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DIVISIÓN DE NUEVAS CREACIONES
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Ref.: Expediente No. ***07-003.318***
Solicitud de Patente a nombre de
IDERA PHARMACEUTICALS, INC.

Yo, ***JIMENA ESCOBAR URIBE***, identificada como aparece al pie de mi firma, domiciliada en Bogotá, en la Carrera 11 No. 86-53, piso 6, obrando como apoderada de la sociedad ***IDERA PHARMACEUTICALS, INC.,*** con domicilio en Cambridge, Massachussets, Estados Unidos, solicitante de la patente de la referencia, doy alcance a mi memorial de 7 de julio de 2008, por medio del cual se pago examen de patentabilidad. Sobre el particular informo que por un error involuntario no se señaló en dicho memorial la sociedad IDERA PHARMACEUTICALS, INC., sino la sociedad HYBRIDON, INC.

Ruego a ese despacho tener en cuenta la siguiente aclaración y continuar con el trámite correspondiente.

Señor Superintendente,

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T.P- 68.228
/srr

Industria y Comercio
SUPERINTENDENCIA

DIRECCIÓN DE NUEVAS CREACIONES

RADICACIÓN EXPEDIENTE No. 07-03318

EL EXTRACTO DE LA PRESENTE SOLICITUD DE **PATENTE DE INVENCIÓN**

FUE PUBLICADO EN LA GACETA DE PROPIEDAD INDUSTRIAL No **587** DE

FECHA **18 DE ENERO DE 2008** EL TÉRMINO HÁBIL SEÑALADO POR LA LEY

EXPIRA EL **16 DE ABRIL DE 2008**

NOTA: Dentro del plazo de los seis (6) meses siguientes a partir de la fecha de publicación en la gaceta de Propiedad Industrial, deberá solicitar y cancelar el examen de patentabilidad so pena de que la solicitud caiga en abandono, de acuerdo con lo establecido en el Artículo 44 de la Decisión 486/2000.

SE PRESENTARON OPOSICIONES POR PARTE DE TERCEROS.

SI _____ NO ____X____

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Nucleic Acids Research, 2003, Vol. 31, No. 9 2393–2400
DOI: 10.1093/nar/gkg343

Divergent synthetic nucleotide motif recognition pattern: design and development of potent immunomodulatory oligodeoxyribonucleotide agents with distinct cytokine induction profiles

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Received December 23, 2002; Revised February 25, 2003; Accepted March 7, 2003

ABSTRACT

Unmethylated CpG dinucleotides present within certain specific sequence contexts in bacterial and synthetic DNA stimulate innate immune responses and induce cytokine secretion. Recently, we showed that CpG DNAs containing two 5′-ends, immunomers, are more potent in both regards. In this study, we show that an immunomer containing a synthetic CpR motif (R = 2′-deoxy-7-deazaguanosine) is a potent immunostimulatory agent. However, the profile of cytokine induction is different from that with immunomers containing a natural CpG motif. In general, a CpR immunomer induced higher interleukin (IL)-12 and lower IL-6 secretion. Compared with conventional CpG DNAs, both types of immunomers showed a rapid and enhanced activation of the transcription factor NF-κB in J774 cells. NF-κB activation by CpG DNA corresponded to degradation of IκBα in J774 cells. All three immunostimulatory oligonucleotides activated the p38 mitogen-activated protein kinase pathway as expected. Immunomers containing CpG and CpR motifs showed potent reversal of the antigen-induced Th2 immune response towards a Th1 type in antigen-sensitized mouse spleen cell cultures. Immunomers containing a CpR motif showed significant antitumor activity in nude mice bearing MCF-7 human breast cancer and U87MG glioblastoma xenografts. These studies suggest the ability for a divergent synthetic nucleotide motif recognition pattern of the receptor involved in the immunostimulatory pathway and the possibility of using synthetic nucleotides to elicit different cytokine response patterns.

INTRODUCTION

The presence of CpG dinucleotides in certain sequence contexts in bacterial and synthetic oligodeoxyribonucleotides (CpG DNAs) is known to activate vertebrate innate immune cells and B cells (1–4). Various cytokines are secreted in the process, including interferon-γ (IFN-γ), interleukin (IL)-12, tumor necrosis factor-α (TNF-α) and IL-6, and the levels of co-stimulatory surface molecules increased (5–8). These activities depend on the sequences flanking the CpG dinucleotide (3,9–11), and we have investigated the structural and chemical characteristics of CpG DNA required for immune stimulation (12–14).

Although direct evidence for physical binding is lacking, it is believed that Toll-like receptor 9 (TLR9) recognizes CpG dinucleotides in bacterial and synthetic DNA and activates intracellular immune signaling pathways (15). Our structure–immunostimulatory activity studies showed that the receptor is highly specific for deoxyribonucleotides in CpG, as ribonucleotides or 2′-*O*-alkyl ribonucleotides abrogate immunostimulation (12). We have identified critical structural features in the pentose sugar (13,16–19), phosphate backbone (13,20), nucleobases (21,22) and nucleosides (23) required for activity. Moreover, through the use of synthetic pyrimidine (Y) and purine (R) bases in YpG and CpR motifs, we showed requirements for a functional group on cytosine and guanine in receptor recognition (24).

More importantly, our recent studies suggest that the receptor reads CpG DNA sequence from the 5′- to the 3′-end, and modifications that block the 5′-end abrogate immunostimulation (25–29). In contrast, CpG oligonucleotides with two accessible 5′-ends (immunomers) show enhanced activity (25–29). Immunomers containing phosphodiester backbones show enhanced nuclease resistance and immunostimulation without the need for palindromic or guanine-rich sequences (28). The size of ligand conjugated at the 5′-end determines accessibility. For example, fluorescein conjugation at the 5′-end does not effect cellular uptake compared with 3′-conjugate, but prevents immunostimulation (26). Immunomers containing as few as 5 or 6 nt in each

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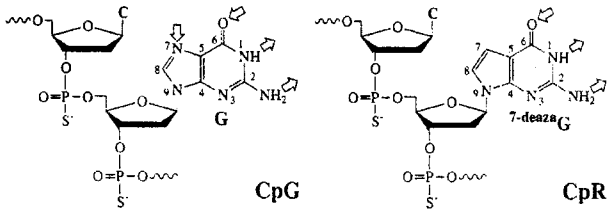

Figure 1. Structures of natural CpG and synthetic CpR immunostimulatory motifs. G and R are shown for comparison. Hydrogen bond donor and acceptor groups are shown with outward and inward arrows, respectively.

segment also show potent immunostimulation in both mouse and human systems (29).

In the present study, we demonstrate that an immunomer shows increased immunostimulatory activity compared with a conventional CpG DNA molecule containing the same 'GACGTT' motif but only one 5′-terminus. Moreover, for the first time, we show that an immunomer containing a synthetic dinucleotide CpR (R = 2′-deoxy-7-deazaguanosine) (Fig. 1) exhibits potent immunostimulatory activity. Interestingly, subtle differences were observed in cytokine secretion profiles induced by immunomers containing CpR and CpG motifs. These studies suggest that receptor response may be determined by nucleotide motif recognition patterns (NMRPs) and the receptor can recognize a variety of structurally similar nucleotide motifs.

MATERIALS AND METHODS

Synthesis and purification

CpG DNA [**1**, 5′-d(CTATCTGACGTTCTCTGT)-3′] and immunomers [**2**, 5′-d(TCTGACGTTCT-X-TCTTGCAG-TCT)-5′; **3**, 5′-d(TCTGACRTTCT-X-TCTTRCAGTCT)-5′ where X and R are glyceryl linker and 2′-deoxy-7-deaza-guanosine, respectively] were synthesized on a 1–2 μmol scale using β-cyanoethylphosphoramidite chemistry on a PerSeptive Biosystem's 8909 Expedite DNA synthesizer as described earlier (27). The phosphoramidites of dA, dG, dC and T were obtained from Applied Biosystems. 2′-Deoxy-7-deazaguanosine phosphoramidite and the solid support attached with di-4,4′-dimethoxytrityl-protected glyceryl linker were obtained from ChemGenes. Beaucage reagent was used as an oxidant to obtain the phosphorothioate backbone modification (30). After the synthesis, immunomers were deprotected using standard protocols, purified by HPLC, and dialyzed against USP quality sterile water for irrigation (Braun). The immunomers were lyophilized and dissolved again in distilled water, and the concentrations were determined by measuring the UV absorbance at 260 nm (31). All the immunomers synthesized were characterized by capillary gel electrophoresis (CGE) and matrix-assisted laser desorption ionization time of flight mass spectrometry (Applied Biosystem's Voyager-DE™ STR Biospectrometry™ Workstation) for purity and molecular mass, respectively. The purity of full-length immunomers ranged from 89 to 95%, with the rest being shorter by 1 or 2 nt (n – 1 and n – 2) as determined by CGE and/or denaturing PAGE. All CpG DNAs contained <0.1 EU/ml of endotoxin as determined by the Limulus assay (Bio-Whittaker).

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 (12,32). Murine J774 macrophage cells (American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum and antibiotics (100 IU/ml of penicillin G/streptomycin). All other culture reagents were purchased from Mediatech (Gaithersburg, MD).

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 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 the cell cultures. The cells were then incubated at 37°C for 24 h and the supernatants were collected for enzyme-linked immunosorbent assays (ELISAs). 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 was measured by sandwich ELISA as described previously (27). The required reagents, including cytokine antibodies and standards, were purchased from PharMingen.

Mouse splenomegaly assay

Female BALB/c mice (4–6 weeks, 19–21 g) were divided into groups of two or three mice. CpG DNAs were dissolved in sterile phosphate-buffered saline (PBS) and administered intraperitoneally (i.p.) to mice at a dose of 10 mg/kg. The mice were sacrificed after 40 h, the spleens were harvested and weighed as described previously (12,32) and the number of mononuclear cells was determined. Blood was withdrawn from animals by retro-orbital puncture before sacrificing for cytokine assays. The IL-12 levels in serum samples were determined by ELISA as described above.

Sensitization of mice with conalbumin

Four- to six-week-old BALB/c female mice were obtained from Taconic (Germantown, NY). The mice were given i.p. injections of 200 μg of conalbumin (Sigma) in 100 μl of PBS mixed with 100 μl of ImjectAlum adjuvant (Pierce) on days 0 and 7, and intranasally challenged on days 14 and 21. The mice were sacrificed 72 h after the last challenge by CO_2 inhalation. Spleens were excised and single cell suspensions were prepared as described above. Spleen cells were treated with immunomers at different concentrations for 2 h followed by treatment with 50 μg/ml of conalbumin. Supernatants were harvested after 72 h, and IL-5, IL-6 and IL-12 levels were measured by ELISA as described above.

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 (27).

Table 1. Induction of cytokine secretion by immunomers in mouse spleen cell cultures

CpG DNA	IL-12 (pg/ml) ± SD					IIL-6 (pg/ml) ± SD				
	0.1 μg/ml	0.3 μg/ml	1.0 μg/ml	3.0 μg/ml	10.0 μg/ml	0.1 μg/ml	0.3 μg/ml	1.0 μg/ml	3.0 μg/ml	10.0 μg/ml
BALB/c										
1	100 ± 3	546 ± 23	1313 ± 69	1775 ± 139	2732 ± 193	90 ± 0	162 ± 16	4316 ± 330	9774 ± 798	10 472 ± 264
2	1142 ± 99	3330 ± 324	3735 ± 180	3604 ± 186	3656 ± 76	240 ± 57	3836 ± 169	8601 ± 266	12 507 ± 177	12 468 ± 252
3	219 ± 9	1151 ± 30	2601 ± 197	2823 ± 134	4351 ± 113	41 ± 3	462 ± 32	4230 ± 31	10 498±438	11 890 ± 291
C	57 ± 4					7 ± 1.6				
C57BL/6										
1	130 ± 8	682 ± 2	787 ± 19	889 ± 71	948 ± 109	332 ± 88	1006 ± 125	6398 ± 536	7257 ± 132	9860 ± 964
2	1526 ± 171	1989 ± 272	1771 ± 41	1608 ± 38	1330 ± 158	1069 ± 59	5944 ± 167	12 390 ± 932	9849 ± 734	12 224 ± 131
3	454 ± 23	1528 ± 56	1478 ± 90	1487 ± 46	1816 ± 107	54 ± 12	1848 ± 154	8770 ± 659	11 149 ± 1556	13 707 ± 1660
C	71 ± 11					108 ± 38				
C3H/HeJ										
1	206 ± 23	781 ± 63	890 ± 53	931 ± 148	1120 ± 43	ND	466 ± 42	2546 ± 204	5465 ± 140	6134 ± 171
2	1394 ± 97	2183 ± 96	1935 ± 115	1770 ± 33	1433 ± 74	222 ± 23	1556 ± 35	7910 ± 182	8887 ± 171	7669 ± 251
3	487 ± 35	1124 ± 160	1428 ± 55	1414 ± 71	1484 ± 101	20 ± 6	405 ± 64	2712 ± 92	6686 ± 189	6953 ± 201
C	25 ± 6					10 ± 3				

C, no added immunomer control; ND, not detected.

Preparation of J774 cell lysates and western blotting

The levels of phosphorylated and total p38 mitogen-activated protein kinases (MAPKs) in J774 cells following CpG DNA treatment were measured by western blotting as described previously (28). All antibodies required for the assay were purchased from Cell Signaling Technology.

In vivo nude mice human cancer models and treatment plan

The animal use and care protocols were approved by the Institutional Committee on Animal Use and Care of the University of Alabama at Birmingham. Female athymic nude mice (nu/nu, 4–6 weeks old) were obtained from the Frederick Cancer Research and Development Center (Frederick, MD, USA) and inoculated with MCF-7 or U87MG cells as described previously (33,34). Tumor-bearing animals were divided randomly into various treatment groups (five/group) and treated by subcutaneous injection with immunomer **3** at a dose of 0.5 mg/kg/injection or saline (control) 3 days per week (days 1, 3 and 5). The MCF-7 study was terminated on day 15 and the U87MG study was terminated on day 42 when the tumor size reached 3000 mg in control group mice. Animals in the treatment group in the MCF-7 study received a total of six injections and those in the U87MG study received a total of 15 injections. The mice were monitored by general clinical observations, body weight and tumor growth. Tumor growth was recorded with the use of calipers, by measuring the long and short diameters of the tumor. Tumor mass (in g) was calculated using the formula $1/2a \times b^2$, where a and b are the long and short diameters (in cm), respectively.

RESULTS

CpG DNA containing two accessible 5′-ends shows greater immune stimulation

Activation of mouse (BALB/c, C57BL/6 or C3H/HeJ) spleen cells in cultures with CpG DNA for 24 h resulted in cytokine induction. In the present studies, two typical cytokines, IL-12 and IL-6, are measured. Both **1** and **2** induced concentration-dependent IL-12 and IL-6 secretion in all three strains of mouse spleen cell cultures (Table 1). Figure 2 shows a typical concentration-dependent IL-12 and IL-6 secretion in BALB/c mouse spleen cell cultures. In general, 3′–3′-linked immunomer **2** induced higher IL-12 secretion than conventional CpG DNA **1**. A control DNA molecule lacking CpG induced similar levels of IL-12 and IL-6 secretion to background values observed with medium alone (data not shown). Previously we showed that two CpG DNAs covalently attached through 5′–5′ linkages induce minimal cytokine secretion (25–28). We also demonstrated that two CpG DNAs attached through 5′–3′ linkage (containing two immunostimulatory motifs) showed no greater activity than a shorter CpG DNA containing one immunostimulatory motif (27). An undecanucleotide that corresponds to one of the segments of immunomer **2** showed minimal activity in previous studies (27). Therefore, 5′–5′-linked and 5′–3′-linked oligonucleotides were not tested further in the present study.

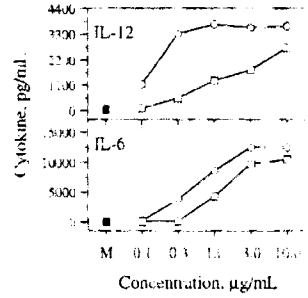

Figure 2. Induction of cytokine secretion by CpG DNAs in BALB/c mouse spleen cell cultures. BALB/c mouse spleen cells were cultured and activated with CpG DNAs **1** (squares) and **2** (circles) at various concentrations for 24 h, and the levels of secreted IL-12 (top) and IL-6 (bottom) in the culture medium were measured by ELISA. See Materials and Methods for cell culture and ELISA protocols. M stands for medium-treated control (filled square). Each value is an average of three replicates, and the results are representative of at least two independent experiments.

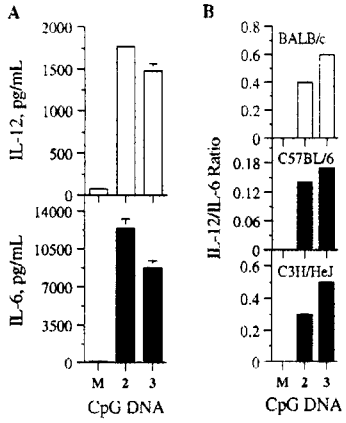

Figure 3. (A) Induction of cytokine secretion by immunomers in C57BL/6 mouse spleen cell cultures. C57BL/6 mouse spleen cells were cultured and activated with immunomers **2** and **3** at various concentrations for 24 h, and the levels of secreted IL-12 (top) and IL-6 (bottom) in the culture medium were measured by ELISA. The results shown are for 1 μg/ml concentration. See Materials and Methods for cell culture and ELISA protocols. M stands for medium-treated control. Each value is an average of three replicates, and the results are representative of at least two independent experiments. (B) The ratio of IL-12 to IL-6 induced at 1 μg/ml concentration of immunomers **2** and **3** in BALB/c, C57BL/6 and C3H/HeJ mouse spleen cell cultures.

A synthetic CpR motif shows potent immunostimulatory activity

We substituted the G of the CpG dinucleotide in **2** with R (2′-deoxy-7-deazaguanosine) to obtain compound **3** containing a synthetic immunostimulatory motif CpR (Fig. 1). In all three strains of mouse spleen cell culture assays, **3** induced IL-12 and IL-6 secretion (Table 1), suggesting that the presence of R in immunomers does not prevent binding to the receptor. Figure 3A shows the results obtained with **2** and **3** in C57BL/6 mouse spleen cell cultures. Surprisingly, activation of spleen cells with **3** resulted in similar levels of IL-12, but lower IL-6, induction compared with that with **2** in all three mouse strains tested (Table 1). The plots of ratios of IL-12 to IL-6 show the differences in cytokine induction profiles by **2** and **3** in all three strains of mouse spleen cells (Fig. 3B). Additionally, similar activity observed in lipopolysaccharide non-responsive C3H/HeJ mouse spleen cell cultures suggest that immunomers do not activate immune cells through TLR4.

In vivo effects of CpG DNAs on splenomegaly and serum IL-12 levels

To evaluate the immunostimulatory effects *in vivo*, CpG DNAs **1–3** were administered to BALB/c mice i.p. at a dose of 10 mg/kg (single dose). The increases in spleen weight, splenocyte number and IL-12 levels in serum were determined 40 h post administration (Table 2). A significant increase in spleen weights was observed in mice injected with CpG DNA compared with mice injected with PBS (86 ± 10 mg) (Fig. 4). The mice treated with CpG DNAs **1–3** showed an average increase of 300–330% in their spleen weights compared with the control group. There were also more mononuclear cells in spleens of treated mice (Table 2). After 40 h, serum IL-12 levels increased with all three CpG DNAs (Table 2). Moreover, as in cell cultures, greater IL-12 induction resulted with immunomers **2** and **3** than with **1**.

Table 2. *In vivo* effects of conventional CpG DNA and immunomers in BALB/c mice[a]

CpG DNA	Spleen weight (mg ± SD)	Mononuclear cells/spleen, ×10⁶ (% expansion)	Serum IL-12 (pg/ml ± SD)
1	259 ± 23	78 (13.0)	2202 ± 220
2	274 ± 27	121 (75.4)	4739 ± 293
3	284 ± 12	86 (24.6)	5447 ± 253
PBS	86 ± 10	69	ND

[a]A single dose of 10 mg/kg was given i.p. and the data were obtained 40 h post-administration.
ND, not detected.

Figure 4. Administration of CpG DNA to mice causes splenomegaly. Photographs of representative spleens of BALB/c mice after 40 h of treatment with a single dose of CpG DNA **1–3** or PBS as described in Materials and Methods.

Activation of NF-κB in J774 cells

The transcription factor NF-κB is activated by CpG DNA treatment of macrophages (35) and is known to induce cytokine secretion. We compared the activation of NF-κB by CpG DNAs **1–3** in J774 cells. Figure 5A shows an EMSA of J774 nuclear extracts treated with an NF-κB-binding oligonucleotide probe. All three CpG DNAs activated the transcription factor, as shown in Figure 5B. However, significant differences are seen in the kinetics of activation of NF-κB (Fig. 5A) by **1** (lane 2) and immunomers **2** (lane 3) and **3** (lane 4). Immunomers **2** and **3** showed strong activation of NF-κB within 30 min, while **1** showed minimal activation in this time. After 1 h, significant activation of NF-κB was found with all three CpG DNAs **1–3**. However, immunomers **2** and **3** showed relatively more potent activation of NF-κB at this time also compared with **1** (Fig. 5A). After 3 h, all three CpG DNAs showed a similarly strong NF-κB presence in J774 cell nuclear extracts. Importantly, both **2** and **3**, containing natural CpG and synthetic CpR motifs, respectively, showed similar levels of NF-κB activation.

Degradation of IκBα in J774 cells

By interacting with NF-κB, IκBs inhibit the translocation of NF-κB to the nucleus and thereby its binding to DNA (36,37). Therefore, the presence of NF-κB in nuclear extracts in

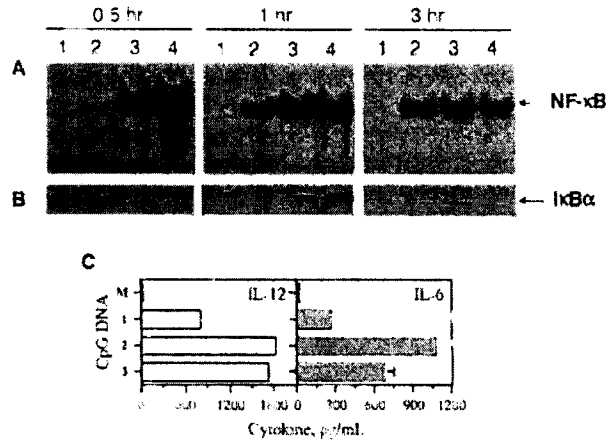

Figure 5. (**A**) Activation of the transcription factor NF-κB in J774 macrophage cells upon treatment with CpG DNAs. J774 cell cultures were treated with PBS (lane 1) or 10 μg/ml of CpG DNAs **1** (lane 2), **2** (lane 3) or **3** (lane 4) for 0.5, 1 or 3 h, and nuclear extracts were isolated, treated with a ^{32}P-labeled DNA probe containing NF-κB-binding sequence and analyzed by EMSA as described in Materials and Methods. (**B**) Degradation of IκBα in J774 macrophage cells upon treatment with CpG DNAs. J774 cell cultures were treated with PBS (lane 1) or 10 μg/ml of CpG DNAs **1** (lane 2), **2** (lane 3) or **3** (lane 4) for 0.5, 1 or 3 h, and cytoplasmic fractions were extracted, and analyzed as described in Materials and Methods. (**C**) Induction of cytokine secretion by immunomers in J774 macrophage cell cultures. J774 cells were cultured and activated with CpG DNAs **1**–**3** at various concentrations for 24 h and the levels of secreted IL-12 (left) and IL-6 (right) in the culture medium were measured by ELISA. The results shown are for 10 μg/ml concentration. See Materials and Methods for cell culture and ELISA protocols. M stands for medium-treated control. Each value is an average of three replicates, and the results are representative of at least two independent experiments.

general should correspond to the degradation of IκBs, although recent studies indicate that NF-κB activation could also occur without IκB degradation (38). We evaluated whether CpG DNA-mediated activation of NF-κB correlates with an enhanced and sustained degradation of IκBα in J774 cells. A western blot assay was performed on cytoplasmic extracts using specific antibodies against murine IκBα. As shown in Figure 5B, IκBα levels were only marginally affected by treatment with **1** for 30 min, corresponding to the minimal activation of NF-κB (Fig. 5A). After 1 h, IκBα had completely disappeared, but it reappeared after 3 h. In the case of treatment with immunomers **2** and **3**, IκBα was rapidly degraded within 30 min and reappeared after 1 h.

Activation of the p38 MAPK pathway in J774 cells

CpG DNA is also known to activate the stress-activated p38 MAPK pathway (39,40). To evaluate whether immunomers **2** and **3** activate the p38 MAPK pathway similarly to **1**, we measured the status of p38 phosphorylation in J774 cells following treatment with CpG DNAs for 30 min. All three CpG DNAs induced phosphorylation of p38 in J774 cells to similar extents (Fig. 6), although significant differences were observed in NF-κB activation.

Consistent with rapid and strong activation of NF-κB by immunomers **2** and **3**, higher levels of IL-12 and IL-6 induction were found in J774 cell cultures (Fig. 5C).

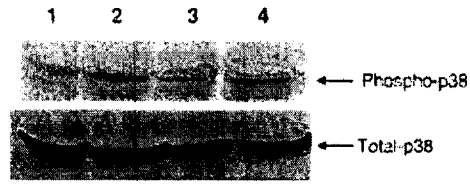

Figure 6. p38 phosphorylation in J774 macrophages. J774 cells were cultured, treated with PBS (lane 1) or 10 μg/ml of CpG DNAs **1** (lane 2), **2** (lane 3) or **3** (lane 4) for 0.5 h, and cell lysates were extracted and analyzed by western blotting using a phospho-p38 MAPK antibody as described in Materials and Methods. Total cellular p38 content is shown in the lower panel.

Effect of immunomers on cytokine induction in conalbumin-sensitized mouse spleen cell cultures

CpG DNA induces predominantly Th1-type immune responses. It has been shown that CpG DNA treatment reverses established antigen-induced Th2 responses to Th1-type responses (41,42). To assess the effects of treatment of immunomers on Th2 cytokines associated with allergic airway responses, we measured IL-5, IL-12 and IL-6 secreted in spleen cell cultures obtained from BALB/c mouse sensitized and challenged with conalbumin. In the absence of immunomer treatment, conalbumin-sensitized mouse spleen cells secreted markedly higher levels of IL-5, and low IL-12, suggesting predominantly a Th2 polarization (Table 3). When the allergen-primed spleen cells were treated with immunomers, a concentration-dependent decrease in IL-5 and increase in IL-12 secretion was observed. Table 3 shows the levels of IL-5, IL-12 and IL-6 induced following treatment with 1.0 μg/ml concentration of immunomers. These data suggest that immunomers not only induce Th1-type cytokine secretion but potently reverse antigen-induced Th2 responses. Significantly, in these assays, immunomer **3** containing the CpR motif also induced lower levels of IL-6 compared with immunomer **2** containing the natural CpG motif.

Antitumor activity in nude mice bearing human tumor xenografts

CpG DNAs have been shown to induce antitumor activity *in vivo* (28,29,43). Since the immunomer containing the synthetic CpR motif (3) induced potent immunostimulatory activity in cell culture assays, we evaluated its antitumor activity *in vivo*. Compound **3** was administered subcutaneously three times a week at a dose of 0.5 mg/kg to nude mice bearing MCF-7 human breast tumor or U87MG human glioblastoma xenografts that express wild-type p53. Growth of MCF-7 tumors was inhibited by 60% on day 15 compared with the saline control (Fig. 7). At the same dose, **3** inhibited growth of the glioblastoma by 63% on day 42 compared with the saline control (Fig. 7). At this dose, **3** did not cause any treatment-related toxicity in mice. There was no difference in body weight gains between treated animals and controls (data not shown). These preliminary antitumor studies suggest that immunomers containing synthetic CpR motifs exhibit potent antitumor activity *in vivo*.

Table 3. Cytokine induction by CpG DNA and immunomers in conalbumin-sensitized BALB/c mouse spleen cell cultures

CpG DNA[a]	IL-5 (pg/ml ± SD)	IL-12 (pg/ml ± SD)	IL-6 (pg/ml ± SD)
1	567 ± 148	647 ± 102	1246 ± 109
2	131 ± 36	1660 ± 56	9567 ± 940
3	325 ± 7	1508 ± 98	4382 ± 694
Medium	1345 ± 437	45 ± 9	396 ± 12

[a]Conalbumin-sensitized BALB/c mouse spleen cells were cultured, treated with different concentrations of CpG DNAs and the culture supernatants were assayed for the cytokines by ELISA as described in Materials and Methods. Data shown are for 1 μg/ml concentration of CpG DNA and immunomers. Each value is an average of three readings.

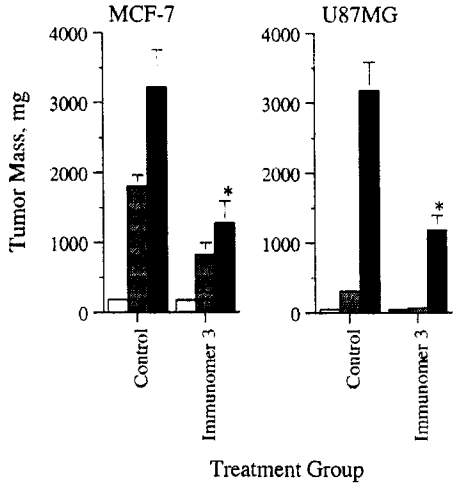

Figure 7. *In vivo* antitumor activity. Nude mice bearing MCF-7 human breast cancer (left panel) or U87MG glioblastoma (right panel) xenografts were treated with saline or immunomer **3** as specified in Materials and Methods. Data are shown for days 0, 9 and 15 for MCF-7, and for days 0, 28 and 42 for U87MG studies. Each group had six mice. Immunomer was given subcutaneously on every other day starting at day 0. *Represents a statistically significant value ($P < 0.01$).

DISCUSSION

Bacterial and synthetic CpG DNAs promote B cell proliferation, immunoglobulin production and the secretion of a number of cytokines, including IL-12, IFN-γ, IL-6 and TNF-α, from B cells, monocytes/macrophages, dendritic cells and natural killer cells. It has been shown that a receptor, TLR9, is engaged in the recognition of CpG DNA and subsequent initiation of the signaling cascade leading to the activation of the transcription factor NF-κB. In this study, we show that immunomers containing two accessible 5′-ends are more immunostimulaotry than a conventional CpG DNA, and the receptor recognizes a synthetic CpR motif in immunomers similarly to the natural CpG motif.

Our recent studies showed that covalent conjugation of molecules such as fluorescein at the 5′-end of a CpG DNA molecule abrogated the immunostimulatory activity, leaving uptake unaffected compared with the 3′-conjugate (26). Earlier studies of CpG DNAs containing multiple copies of CpG motifs showed no increased activity (25–27). From these results and those reported here, we conclude that the increased activity of **2** and **3** is not due to the presence of two copies of the CpG or CpR dinucleotide motif but to the presence of two 5′-accessible ends in the molecule. If this were not so, then similar cytokine induction would be expected if **1** were used at double the concentration of **2**, giving the same concentration of CpG dinucleotide motifs. However, this is not the case. Additionally, immunomers linked 5′–5′ are inactive or much less active than 3′–3′-linked analogs or conventional CpG DNAs (25–27).

Compared with conventional CpG DNAs, a single administration of immunomer to mice resulted in significantly greater spleen enlargement, mononuclear cell expansion in spleen and serum IL-12 levels (44). A control immunomer lacking CpG was inactive, showing the need for this motif (data not shown). The greater systemic effects of immunomers could be due to (i) the presence of two accessible 5′-ends (25–29), and (ii) increased *in vivo* metabolic stability from a lack of 3′-ends for nuclease degradation (28,29,45,46).

Following recognition of CpG DNA by TLR9, a number of cellular events are initiated, including recruitment of MyD88, IRAK and TRAF6, and activation of IκB kinase, MAPK, the stress kinases N-terminal c-Jun kinases JNK-1 and -2, and p38, leading to the activation of multiple transcription factors ATF-2, AP-1 and NF-κB. The transcription factors activated ultimately stimulate the synthesis of a number of cytokines and expression of co-stimulatory molecules. Like CpG DNA **1**, immunomers activated NF-κB and p38 MAPK pathways in J774 cells. However, the kinetics of activation in the two cases are distinctly different. Generally, activation of NF-κB in J774 cells is more rapid and sustained with immunomers **2** and **3** than with **1**. However, similar levels of p38 phosphorylation are observed with both immunomers and **1** after 1 h. Further kinetic studies are underway to distinguish any differences in the activation of the stress-induced MAPK pathway.

Only unmethylated CpG dinucleotide motifs have been shown to induce immunostimulatory effects. We and others have shown that the presence of a methyl group at the 5-position of cytosine abrogates immunostimulatory activity (12). Through the use of synthetic pyrimidines in place of cytidine, we showed that 5-hydroxyl and *N*4-ethyl substituents do not interfere with receptor recognition and immunostimulation (24). Similar studies with guanosine analogs showed the need for hydrogen bond donors at positions 1 and 2, and a hydrogen bond acceptor at position 6. However, the hydrogen bond-accepting N7 atom is not essential, and a 2′-deoxy-7-deaza-guanosine can be used in the synthetic immunostimulatory motif CpR (see Fig. 1 for structures) (24).

Here, we provide the first evidence that the CpR motif in immunomers is not only recognized by the receptor but induces a different cytokine secretion profile from that of the natural CpG motif. In both *in vitro* and *in vivo* studies, CpG immunomer **2** induced higher IL-12 and IL-6 than did conventional CpG DNA **1**. Importantly, compared with **2**, the CpR immunomer **3** consistently induced significantly lower levels of IL-6, but similar levels of IL-12 *in vitro* and *in vivo*. Elevated levels of IFN-γ expected with increased IL-12 expression were also observed [(27), and data not shown]. In allergen-sensitized mouse spleen cell cultures, both immunomers showed a potent inhibition of IL-5 secretion

compared with CpG DNA **1**. Immunomers showed similar IL-12 and IL-6 secretion profiles in Th2 polarized mouse spleen cell cultures to those found in normal mouse spleen cell cultures and *in vivo*. In addition to different cytokine secretion profiles, immunomer **3** showed a greater antigen-specific IgG2a/IgG1 antibody ratio compared with immunomer **2** (D.Wang and S.Agrawal, unpublished data), suggesting that the CpR motif is recognized by the receptor but results in distinct downstream effects compared with the normal CpG motif-containing immunomer. The kinetics of activation of NF-κB are similar with both immunomers, and it is not clear how they produce distinct cytokine expression profiles. Detailed mechanistic studies are required with different subsets of immune cells to distinguish the downstream events. Nonetheless, recognition of both natural CpG and synthetic CpR motifs suggests alternative NMRPs for the receptor, although the involvement of a related receptor cannot be ruled out. TLR3 that belongs to the same family specifically recognizes viral dsRNA and synthetic poly(I)·poly(C) (47). Greater IL-12 induction by immunomers could be of importance for cancer therapy (43,48) as well as for adjuvant action (49,50) with vaccines, allergens and monoclonal antibodies in therapy and prophylaxy. Induction of lower IL-6 and TNF-α levels [(27), and data not shown] by synthetic CpR immunomers may permit development of immunotherapeutic agents with reduced cytokine-related toxicities (51,52).

In conclusion, we have shown that immunomers induce rapid and sustained immunostimulation with distinct cytokine secretion profiles both *in vitro* and *in vivo*. Their increased activity results from two accessible 5′-ends and improved metabolic stability *in vivo*. For the first time, we have shown that the receptor recognizes a synthetic CpR motif and that alternative NMRPs produce different cytokine secretion profiles. The ability of the novel synthetic motif to induce immune stimulation resulted in potent antitumor activity in nude mice bearing MCF-7 human breast tumor and U87MG human glioblastoma xenografts. Additionally, no treatment-related toxcity was observed in mice at the dose studied. A number of other purine and pyrimidine analogs that are recognized by the receptor are under study currently for their distinct immunological properties. These studies will not only permit development of tailor-made immunotherapeutic agents for specific diseases but also illuminate receptor–CpG DNA recognition and signaling at the molecular level.

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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.[2–4]

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

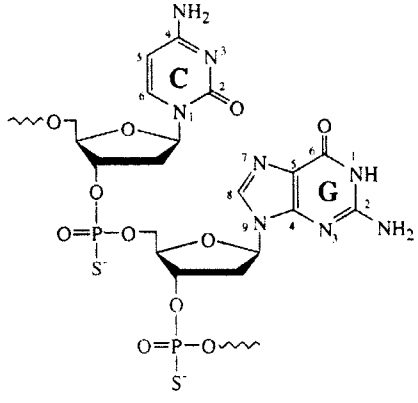

Figure 1. Chemical structure of a CpG-motif showing functional groups on cytosine and guanine that serve as hydrogen bond acceptor and donor groups.

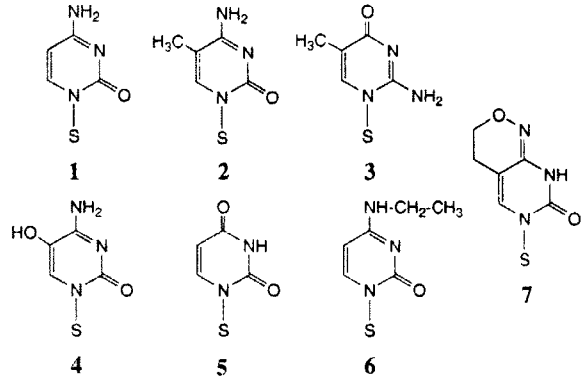

Figure 2. Chemical structures of cytosine (**1**) and its analogues (**2–7**) used in the study. S represents deoxyribose.

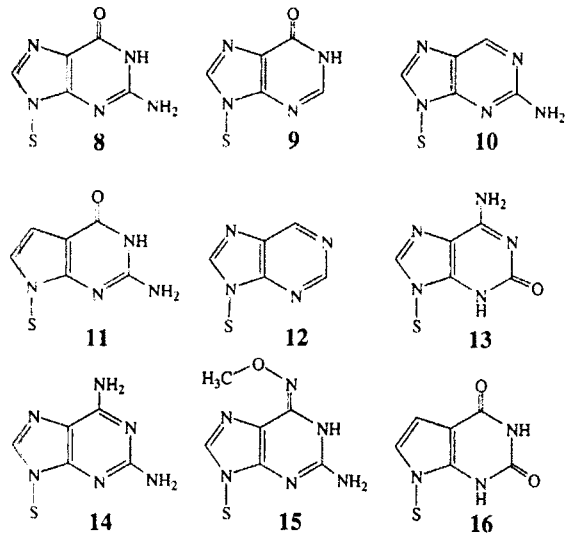

Figure 3. The structures of natural guanine (1) and the other purine analogues 2–9 used in the study. S represents 2′-deoxyribose.

(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(CTATCTGACGTTCTCTGT)	5704	5705
2	d(CTATCTGAYGTTCTCTGT)	5720	5721
3	d(CTATCTGACYTTCTCTGT)	5681	5681
4	d(CTATCTGAYGTTCTCTGT)	5733	5733
5	d(CTATCTGACYTTCTCTGT)	5694	5693
6	d(CTATCTGACRTTCTCTGT)	5704	5704
7	d(CTATCTGARCTTCTCTGT)	5705	5704
8	d(TCTCCCAGCGTGCGCCAT)	5684	5685
9	d(TCTCCCAGCRTGCRCCAT)	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

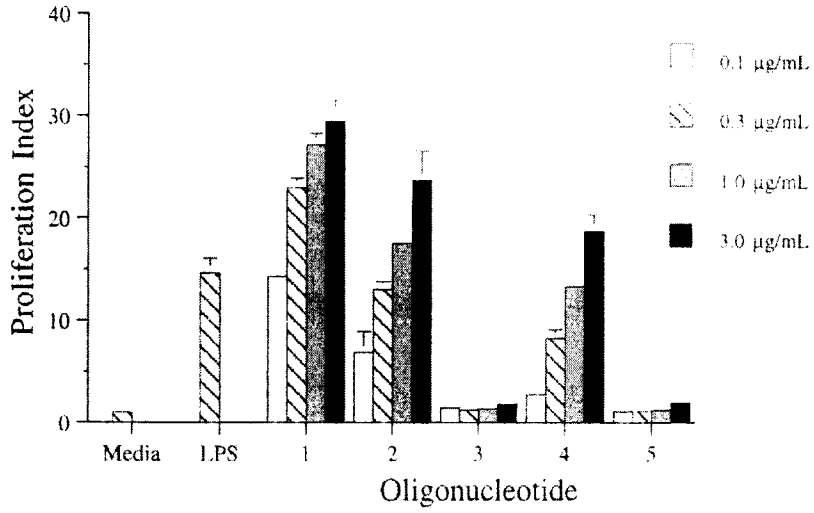

Figure 4. Lymphocyte proliferation of YpG-PS-oligos in mouse spleen lymphocyte cultures at indicated concentrations. LPS stands for lipopolysaccharide.

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.

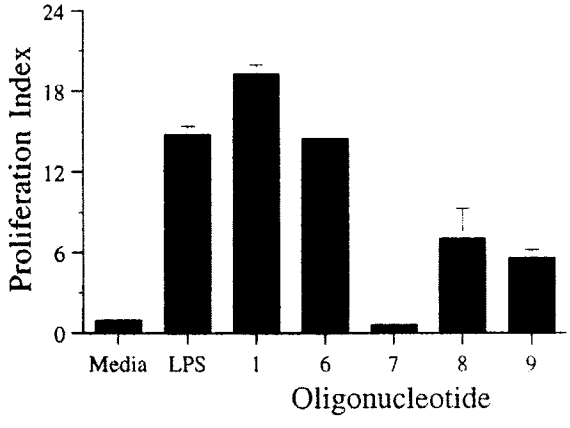

Figure 5. Lymphocyte proliferation of CpR-PS-oligos in mouse spleen lymphocyte cultures at 1.0 μg/mL concentration. LPS stands for lipopolysaccharide.

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,

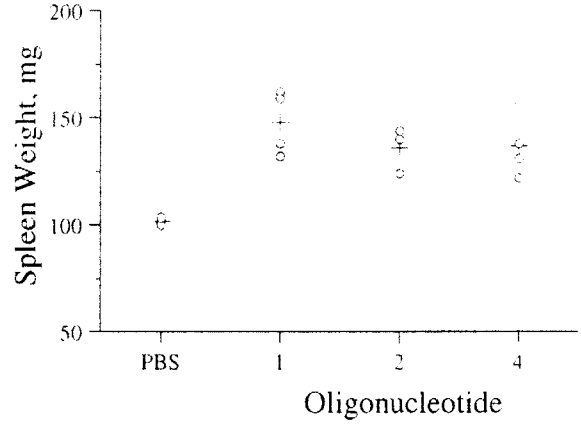

Figure 6. Spleen enlargement of mice following administration of YpG-PS-oligos. Each circle represents the spleen weight of an individual mouse and the – represents the mean spleen weight for each group.

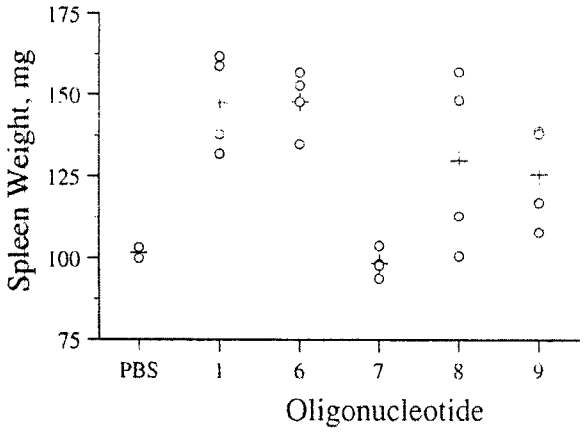

Figure 7. Spleen enlargement of mice following administration of CpR-PS-oligos. Each circle represents the spleen weight of an individual mouse and the + represents the mean spleen weight for each group.

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 contained 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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