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Biochemical and Biophysical Research Communications 306 (2003) 948–953
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Secondary structures in CpG oligonucleotides affect immunostimulatory activity

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Received 1 May 2003

Abstract

Oligodeoxynucleotides containing CpG dinucleotides in specific sequence contexts activate the vertebrate immune system. Our previous studies showed that the 5′-end of a CpG oligonucleotide should be accessible for receptor recognition and subsequent immune stimulation. Activity is abrogated if this end is blocked by joining two CpG oligos through 5′–5′ linkage. It was not known whether a similar effect would arise from secondary structures at either end of a CpG oligo, such as hairpin loops or terminal dimers. In the present study we found that 5′-terminal secondary structures affect activity significantly more than those at the 3′-end. The need for an open 5′-end suggests that the receptor responsible for immune stimulation reads the DNA sequence from this end. These results may also provide insights to place CpG motifs appropriately in DNA vaccines to induce additional Th1 type responses.

Keywords: CpG DNA; DNA secondary structures; Hairpin-loop; Immune stimulation; NF-κB activation; Terminal dimers

Bacterial DNA, synthetic oligodeoxynucleotides, and DNA vaccines containing unmethylated CpG-dinucleotides in specific sequence contexts (CpG DNA) activate the vertebrate immune system leading to proliferation of B cells and activation of macrophages, monocytes, NK cells, and dendritic cells [1–5]. In response to CpG DNA activation, immune cells secrete a number of cytokines including IL-12, IFN-γ, IFN-α, IL-6, and TNF-α and express several costimulatory molecules [1–5]. The ability of CpG DNA to induce Th1 cytokine production that promotes CTL responses with enhanced immunoglobulin production has been used for treating a broad spectrum of disease indications including cancers, viral and bacterial infections, and inflammatory disorders and as an adjuvant in immunotherapy [1,5–8].

The presence and position of a CpG-dinucleotide and the sequences that flank it are critical for immunostimulatory activity [1–8]. Our laboratory has shown significant effects due to ribose modifications in the flanking sequences. These depend on the position and nature of substituents including 2′-*O*-methoxyethoxy and 2′- or 3′-*O*-methyl groups [9–12]. Phosphate modifications can also increase or decrease immunostimulatory activity depending on their position [13]. In addition activity can be increased by deletion of certain nucleobases or substitution of certain flanking nucleotides with linkers [14–16]. Presumably these nucleosides are not involved in immunostimulation. Although phosphorothioate-modified single-stranded CpG DNAs were extensively studied, the first reports of immune stimulation by DNA came from studies using bacterial DNA and short fragments of DNA containing palindromic sequences, which were both double-stranded structures and phosphodiester backbones [1–3]. Additionally, the presence of CpG dinucleotides in plasmid DNA has been shown to contribute significantly to the immune response produced by DNA vaccines [5,17].

Our previous studies showed that the 5′-terminus is involved in receptor recognition and that accessibility of this end is critical for activity [18–22]. This accounts for loss of activity following 5′-terminal conjugation of ligands larger than fluorescein or a 5′–5′ linked dinucleotide [23]. As 3′-conjugation is without effect, changes in

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doi:10.1016/S0006-291X(03)01080-5

uptake cannot account for the results [23]. However, there have not been any systematic studies to elude the role of secondary structure of DNA on the resulting immune response. In the present study, we investigated immunostimulation by CpG DNA with 5′- and 3′-hairpin loops or sticky ends that can form duplexes.

Materials and methods

Oligonucleotide synthesis and purification. CpG oligos were synthesized on a 1–2 μmol scale using β-cyanoethylphosphoramidites on a PerSeptive Biosystem's 8909 Expedite DNA synthesizer as described earlier. The phosphoramidites of dA, dG, dC, and T were obtained from PE Biosystems. Beaucage reagent was used as an oxidant to obtain the phosphorothioate backbone modification [24]. All oligos were deprotected using standard protocols, purified by HPLC, and dialyzed against USP quality sterile water for irrigation (Braun). The oligos were lyophilized and dissolved again in distilled water and the concentrations were determined from UV absorbance at 260 nm. All oligos were characterized by CGE and MALDI–TOF mass spectrometry (Applied Biosystem's Voyager-DE STR Biospectrometry Workstation) for purity and molecular mass, respectively. The purity of full-length oligos ranged from 90% to 96% with the rest being shorter by one or two nucleotides (n-1 and n-2) as determined by CGE and/or denaturing PAGE. All oligos contained less than 0.075 EU/mL of endotoxin as determined by the Limulus assay (Bio-Whittaker).

UV thermal melting studies. Thermal melting studies were carried out in 1 mL solution of 10 mM disodium hydrogen phosphate, pH 7.2 ± 0.2, containing 150 mM NaCl and 2 mM $MgCl_2$. The solutions were heated to 95 °C for 10 min and allowed to come to room temperature slowly before being stored at 4 °C overnight. The final concentration of oligonucleotide strand was 2.0 μM. UV thermal melting measurements were performed at 260 nm on a Perkin–Elmer Lambda 20 Spectrophotometer attached to a peltier thermal controller and a personal computer using 1 cm path length quartz cuvettes at a heating rate of 0.5 °C/min. Melting temperatures (T_m) were taken as the temperature of half-dissociation and were obtained from first derivative plots. Each T_m value is an average of two or three independent experiments and the values were within ±0.7 °C.

Cell culture conditions and reagents. Spleen cells from 4- to 8-week-old BALB/c, C57BL/6 or C3H/HeJ mice were cultured in RPMI complete medium as described earlier [9,25]. Murine J774 macrophages (American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FCS and antibiotics (100 IU/mL of penicillin G/streptomycin). All other culture reagents were purchased from Mediatech (Gaithersburg, MD).

Spleen cell proliferation assay. Typically, mouse (BALB/c) spleen cells were cultured with CpG oligos at concentrations of 0.1, 1.0, and 10.0 μg/mL for 48 h and cell proliferation was determined by [^{3}H]uridine incorporation, as described previously [9].

Cytokine ELISAs. Mouse spleen or J774 cells were plated in 24-well dishes using 5×10^6 or 1×10^6 cells/mL, respectively. The CpG oligos dissolved in TE buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA) were added to a final concentration of 0.03, 0.1, 0.3, 1.0, 3.0, or 10.0 μg/mL to the 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 oligo in triplicate for each concentration. The secretion of IL-12 and IL-6 was measured by sandwich ELISA as described previously [21]. The required reagents, including cytokine antibodies and standards, were purchased from PharMingen.

Mouse splenomegaly assay. Female BALB/c mice (4–6 weeks, 19–21 gm) were divided into groups of three mice. CpG oligos were dissolved in sterile PBS and administered intraperitoneally (ip) to mice at a dose of 5 mg/kg. The mice were sacrificed after 48 h and the spleens were harvested and weighed [9,25].

Preparation of J774 cell nuclear extracts and EMSA. NF-κB activation in J774 cells treated with CpG DNAs was carried out and analyzed by EMSA as described previously [20,21].

Results

A 17-mer phosphorothioate oligonucleotide (**1**) containing a 'GACGTT' hexameric motif was used as a positive control (Table 1). Oligonucleotides **2–7** contain additional sequences of 5, 11, or 17 nucleotides complementary to parts of **1** (Table 1). These extensions are linked either at the 3′- (**2–4**) or 5′-end (**5–7**) and contain a GAA trimer that allows formation of a stable stem-loop [26]. Formation of hairpins by **2–7** was determined by UV thermal melting experiments. The T_m values of 40–66 °C in 10 mM sodium phosphate, pH 7.2, containing 150 mM NaCl and 2 mM $MgCl_2$ suggest that **2–7** formed stable secondary structures under the experimental conditions (Table 1).

In vitro mouse splenocyte proliferation

Initially, oligos **1**, **2**, and **5** were examined for their ability to induce proliferation of BALB/c mouse spleen cells in cultures. Oligos **1** and **2** induced a dose-dependent spleen cell proliferation. The parent oligo **1**, which did not have a stem-loop structure, showed a proliferation index of 6.0 ± 0.3 at a concentration of 1.0 μg/mL (Fig. 1A). Oligo **2**, which forms a stem-loop structure at its 3′-end, gave a proliferation index of 9.0 ± 2.5 at the same concentration. Oligo **5**, which forms a stem-loop at its 5′-end, however, showed a lower proliferation index of 1.5 ± 0.3 at the same concentration, which is similar to that of PBS control (Fig. 1A).

In vivo mouse spleen enlargement

The same three oligos **1**, **2**, and **5** were administered to BALB/c mice at a dose of 5 mg/kg to determine if they induce spleen enlargement in vivo. The increase in spleen weight in response to oligo treatment compared with the control group of mice injected with PBS is considered to be a result of immunostimulatory activity of CpG oligos [9,25]. The results of in vivo studies are presented in Fig. 1B. Oligo **1**, which did not have a stem-loop structure, and oligo **5**, which had a stem-loop forming sequence at the 5′-end, increased spleen weight by about 29% and 15%, respectively, compared with the control group that received PBS. In contrast, oligo **2**, which had a stem-loop structure at the 3′-end, caused about 48% increase in spleen weight compared with the control group.

Table 1
Sequences, secondary structures, and T_ms of CpG oligonucleotides

Number	Sequence (5′ → 3′)[a]	Length	T_m (°C) [b]	Structure[c]
1	CTGTCTGACGTTCTCTG	17-mer	—	
2	CTGTCTGACGTTCTCTGGAACAGAG	25-mer	41.2	
3	CTGTCTGACGTTCTCTGGAACAGAGAACGTC	31-mer	57.7	
4	CTGTCTGACGTTCTCTGGAACAGAGAACGTCAGACAG	37-mer	66.3	
5	GACAGGAACTGTCTGACGTTCTCTG	25-mer	39.6	
6	AACGTCAGACAGGAACTGTCTGACGTTCTCTG	31-mer	56.3	
7	CAGAGAACGTCAGACAGGAACTGTCTGACGTTCTCTG	37-mer	63.2	
8	CTATCTGACGTTCTCTGT	18-mer	—	
9	CTATCTGACGTTCTCTGT-gtgatcac	26-mer	35.1	
10	gtgatcac-CTATCTGACGTTCTCTGT	26-mer	34.3	

[a] Sequences shown in uppercase indicate phosphorothioate backbone modification; CpG motifs are shown underlined; nucleotides shown in bold represent loop region; sequences shown in lowercase indicate phosphodiester modification.
[b] Measured as described in the text.
[c] Possible secondary structures formed by each oligonucleotide; box represents CpG motif and arrow indicates 5′ → 3′ directionality of the CpG motif.

A stable hairpin loop at the 5′-end, but not at the 3′-end, blocks immunostimulatory activity

CpG oligos induce a number of cytokines including IL-12 and IL-6. In BALB/c mouse spleen cell cultures test compounds **1** and **2** induced IL-12 and IL-6 by a concentration-dependent mechanism. Parent oligo **1** induced 1514 ± 113 pg/mL of IL-12 and 7681 ± 839 pg/mL of IL-6 at 3.0 μg/mL concentration (Fig. 2). Oligo **2**, containing a 3′-hairpin, induced slightly more IL-12 (1771 ± 286 pg/mL) and less IL-6 (2582 ± 300 pg/mL). However, oligo **5**, containing a 5′-hairpin, failed to induce cytokine secretion. It appears that the hairpin at this end blocks recognition just like the 5′-terminal modifications studied previously [18–23].

Duplex stem structure interferes with immune stimulation

CpG DNAs **3** and **4** have 3′-hairpin stems that extend over the GACGTT motif or reach all the way to the 5′-end. As a result, they contain two CpG motifs, with GACGTT in the top strand and its complementary,

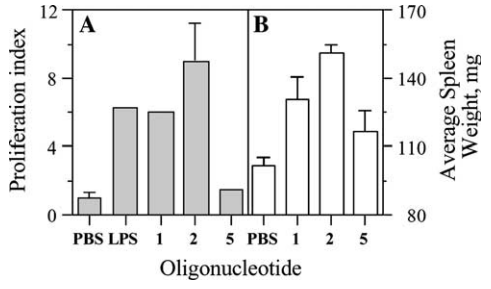

Fig. 1. (A) Cell proliferation induced by oligos **1**, **2**, and **5** in BALB/c mouse spleen cell cultures at a concentration of 1.0 μg/mL. (B) Splenomegaly induced by oligos **1**, **2**, and **5** at a dose of 5 mg/kg administered intraperitoneally to BALB/c mice. LPS : lipopolysaccharide.

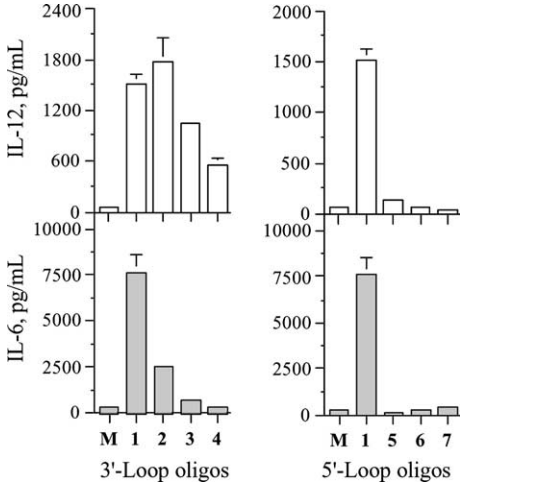

Fig. 2. Induction of cytokines IL-12 and IL-6 in BALB/c mouse spleen cell cultures after 24 h of incubation with oligonucleotides **1–7** at a concentration of 3 μg/mL.

AACGTC, in the bottom (Table 1). Oligos **6** and **7** have similarly long but 5′-hairpins. Despite having two CpG motifs, **3** and **4** induced lower IL-12 and minimal or no IL-6 compared with **1** (Fig. 2). Both **6** and **7**, with 5′-hairpins, failed to induce cytokine secretion under the same experimental conditions. Minimal cytokine induction by **4** suggests that the extension of stem structure to the 5′-end is detrimental and perhaps interferes with recognition and subsequent immune stimulation.

A duplex at the 5′- but not the 3′-end interferes with immunostimulation

As base-pairing at the 5′-end of **4** inhibited immune stimulation, duplex formation at both ends was investigated using CpG DNAs **8–10**. Oligo **8** contains 18 nucleotides and the same GACGTT motif as **1**. Self-complementary 3′- or 5′-extensions in **9** and **10** act as sticky ends to form dimers of 8 bp (Table 1). These duplexes contain phosphodiester linkages to reduce any length-dependent phosphorothioate effect on immune stimulation. Oligo **9** dimerizes at the 3′-end and showed similar IL-12 and IL-6 induction as its parent, **8** (Fig. 3). However, **10**, which forms a 5′-duplex, induced minimal IL-12 and IL-6 (Fig. 3). Thus, the ability to form a 5′-duplex strongly correlates with loss of immune stimulation.

Activation of NF-κB in J774 cells

Toll-like receptor 9 (TLR9) has been shown to recognize unmethylated CpG-dinucleotides in bacterial, plasmid, and synthetic DNAs [27] and activate stress kinase [28] and NF-κB pathways [29]. We examined the activation of NK-κB in J774 murine macrophage cell nuclear extracts by oligos **1–8** using EMSA. Fig. 4 shows the results obtained. Both parent oligos **1** and **8** activated NF-κB as indicated by the presence of a complex. Oligos **2–4**, which have loop at the 3′-end, also activated NF-κB as indicated by the presence of the appropriate complex. In contrast, 5′-end loop oligos **5–7** failed to activate the transcription factor NF-κB in J774 cells (Fig. 4). A control non-CpG oligo failed to activate NF-κB under the same experimental conditions (lane C). These results are consistent with the data obtained in mouse spleen cell culture assays.

Cytokine induction in J774 cells

We further examined the ability of oligos **1–7** to induce cytokine secretion in J774 cell cultures. The IL-12 and IL-6 data obtained at 10 μg/mL concentration of oligos (Fig. 5) complement the results obtained in splenocyte cultures. These results further confirm that the receptor reads the CpG DNA sequence from its 5′-end, and an accessible 5′-end is required for CpG DNA activity. The presence of secondary structures in oligos can interfere with receptor reading and thereby immunostimulatory activity.

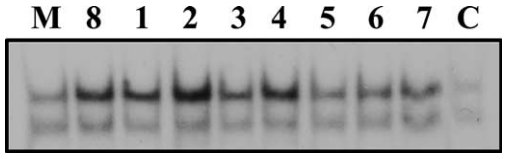

Fig. 4. Activation of NF-κB in J774 macrophages after 1 h stimulation with 10 μg/mL of oligos **1–8** as labeled with oligo numbers. M and C stand for cells treated with media only and non-CpG oligo, respectively.

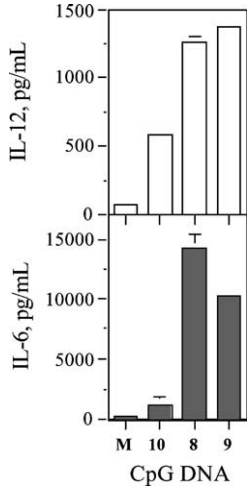

Fig. 3. Induction of cytokines IL-12 and IL-6 in BALB/c mouse spleen cell cultures after 24 h of incubation with oligonucleotides **8–10** at a concentration of 3 μg/mL.

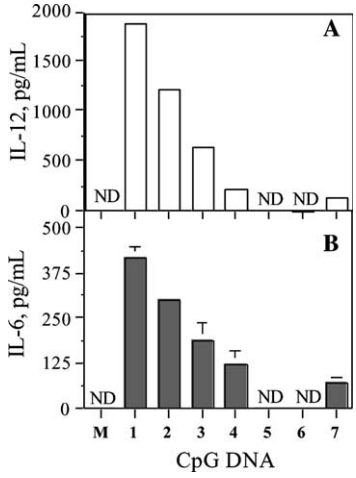

Fig. 5. Induction of cytokines IL-12 and IL-6 in J774 macrophage cell cultures at 10 μg/mL concentration of oligos. M stands for control treated with PBS. ND denotes not detected.

Discussion

Toll-like receptor 9 (TLR9) has been proposed to recognize unmethylated CpG DNA [27] and activate several signaling pathways, including stress kinase [28] and NF-κB pathways [29]. However, a direct interaction of CpG DNA with its receptor has not been established yet. Alternately, DNA protein kinase and Hsp90 have been shown to involve in CpG DNA immune stimulation [30,31], which may serve as co- or alternate receptors. CpG DNAs with different backbones, sequences, and structures activate the immune cells [3–5,32,33]. However, TLR9 in different vertebrate species exhibits selectivity for the bases flanking the CpG dinucleotide [27,34,35]. It is not known what are the precise sequence and structural requirements of the CpG DNA for this recognition and binding to the receptor factors.

We have shown previously that linking two CpG oligos through their 3′-ends facilitates increased immunostimulatory activity while activity is lost if they are linked through their 5′-ends [18–23]. This suggested that the receptor reads the CpG DNA from the 5′-end and an accessible 5′-end is required for immunostimulation. Further studies showed that 5′-accessibility is determined by the size of ligand attached at the 5′-end [23]. In the present study, we found that secondary structures in oligos such as terminal stem-loops and duplexes can significantly affect both receptor recognition and downstream cytokine induction.

The 3′-stem-loop in **2** reduced levels of IL-6 but not IL-12 compared with parent oligo **1**. As both oligos induce cytokines but with different profiles, it appears that the 3′-terminal hairpin does not interfere with CpG oligo recognition but rather modulates signal transduction. The longer stem duplexes in oligos **3** and **4**, however, appear detrimental to recognition, even though they contain an additional CpG motif. Oligos **5–7**, with 5′-stem-loop structures, induced minimal or no IL-12 and IL-6 secretion in the concentration range studied, suggesting that the receptor recognizes or reads the sequence from the 5′-end which must be accessible.

Further evidence that a 5′-terminal duplex impedes recognition and activity comes from comparing the results of oligo **10** with those of oligo that does not form duplex (**8**) or that which forms a 3′-terminal duplex (**9**). The present studies suggest that the two CpG motifs in duplex structures do not equally contribute to the activity. The additional AACGTC motif in **6** and **7** is either not recognized by the mouse cells or else is unable to overcome the inhibition due to the terminal hairpin. Oligo containing AACGTC motif in a single-stranded oligo showed minimal activity (data not shown), suggesting that the activity seen with oligos **3** and **4** comes from GACGTT motif. This may apply to DNA vaccines also in which the activity comes from only appropriate motifs and the duplex structure provides nuclease stability.

Increased resistance to degradation by 3′-exonucleases due to a 3′-hairpin might also contribute to the greater activity of **2** and **3** over **5–7** [36,37]. However, oligos **5** and **6**, which have single-stranded structures at the 3′-end, should have similar nuclease stability and therefore should have exhibited similar immunostimulatory activity as that of **1**. The results presented here, however, suggest that they do not elicit similar levels of immune responses, suggesting that the differences observed in the immunostimulatory activity of these oligos are not related to their metabolic stabilities, but to their structures.

Taken together these results suggest that the receptor recognizes or reads the CpG oligo sequence from its 5′-end which must be accessible for immunostimulation. The immunostimulatory activity of CpG oligos is minimized significantly by the presence of secondary structures at the 5′-end flanking sequence. In double-stranded DNA, GACGTT/AACGTC motifs in complementary strands are not equally immunostimulatory. Hairpin or duplex structures block access and, depending on their position and stability, they can completely prevent receptor binding or modulate signal transduction. It may be possible to change cytokine secretion profiles by optimal placement of a secondary structure at the 3′-end of the CpG oligo. Additionally, secondary structures formed at the 3′-end are also known to increase the metabolic stability of oligonucleotides [36,37].

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