at position 6 were switched to postions 6 and 2, respectively, showed no immunostimulatory activity compared with the parent PS-oligo 1. This result suggests that one or more of the functional groups at positions 1, 2, and 6 in natural guanine are critical for the immunostimulatory activity of CpG-oligos.

Replacing the hydrogen bond acceptor keto oxygen at position 6 of guanine with a hydrogen bond donor amino group (**14**) resulted in the loss of immunostimulatory activity, suggesting that the keto group at position 6 of guanine is necessary for immune stimulation. Placement of a methoxy substitution on the amino group at position 6 of 2-aminoadenine (**14**) resulted in a K-base (**15**) with a hydrogen bond acceptor group at position 6. The substitution of the K-base (**15**) for natural guanine in the CpR-motif resulted in complete loss of immunostimulatory activity, suggesting that the -*O*-Me group at this position causes steric hindrance of the binding and/or is not recognized by the protein factors, although N6 can serve as a hydrogen bond acceptor.

The PS-oligo that had 7-deazaxanthine (**16**) (which is similar to 7-deazaguanine except that the amino group at position 2 was replaced with a keto group) in place of natural guanine in the CpR-motif induced no cell proliferation. These results suggest that the presence of a keto group at position 2 is not tolerated, though the abscence of an amino group at this position is tolerated to certain extent (see Discussion for inosine (**9**) containing PS-oligos). The present studies represent the first step towards understanding the mechanism of CpG-oligo recognition of and interaction with protein/receptor factors in the immunostimulatory cascade. These studies also help in the development of non-natural CpG-motif-containing (YpG- and CpR-motifs) oligos for immunomodulation. Several other purine modifications (R) with or without N7, and pyrimidine modifications (Y) with required hydrogen bond acceptor and donor groups at appropriate positions are under study for their immunostimulatory activity in place of natural guanine and cytosine in the CpG-motif. The next logical step in the development of YpR-motif-containing-PS-oligos for immunomodulatory studies are in progress.

In conclusion, the results presented here show that the N7 of guanine is not critical for immunostimulatory activity exhibited by CpG-PS-oligos. An unnatural motif such as Cp7-deazaG can be used in PS-oligos to induce immune-related effects in vitro and in vivo. The results also show that the functional groups at the 2, 3, and 4 positions of cytosine are important for CpG-related immunostimulatory activity. A hydrophobic substitution at the 5-position of cytosine completely suppresses immunostimulatory activity of a CpG-oligo, while a hydrophilic group at this position is tolerated well. The results also indicate that a hydrogen bond donor group is more favorable at the 4-position of cytosine than a hydrogen bond acceptor group. The immunostimulatory activity of YpG- and CpR-motifs containing oligos can be modulated further as desired by incorporating appropriate chemical modifications in the 5′- and/or 3′-flanking sequence as reported recently for natural CpG-motif containing PS-oligos,[20,21] suggesting that YpG- and CpR-motifs are recognized as natural CpG-motifs in DNA.

Experimental

Oligodeoxynucleotide synthesis and purification

Oligonucleotides were synthesized using β-cyanoethyl-phosphoramidite chemistry on a PerSeptive Biosystem's 8900 Expedite DNA synthesizer on 1 μmol scale. Phosphoramidites of dA, dG, dC and T were obtained from PerSeptive Biosystems. All other modified base phosphoramidites were purchased from Glen Research or ChemGenes. Beaucage reagent was used as oxidant to obtain phosphorothioate backbone modification. Synthesis with modified bases was carried out as per specifications recommended by phosphoramidite manufacturer. After the synthesis, oligos were deprotected as required, purified by HPLC, converted to sodium form 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 (Brucker Proflex III MALDI-TOF mass spetrometer with 337 nm N2 laser). Molecular weights observed and calculated for each oligonucleotide are shown in Table 1.

Mouse lymphocyte proliferation assay

Lymphocytes obtained from BALB/c mouse (4–8 weeks) spleens were cultured in RPMI complete medium as described earlier.[16] 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-oligos or LPS (lipopolysaccharide, a positive control) were added to the cell culture in 10 μL of TE buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA) at a final concentration 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 in triplicate. The averages were calculated, normalized and presented as proliferation index.

In vivo assay of YpG- and CpR-oligonucleotides

Female BALB/c mice (4–6 weeks, 19–21 g) were divided in to different groups with four mice in each group. Oligonucleotides were dissolved in sterile PBS and administered ip to mice at a dose of 10 mg/kg. After 72 h, mice were sacrificed and spleens were harvested and weighed.

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