'Immunomers'—novel 3′–3′-linked CpG oligodeoxyribonucleotides as potent immunomodulatory agents

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ABSTRACT

Oligodeoxyribonucleotides containing CpG di-nucleotides (CpG DNAs) are currently being evaluated as novel immunomodulators in clinical trials. Recently, we showed that an accessible 5′ end is required for immunostimulatory activity and blocking the 5′ end of CpG DNA by conjugation of certain ligands abrogates immunostimulatory activity. Based on these results, we designed and synthesized 3′–3′-linked CpG DNAs that contained two or more identical CpG DNA segments, referred to here as 'immunomers'. The use of solid support bearing diDMT-glyceryl-linker permitted convenient synthesis of immunomers with both segments synthesized simultaneously, giving better yields and purity. The *in vitro* and *in vivo* studies suggest that as a result of accessibility to two 5′ ends for recognition, immunomers show an enhanced immunostimulatory activity compared with linear CpG DNAs. We also studied the suitability of a number of different linkers for attaching the two segments of immunomers. A C3-linker was found to be optimal for joining the two segments of immunomers. Incorporation of multiple linkers between the two segments of immunomers resulted in different cytokine profiles depending on the nature and number of linkers incorporated. Additionally, the length of immunomer also plays a significant role in inducing immune responses. An immunomer containing 11 nt in each segment showed the highest activity and an 11mer linear CpG DNA failed to stimulate an immune response. These results suggest that immunomers have several advantages over conventional linear CpG DNAs for immunomodulatory activity studies.

INTRODUCTION

The presence of CpG dinucleotides in certain sequence contexts in bacterial and synthetic oligonucleotides (CpG DNAs) are known to activate vertebrate innate immune system cells and B cells (1–4). The activation of immune cells by CpG DNA induces secretion of a myriad of cytokines, including IL-6, IL-12, TNF-α and IFN-γ, and stimulates expression of co-stimulatory surface molecules (5–8). The presence of a CpG dinucleotide and the sequences flanking it play a critical role in determining the immunostimulatory activity of CpG DNAs (3,9–11). The use of CpG DNAs as antitumor, anti-infectious, anti-asthmatic and anti-inflammatory agents, and as adjuvants in immunotherapy has been reported (12–14).

Evidence suggests that TLR9, a molecular pattern recognition receptor, recognizes CpG dinucleotides present in DNA and activates intracellular immune signaling pathways (15). Substitution of C and G nucleotides in a CpG dinucleotide with ribonucleotides or 2′-*O*-alkyl substituted ribonucleotides neutralizes immune responses, suggesting that the receptor has high specificity for deoxyribonucleotides in a CpG dinucleotide (16). Our laboratory has been studying sequence and structural changes in CpG DNA that might potentiate or neutralize immunostimulatory activities in a predictable fashion (17). In addition, these studies could lead to the understanding of the molecular basis of recognition between CpG DNA and its receptor(s). Our studies have shown a number of critical structural and functional group requirements of the pentose sugar (16,18–20), phosphate backbone (16,21) and nucleobases (22) for the immunostimulatory activity of CpG DNA. Moreover, we demonstrated the importance of functional groups on cytosine and guanine required for receptor recognition by substitution of natural cytosine and guanine with synthetic pyrimidine and purine bases in a CpG dinucleotide, thereby introducing synthetic motifs referred to as YpG and CpR motifs (23).

Recently, we showed that when two CpG DNA molecules were linked through their 5′ ends, immunostimulatory activity was abrogated compared with a 5′→3′ CpG DNA containing the same number of CpG dinucleotides (24,25). In contrast, an increased immunostimulatory activity was observed when they were linked through their 3′ ends. Based on these observations, we postulated that an accessible 5′-end of CpG DNA was required for recognition by receptor(s) and subsequent initiation of the immunostimulatory signaling cascade. In the present paper, we describe a number of 3′–3′-linked CpG DNAs, referred to here as 'immunomers', to establish the

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Table 1. Phosphorothioate immunomers and related CpG DNAs showing the number and position of linker

CpG DNA number	Sequence	Linker (**X**)[a]	Length[b]	Molecular Weight Calculated	Found[c]
10	5′-CTATCTGA<u>CG</u>TTCTCTGT-3′	None	18mer	5704	5705
11	(5′-CTATCTGA<u>CG</u>TTCTCTGT)₂-**X**-C-5′	Glyceryl linker (**2**)	18mer	11945	11943
12	(5′-CTATCTGA<u>CG</u>TTCTCTGT)₂-**X**-C-5′	Long linker (**3**)	18mer	12158	ND
13	(5′-CTATCTGA<u>CG</u>TTCTCTGT)₃-**X**	Long linker (**3**)	18mer	17635	ND
14	5′-CTGA<u>CG</u>TTCTCTGT-3′	None	14mer	4429	4428
15	5′-CTGA<u>CG</u>TTCTCTGT-**X**-TGTCTCTT<u>GC</u>AGTC-5′	Glyceryl linker (**2**)	14mer	9106	ND
16	(5′-CTGA<u>CG</u>TTCTCTGT)₂-**X**-C-5′	Long linker (**3**)	14mer	9609	ND
17	5′-TCTGA<u>CG</u>TTCTCTGT-3′	None	15mer	4749	4746
18	5′-TCTGA<u>CG</u>TTCT-3′	None	11mer	3458	3457
19	5′-GA<u>CG</u>TTCT-3′	None	8mer	2513	2512
20	5′-CTGA<u>CG</u>TTCTCTG-**X**-GTCTCTT<u>GC</u>AGTC-5′	Glyceryl linker (**2**)	13mer	8465	8455
21	5′-CTGA<u>CG</u>TTCTCT-**X**-TCTCTT<u>GC</u>AGTC-5′	Glyceryl linker (**2**)	12mer	7775	7772
22	5′-TCTGA<u>CG</u>TTCT-**X**-TCTT<u>GC</u>AGTCT-5′	Glyceryl linker (**2**)	11mer	7164	7166
23	5′-CTGA<u>CG</u>TTCT-**X**-TCTT<u>GC</u>AGTC-5′	Glyceryl linker (**2**)	10mer	6524	6522
24	5′-TGA<u>CG</u>TTCT-**X**-TCTT<u>GC</u>AGT-5′	Glyceryl linker (**2**)	9mer	5914	5913
25	5′-GA<u>CG</u>TTCT-**X**-(T-5′)TCTT<u>GC</u>AG-5′	Glyceryl linker (**2**)	8mer	5594	5593
26	5′-TCTGAGCTTCT-**X**-TCTTCGAGTCT-5′	Glyceryl linker (**2**)	11mer	7164	7166
27	5′-CTGA<u>CG</u>TTCT-**X**-TCTT<u>GC</u>AGTC-5′	Phosphorothioate (**1**)	10mer	6354	6354
28	5′-CTGA<u>CG</u>TTCT-**X**-TCTT<u>GC</u>AGTC-5′	C2-linker (**4**)	10mer	6494	6519
29	5′-CTGA<u>CG</u>TTCT-**X**-TCTT<u>GC</u>AGTC-5′	C3-linker (**5**)	10mer	6508	6503
30	5′-CTGA<u>CG</u>TTCT-**X**-TCTT<u>GC</u>AGTC-5′	C4-linker (**6**)	10mer	6522	6515
31	5′-CTGA<u>CG</u>TTCT-**X**-TCTT<u>GC</u>AGTC-5′	C12-linker (**7**)	10mer	6634	6629
32	5′-CTGA<u>CG</u>TTCT-**X**-TCTT<u>GC</u>AGTC-5′	Spacer18 (**8**)	10mer	6714	6710
33	5′-CTGA<u>CG</u>TTCT-**X**-TCTT<u>GC</u>AGTC-5′	Abasic linker (**9**)	10mer	6550	6547
34	5′-CTGA<u>CG</u>TTCT-**XX**-TCTT<u>GC</u>AGTC-5′	C3-linker (**5**)	10mer	6662	6660
35	5′-CTGA<u>CG</u>TTCT-**XXX**-TCTT<u>GC</u>AGTC-5′	C3-linker (**5**)	10mer	6816	6814
36	5′-CTGA<u>CG</u>TTCT-**XX**-TCTT<u>GC</u>AGTC-5′	Abasic linker (**9**)	10mer	6746	6743
37	5′-CTGA<u>CG</u>TTCT-**XXX**-TCTT<u>GC</u>AGTC-5′	Abasic linker (**9**)	10mer	6942	6939
38	3′-C-**X**-(CTATCTGA<u>CG</u>TTCTCTGT-3′)₂	Glyceryl linker (**2**)	18mer	11945	ND
39	5′-CTGA<u>CG</u>TTCTCTGTCTTGTCCTGA<u>CG</u>TTCTCTGT-3′	None	34mer	10852	ND

[a]See Figure 1A for chemical structures of linkers **1**–**9**.
[b]length of each segment in 3′–3′- or 5′–5′-linked CpG DNA excluding linker.
[c]ND, not determined.

nature and length of linker required for tethering CpG DNAs and report the optimal length of the immunomers required for immunostimulatory activity.

MATERIALS AND METHODS

CpG DNA and immunomer synthesis and purification

CpG DNAs and immunomers were synthesized on a 1–2 μmol scale using β-cyanoethylphosphoramidite chemistry on a PerSeptive Biosystem's 8909 Expedite DNA synthesizer as described earlier (24–27). The 3′-phosphoramidites of dA, dG, dC and T were obtained from PE Biosystems. The 5′-phosphoramidites of dA, dG, dC, and T were obtained from Glen Research Corporation. DiDMT protected glyceryl linker attached to CPG-solid-support was obtained from ChemGenes. Symmetric doubler (long linker, **3**) phosphor-amidite was obtained from Glen Research Corporation. Beaucage reagent was used as an oxidant to obtain the phosphorothioate backbone modification (28). After the synthesis, immunomers were deprotected using standard protocols, purified by HPLC and dialyzed against United States Pharmacopea 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 (29). All the immunomers synthesized were characterized by CGE and MALDI–TOF mass spectrometry (Applied Biosystem's

Voyager-DE™ STR Biospectrometry™ Workstation) for purity and molecular mass (Table 1), respectively. The purity of full-length immunomers ranged from 89 to 95% with the rest being shorter by one or two nucleotides (n–1 and n–2) as determined by CGE and/or denaturing PAGE.

Cell culture conditions and reagents

Spleen cells from 4–8 week old BALB/c mice were cultured in RPMI complete medium as described earlier (16,30). Murine macrophage-like cells, J774 (American Type Culture Collection, Rockville, MD), were cultured in Dulbecco's modified Eagles 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).

ELISAs for IL-12, IFN-γ, IL-10, TNF-α and IL-6

BALB/c mouse spleen or J774 cells were plated in 24-well dishes at a density of 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 mouse spleen cell cultures to give final concentrations of 0.03, 0.1, 0.3, 1.0, 3.0 or 10.0 μg/ml. With J774 cell cultures, final concentrations of CpG DNA were 1.0, 3.0 or 10.0 μg/ml. The cells were then incubated at 37°C for 24 h and the supernatants were collected for ELISAs. The experiments with each CpG DNA were repeated two or three times using triplicates of each concentration. The secretion of IL-12, IFN-γ, IL-10, TNF-α

and IL-6 was measured by sandwich ELISA. The required reagents, including cytokine antibodies and standards were purchased form BD PharMingen. ELISA plates (Costar) were incubated with appropriate antibodies at 5 µg/ml in PBSN buffer (PBS/0.05% sodium azide, pH 9.6) overnight at 4°C and then blocked with PBS/1% BSA at 37°C for 30 min. Cell culture supernatants and cytokine standards were appropriately diluted with PBS/1% BSA, added to the plates in triplicate, and incubated at 25°C for 2 h. Plates were washed and incubated with 1 µg/ml of appropriate biotinylated antibody and incubated at 25°C for 1.5 h. The plates were washed extensively with PBS/0.05% Tween-20 and then further incubated at 25°C for 1.5 h after the addition of streptavidine-conjugated peroxidase (Sigma). The plates were developed with Sure Blue™ (Kirkegaard and Perry) chromogenic reagent and the reaction was terminated by adding Stop Solution (Kirkegaard and Perry). The color change was measured on a Ceres 900 HDI Spectrophotometer (Bio-Tek Instruments) at 450 nm. The levels of IL-12, IFN-γ, IL-10, TNF-α and IL-6 in the cell culture supernatants were calculated from the standard curve constructed under the same experimental conditions for IL-12, IFN-γ, IL-10, TNF-α and IL-6, respectively.

Mouse splenomegaly assay

Female BALB/c mice (4–6 weeks, 19–21 g) were divided into groups of three mice. CpG DNAs were dissolved in sterile PBS and administered subcutaneously (SC) to mice at a dose of 5 mg/kg. After 4 days, mice were sacrificed and the spleens were harvested and weighed.

Preparation of J774 cell nuclear extracts and EMSA

For NF-κB activation studies, J774 cells were plated at a density of 5×10^6 cells/well in six-well plates and nuclear extracts were prepared as described earlier (31). Briefly, after stimulation of J774 cells for 1 h with CpG DNAs at a concentration of 10 µg/ml, cells were washed three times in ice-cold PBS, harvested, and suspended in 0.24 ml of buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT and protease inhibitor cocktail) (Boehringer-Mannheim) and allowed to swell on ice for 15 min. Then 16 µl of 10% Nonidet-P40 was added and the tubes were vortexed vigorously for 10 s. Nuclei were separated from cytosol by centrifugation at 500 *g* for 5 min and were resuspended in 60 µl of buffer B (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA and protease inhibitor cocktail). After 30 min at 4°C, lysates were separated by centrifugation (14 000 *g*, 10 min) and supernatants containing nuclear proteins were transferred to new vials. Protein concentrations were determined (32) and the samples were either used immediately or stored frozen at –70°C.

The oligonucleotide probe d(AGTTGAGGGGACTTTC-CCAGGC) containing the κB binding motif was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Invitrogen) and purified over two successive G-50 mini columns (Amersham-Pharmacia Biotech). Reaction mixtures (25 µl) containing 10 µg of nuclear protein, 5 mM Tris pH 7.5, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 4% glycerol (v/v) and 0.08 mg/ml salmon sperm DNA were preincubated on ice for 15 min. Then, 1×10^6 c.p.m. of the probe was added and the binding reaction was allowed to proceed for 20 min at

room temperature. DNA–protein complexes were then resolved in a 6% native polyacrylamide gel in TBE buffer (22.5 mM Tris, 22.5 mM boric acid and 0.5 mM EDTA, pH 8.3) at 140 V for 2–3 h. Gels were dried and exposed to Kodak Biomax MR film at –70°C. Films were scanned and the images were processed using Adobe imaging software.

RESULTS

Design and synthesis of immunomers

An 18mer CpG DNA sequence **10**, which has a 'GACGTT' hexameric motif, was used in the present study (Table 1). This sequence has been shown to induce immunostimulatory activity in mice in earlier studies (21,22). The immunomers used in the present study contain two identical segments that are tethered through their 3′ends via a non-nucleoside linker or a phosphorothioate linkage (see Fig. 1A for linkers used in the study). Since they contain two identical sequence segments (Fig. 1A), they can be synthesized on an automated DNA synthesizer using two different synthetic strategies, referred to here as linear and parallel synthesis (Fig. 1B). In a linear synthetic strategy, as reported earlier, synthesis is started from one end of the immunomer using a solid support linked 5′-nucleoside and nucleoside-5′-β-cyanoethylphosphoramidites (24–27). When the synthesis of first oligonucleotide segment is complete, it is coupled with a DMT-protected-β-cyano-ethylphosphoramidite derivatized linker and the synthesis is continued using nucleoside-3′-β-cyanoethylphosphoramidites to construct the second segment. The second oligonucleotide segment could be identical or different from the first segment in terms of its length, base composition and/or chemical modifications incorporated. In the present study, however, we used identical sequences and modifications in both the segments of immunomers **27**–**37** (Table 1), which are synthesized using linear synthesis strategy (route i, Fig. 1B).

Alternately, immunomers containing identical sequence segments can be synthesized using a parallel synthesis strategy as shown in Figure 1B (routes ii and iii). This strategy requires a solid support derivatized with a linker having two reactive sites protected with DMT (route iii, Fig. 1B) or its corresponding phosphoramidite (route ii, Fig. 1B). This enables simultaneous synthesis of two identical sequence segments using nucleoside-3′- (or 5′-)β-cyanoethylphosphoramidites. Parallel synthesis of immunomers has certain advantages over linear synthesis: (*i*) it permits incorporation of two or more identical sequence segments; (*ii*) the segments are synthesized concurrently, thereby halving the number of synthetic steps and the time required; and (*iii*) with fewer synthetic cycles, the yield and purity of the final product improves. Immunomers **11**–**13**, **15**, **16** and **20**–**26** (Table 1) were synthesized using the parallel synthesis strategy. Out of the immunomers synthesized by parallel synthesis strategy, immunomers **11**–**13** and **16** were synthesized using 5′-nucleoside attached solid support and diDMT-protected linker phosphoramidite (route ii, Fig. 1B). Note that immunomer **13** contains three identical oligonucleotide segments. The remaining immunomers, **15** and **20**–**26** were synthesized using solid support bearing diDMT glyceryl linker (route iii, Fig 1B).
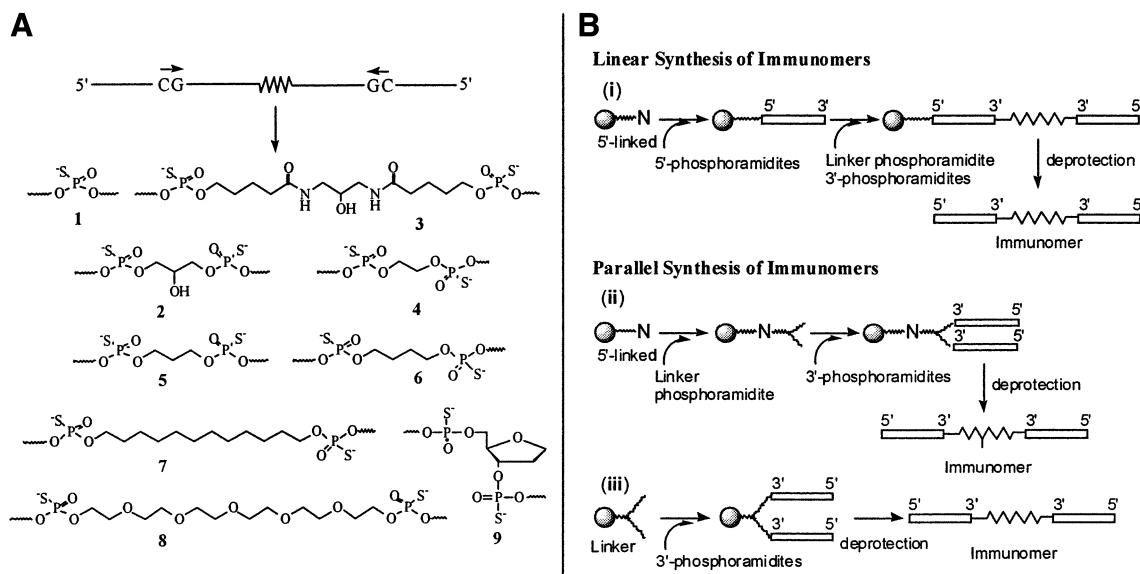
Figure 1. (**A**) A line drawing of immunomer showing CpG dinucleotide directionality and different linkers used to tether two CpG DNAs for synthesis of immunomers. (**B**) Schemes showing (i) linear synthesis of CpG DNA and immunomers, and parallel synthesis of immunomers using (ii) diDMT protected brancher phosphoramidite or (iii) solid support attached diDMT protected brancher. N stands for any nucleoside.

Effect of a linker on immunostimulatory activity of immunomers

Previously, we showed that immunomers having the two segments 3′–3′-linked through a phosphorothioate group (**1**) are more immunostimulatory than those 5′–5′-linked (24,25). To examine the effect of different length linkers, we synthesized two immunomers **11** and **12** incorporating linkers **2** and **3** (Table 1), respectively. Their immunostimulatory activities were determined by induction of cytokines IL-12, IL-6 and IL-10 secretion in BALB/c mouse spleen cell cultures. Both immunomers **11** and **12** induced higher levels of IL-12 and IL-6, but similar levels of IL-10, compared with its parent CpG DNA **10** at a concentration of 1.0 µg/ml (Fig. 2). These results suggest that neither of these linkers interfered with activity of immunomers. Immunomer **13**, which has three identical 18mer segments branching from the same linker (a 'Y'-shaped molecule), produced similar or slightly lower levels of cytokine secretion compared with CpG DNA **10** (data not shown), suggesting that more than two segments do not enhance activity. We, therefore, used immunomers containing two identical sequence segments in all subsequent studies.

To examine whether each segment of immunomer should have the same length as that of CpG DNA **10** (18 nt), we synthesized immunomers **15** and **16** (Table 1) with linkers **2** and **3**, respectively, but with each sequence segment containing 14 nt, and studied immunostimulatory activity in BALB/c mouse spleen cell cultures. As shown in Figure 2, immunomer **15** induced similar levels of IL-12 and lower levels of IL-6 compared with immunomer **11** at a concentration of 1.0 µg/ml. In contrast, truncated immunomer **16**, which contained a longer linker **3**, induced lower IL-12 and higher IL-6 compared with immunomer **12** at the same concentration (Fig. 2A and B). Both immunomers **15** and **16** produced higher levels of IL-10 compared with **11** and **12**, respectively (Fig. 2C). As these results suggested that immunomer length
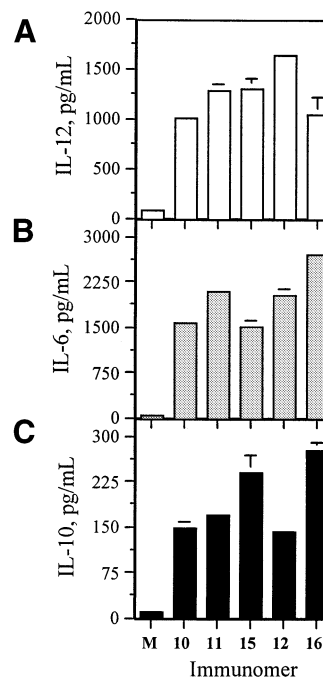

Figure 2. (**A**) IL-12, (**B**) IL-6 and (**C**) IL-10 secretion in BALB/c mouse spleen cell cultures induced by immunomers at a concentration of 1.0 µg/ml. M represents control in the absence of CpG DNA.

affects its activity, we carried out a systematic study to find the optimal length.

Effect of length of immunomer on immunostimulatory activity

Immunomers **11** and **12**, with segments of 18 nt, were compared with **20–25** (Table 1) having shorter segments of 8–14 nt. Immunomers **20–25** contain glyceryl-linker **2** that,

Table 2. Concentration-dependent cytokine secretion in BALB/c mouse spleen cell cultures by immunomers

| CpG DNA | IL-12 (pg/ml) $\pm$ SD | | | | IL-6 (pg/ml) $\pm$ SD | | | |
	0.1 µg/ml	0.3 µg/ml	1.0 µg/ml	3.0 µg/ml	0.1 µg/ml	0.3 µg/ml	1.0 µg/ml	3.0 µg/ml
10	70 ± 13	444 ± 33	1142 ± 100	2570 ± 46	150 ± 8	211 ± 26	3979 ± 514	21491 ± 1951
15	189 ± 24	1404 ± 189	2972 ± 275	2910 ± 191	314 ± 56	456 ± 21	2550 ± 356	20923 ± 103
20	70 ± 8	1025 ± 36	2135 ± 96	2155 ± 128	211 ± 51	533 ± 4	2696 ± 138	17918 ± 1495
21	217 ± 56	1397 ± 42	2443 ± 48	2921 ± 64	183 ± 22	452 ± 15	3812 ± 96	23894 ± 713
22	504 ± 58	1609 ± 132	2428 ± 226	2743 ± 269	233 ± 5	1965 ± 1	9307 ± 95	26825 ± 1239
23	103 ± 22	1417 ± 76	2385 ± 168	3482 ± 81	198 ± 57	376 ± 82	7537 ± 964	24827 ± 1917
24	34 ± 3	135 ± 18	1351 ± 106	3282 ± 115	NT			
Culture medium	71 ± 1				155 ± 28			

NT, not tested.

earlier, increased the activity of **15**, with 14mer segments, relative to CpG DNA **14** (data not shown). Immunomers **15** and **20–25** were tested for their immunostimulatory properties in BALB/c mouse spleen cell cultures.

Table 2 shows the concentration-dependent induction of IL-12 and IL-6 secretion by immunomers **15** and **20–24** in BALB/c mouse spleen cell cultures. In general, a trend of increased secretion of IL-12 and IL-6 was observed as immunomer length reduced from 14 nt (**15**) to 11 nt (**22**) in repeat experiments. However, immunomer **20** showed a lower activity in this particular assay compared with **15**. Further reduction, to 10 nt or less, resulted in a decreased cytokine secretion compared with 11mer immunomer **22**, but maintained a similar or higher activity compared with 18mer parent CpG DNA **10** (Table 2). Similar results were observed with IFN-γ secretion in BALB/c mouse spleen cell cultures (Fig. 3A). However, a different profile was found for TNF-α secretion (Fig. 3B). TNF-α secretion increased as chain-lengths decreased from 18mer (**11**) to 13mer (**20**), dropped as the length decreased further to 12mer (**21**) and 11mer (**22**), then rose again as the length continued to decrease in immunomers **23** and **24** (Fig. 3B). The control immunomer **26**, which has a GpC dinucleotide, induced minimal levels of cytokines in the same assays (Fig. 3 and data not shown), suggesting that a CpG dinucleotide is required for immunostimulatory activity.

We also tested immunomers **10**, **11**, **15**, and **20–25** for their ability to induce spleen enlargement in BALB/c mice. Following subcutaneous administration at a dose of 5 mg/kg, spleen weights were measured after 4 days as described in the Materials and Methods. Increases in spleen weight in excess of that produced by vehicle (PBS) are considered the result of immunostimulatory activity of immunomers (16,29). The splenomegaly data are presented in Figure 3C. CpG DNA **10** caused a 40% increase in spleen weight compared with control mice treated with vehicle. Immunomers containing glyceryl-linker (**2**) and different chain lengths are more potent immunostimulators and caused 83–135% increases in spleen weights. These splenomegaly assays also suggest that immunomer **22** with 11mer segments is optimal for inducing higher immunostimulatory activity as determined by *in vitro* assays.

In addition, we also examined the ability of immunomers **20–26** to induce IL-12 and IL-6 secretion in macrophage-like J774 cell cultures. All immunomers tested induced a
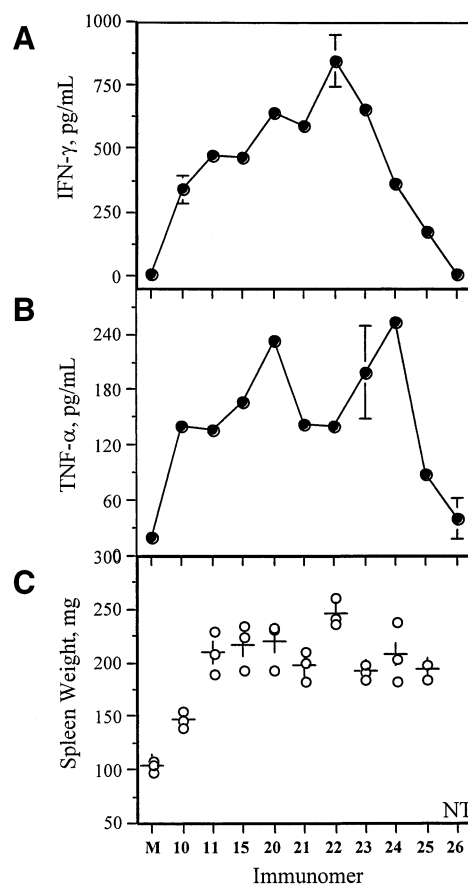

Figure 3. (**A**) IFN-γ and (**B**) TNF-α secretion in BALB/c mouse spleen cell cultures induced by immunomers at a concentration of 1.0 µg/ml. M represents control in the absence of CpG DNA. (**C**) Splenomegaly in BALB/c mice at a dose of 5 mg/kg dose of immunomer administered SC. Spleen weight of each mouse is represented by open circles. Plus sign indicates average spleen weight. M indicates control mice administered with vehicle (PBS). NT stands for not tested.

concentration-dependent cytokine secretion (data not shown). As shown in Figure 4, immunomer **22** induced the highest levels of IL-12 and IL-6 secretion at 3 µg/ml concentration in J774 cell cultures further confirming the results obtained from BALB/c mouse spleen cell culture assays. Importantly, shorter immunomers with 9- and 8-nt segments also showed
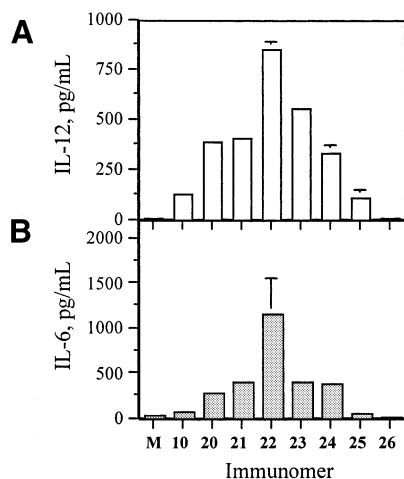
Figure 4. (**A**) IL-12 and (**B**) IL-6 secretion in J774 cell cultures induced by immunomers at a concentration of 3.0 µg/ml. M represents control in the absence of CpG DNA.

Figure 5. (**A**) IL-12 and (**B**) IL-6 secretion in BALB/c mouse spleen cell cultures induced by immunomers at a concentration of 1.0 µg/ml. M represents control in the absence of CpG DNA.

immunostimulatory activity in BALB/c mouse spleen and J774 cell culture and splenomegaly assays compared with parent linear CpG DNA **10**, whereas the linear CpG DNAs of the same length completely failed to show activity in these assays.

Effect of nature and length of linker on immunostimulatory activity

After establishing the optimal length for immunomers, we used linkers **1** and **4**–**9** (Fig. 1A) for tethering the two segments of immunomers to compare immunostimulatory activity. All immunomers (**27**–**33**), had 10-nt long segments (Table 1) and induced concentration-dependent cytokine secretion in BALB/c mouse spleen cell cultures (Table 3). The levels of IL-12 and IL-6 induced by immunomers **10**, **18** and **27**–**33** in spleen cell cultures at a concentration of 1.0 µg/ml are shown in Figure 5 for comparison. CpG DNA **18**, which is an 11mer, failed to induce cytokine secretion in these assays suggesting CpG DNAs as short as 11mers do not induce immune responses. All seven immunomers with different linker groups (**27**–**33**) induced increased cytokine secretion. As shown in Figure 5A, IL-12 secretion increased on going from immunomer **27** with no linker, to a C2-linker
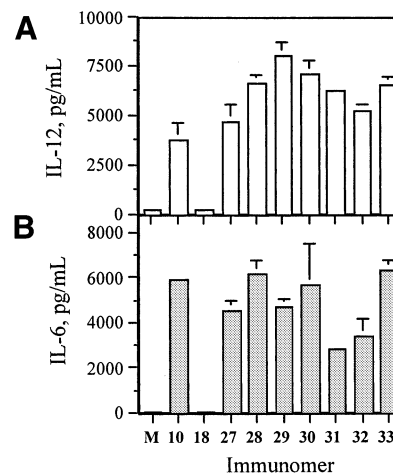
containing **28** and then to a C3-linker (**5**) containing **29**. Other immmunomers **30**–**32**, which have longer linkers [C4- (**6**), C12- (**7**) and hexaethylene glycol-linkers (**8**)], induced lower IL-12 secretion compared with **28** and **29**. Importantly, all seven immunomers induced higher IL-12 than did CpG DNA **10**. Immunomer **33**, which has a more constrained abasic linker (**9**), but maintains a similar distance between the two phosphates as in the C3-linker, induced comparable levels of IL-12 secretion as **29**. All seven immunomers induced similar or lower IL-6 secretion compared with CpG DNA **10** (Fig. 5B). These results suggest that a C3-linker is optimal for immunomer construction.

Effect of multiple linkers in immunomers on immunostimulatory activity

Since the use of longer linkers showed reduced immuno-stimulatory activity, we tested whether incorporation of multiple linkers would be preferable. We synthesized immunomers **34** and **35** with two and three copies of C3-linker (**5**), respectively, and **36** and **37** with two and three copies of the abasic linker (**9**), respectively (Table 1). All four immunomers induced concentration-dependent IL-12 and IL-6 secretion in BALB/c mouse spleen cell cultures (Table 4).

Table 3. Effect of linkers on cytokine secretion in BALB/c mouse spleen cell cultures

CpG DNA	IL-12 (pg/ml) ± SD				IL-6 (pg/ml) ± SD			
	0.1 µg/ml	0.3 µg/ml	1.0 µg/ml	3.0 µg/ml	0.1 µg/ml	0.3 µg/ml	1.0 µg/ml	3.0 µg/ml
10	310 ± 1	1962 ± 78	3780 ± 805	6084 ± 1390	181 ± 57	582 ± 143	5981 ± 138	13879 ± 612
18	203 ± 76	148 ± 26	310 ± 35	1229 ± 45	43 ± 6	35 ± 4	71 ± 3	2562 ± 157
27	1826 ± 230	4770 ± 301	4698 ± 881	6052 ± 372	257 ± 44	1184 ± 111	4551 ± 389	10613 ± 507
28	1419 ± 455	5113 ± 870	6697 ± 346	7558 ± 1139	233 ± 24	1385 ± 75	6233 ± 549	16011 ± 924
29	4118 ± 1099	5768 ± 1250	8042 ± 632	7414 ± 457	421 ± 30	806 ± 131	4725 ± 291	15093 ± 636
30	930 ± 28	7083 ± 297	7135 ± 651	9045 ± 421	127 ± 39	1570 ± 767	5718 ± 1746	14559 ± 1774
31	2177 ± 254	5735 ± 308	6254 ± 190	7443 ± 424	779 ± 64	747 ± 130	2840 ± 140	15311 ± 2459
32	365 ± 109	4731 ± 857	5254 ± 279	7283 ± 905	69 ± 19	480 ± 57	3414 ± 717	13954 ± 151
33	778 ± 136	4854 ± 498	6563 ± 358	8803 ± 666	294 ± 107	587 ± 56	6359 ± 372	17006 ± 1274
Culture medium	256 ± 42				82 ± –17			

Table 4. Effect of multiple linkers on cytokine secretion in BALB/c mouse spleen cell cultures

CpG DNA	IL-12 (pg/ml) ± SD				IL-6 (pg/ml) ± SD			
	0.1 µg/ml	0.3 µg/ml	1.0 µg/ml	3.0 µg/ml	0.1 µg/ml	0.3 µg/ml	1.0 µg/ml	3.0 µg/ml
10	310 ± 1	1962 ± 78	3780 ± 805	6084 ± 1390	181 ± 57	582 ± 143	5981 ± 138	13879 ± 612
29	4118 ± 1099	5768 ± 1250	8042 ± 632	7414 ± 457	421 ± 30	806 ± 131	4725 ± 291	15093 ± 636
34	4005 ± 174	6488 ± 1123	8112 ± 270	6858 ± 251	276 ± 26	2581 ± 285	7033 ± 743	15958 ± 2371
35	3917 ± 677	6156 ± 728	5806 ± 365	6782 ± 539	660 ± 144	1908 ± 268	8223 ± 378	12319 ± 468
33	778 ± 136	4854 ± 498	6563 ± 358	8803 ± 666	294 ± 107	587 ± 56	6359 ± 372	17006 ± 1274
36	1812 ± 150	4879 ± 432	4874 ± 170	6126 ± 253	331 ± 18	1348 ± 128	5823 ± 330	12888 ± 1186
37	2994 ± 46	5293 ± 544	5341 ± 456	5602 ± 91	408 ± 8	1310 ± 82	5764 ± 748	12470 ± 1373
Culture medium	256 ± 42				82 ± −17			

When compared at 1.0 µg/ml concentration, the presence of two and three C3-linkers in immunomers **34** and **35** maintained or reduced IL-12 secretion but increased IL-6 secretion considerably compared with one C3-linker containing **29** (Fig. 6). These results suggest that it may be possible to modulate cytokine secretion by incorporation of an appropriate number of C3-linkers in immunomers.

The incorporation of multiple abasic linkers in immunomers (**36** and **37**) reduced both IL-12 and IL-6 secretion compared with **33**, which contained a single abasic linker (Fig. 6).

5′ end of CpG DNA is required for activation of NF-κB and subsequent immunostimulatory effects

CpG DNA has been shown to activate a number of signaling pathways, including the NF-κB pathway, which plays a critical role in up-regulation of cytokine gene expression (33). We studied the activation of NK-κB in J774 cell nuclear extracts by immunomers **10**, **22**, **38**, and **39** (Table 1) using EMSA. Figure 7A shows the results obtained. CpG DNA **10** activated NF-κB as indicated by the presence of a complex in lane 3 as is the case with LPS treated cells in lane 2 (Fig. 7A). Immunomer **22**, which has two 5′-accessible ends, also activated NF-κB as indicated by the presence of the appropriate complex in lane 5. In contrast, 5′–5′-linked immunomer **38** failed to activate the transcription factor NF-κB in J774 cells (lane 4, Fig. 7A). A 34mer linear CpG DNA (**39**) with two CpG dinucleotides separated by 18 nt also activated the transcription factor NF-κB (see complex band in lane 6, Fig. 7A). This suggests that the enhanced activity of immunomer(s) (**22**) is not as a result of the presence of multiple CpG dinucleotides, but of the presence of accessible 5′ ends.

Immunomer **38**, lacking an accessible 5′ end, induced 11 ± 1 and 7 ± 5 pg/ml of IL-12 and IL-6 secretion, respectively, in J774 cell culture assays at 3 µg/ml concentration (Fig. 7B). This result further confirms the inability of **38** to activate NF-κB in this cell culture system. CpG DNA **39**, which has two CpG dinucleotides, induced 35 ± 14 and 125 ± 10 pg/ml of IL-12 and IL-6, respectively, at a concentration of 3 µg/ml in J774 cell cultures.

DISCUSSION

CpG DNAs have been shown as potential immune adjuvants (34,35). Their use as antiallergens (36,37) and for the treatment of infectious diseases (38,39) and cancer (40,41)
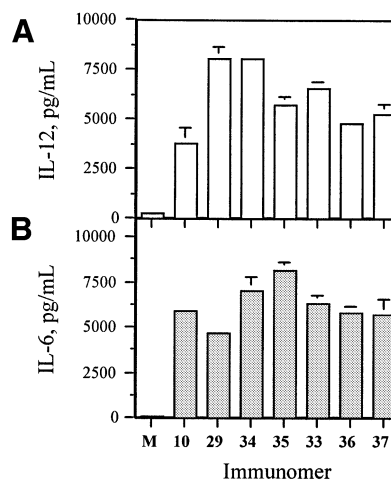

Figure 6. (**A**) IL-12 and (**B**) IL-6 secretion in BALB/c mouse spleen cell cultures induced by immunomers at a concentration of 1.0 µg/ml. M represents control in the absence of CpG DNA.
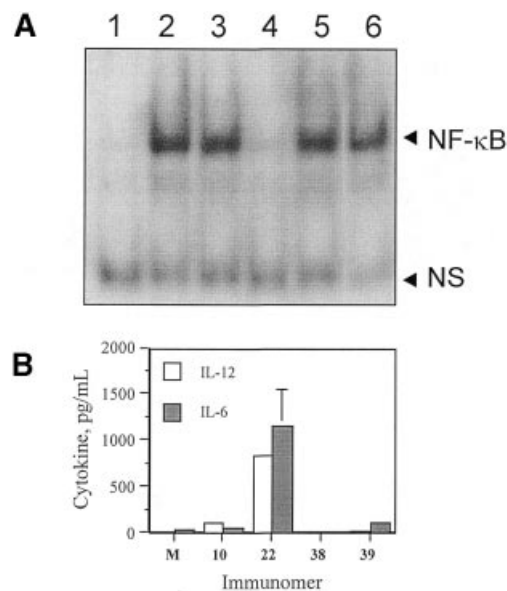

Figure 7. (**A**) Activation of NF-κB in J774 cells by different CpG DNAs. J774 cells were stimulated with LPS (lane 2), CpG DNAs **10** (lane 3), **38** (lane 4), **22** (lane 5) and **39** (lane 6), nuclear extracts were prepared and analyzed on a native polyacrylamide gel as described in Materials and Methods. Lane 1 represents medium treated control. NS stands for non-specific band. NF-κB band is indicated with an arrow. (**B**) Induction of IL-12 and IL-6 secretion by immunomers **10**, **22**, **38** and **39** in J774 cell cultures. M represents control in the absence of CpG DNA.

has been demonstrated. Our earlier studies demonstrated that an accessible 5′ end is required for immunostimulatory activity and the blocking of the 5′ end by attaching two CpG DNAs via their 5′ ends abrogates immunostimulatory activity (24,25). In the present studies, we examined the use of linkers between CpG DNA strands and the required length of immunomers for optimal immunostimulatory activity. The use of linkers for tethering immunomers permitted development of a fast and convenient parallel synthesis approach for producing immunomers. The use of the parallel synthesis approach resulted in higher yields of the immunomer products with improved purity.

The present results support our earlier finding that a 3′–3′-linked immunomer is a more potent immunostimulator than linear CpG DNA containing one or two CpG dinucleotides (24,25). In addition, these results also provided further insight into the immunological effects of CpG DNA and possible molecular basis for recognition of CpG DNA by its receptor. Several studies showed that the presence of multiple CpG dinucleotides increased immunostimulatory activity (42). Immunomers used here inherently contain at least twice as many CpG dinucleotides per molecule as the parent CpG DNA **10**. The higher immunostimulatory activity of immunomer **22** compared with CpG DNA **10** could be as a result of the increased number of CpG dinucleotides per molecule. However, the different activities of 3′–3′- and 5′–5′-immunomers (with two or three branches) and 34mer CpG DNA **39**, containing two or more CpG dinucleotides, in J774 cell cultures for NF-κB activation and cytokine secretion cannot be explained by the multiple CpG dinucleotides in immunomers. Immunomers **13**, **38** and CpG DNA **39** (34mer) also contained two or more CpG dinucleotides but showed lower or no activation of NF-κB and less or no induction of cytokine secretion in J774 cell cultures. Taken together, enhanced activity of immunomers results from the 3′–3′-linked immunomer structure rather than the number of CpG dinucleotides present.

Upon internalization, CpG DNA is recognized by TLR9 with initiation of the signaling cascade that activates NF-κB and expression of cytokines (43,44). However, the molecular mechanism of recognition between CpG DNA and TLR9 is not known. The present results, at least to some extent, suggest that TLR9 reads the CpG DNA sequences from the 5′ to the 3′ end. Blocking the 5′ end by 5′–5′-linking of two CpG DNAs abrogates recognition of CpG DNA by TLR9 and subsequent events such as NF-κB activation and cytokine secretion. The blocking of the immunostimulatory activity also depends on the size of the blocking group conjugated at the 5′ end of the CpG DNA as shown recently (25). Immunomers having two free 5′ ends might be recognized by TLR9 at either or both 5′ ends. The increased immunostimulatory activity of immunomers, however, suggests that they are recognized by the receptors at both 5′ ends. The failure of 5′–5′-linked CpG DNA to activate NF-κB and induce cytokine secretion suggests that the receptor is unable to recognize the molecule lacking an accessible 5′ end even though it has two appropriate CpG dinucleotides.

In the case of CpG DNA **39**, though it has two CpG dinucleotides, it is no more immunostimulatory than a CpG DNA with only a single copy. It appears that the recognition of one of the CpG dinucleotides by the receptor may preclude recognition of the second CpG dinucleotide by a second receptor, because the molecule has a single accessible 5′ end.

The immunostimulatory activity of different immunomers may be affected by the impact of 3′–3′- and 5′–5′-linkages on cellular uptake. Our recent cellular uptake studies using the 5′- or 3′-end fluorescein-labeled CpG DNAs in J774 cell cultures suggested that the uptake is not affected by whether the DNA molecule has a free 3′ or 5′ end (25). Therefore, it is reasonable to assume both 3′–3′- and 5′–5′-linked immunomers are taken up by cells similarly and differences in immunostimulatory activity result from structural effects on receptor recognition and/or binding.

Degradation of oligonucleotides *in vivo* occurs primarily by 3′-exonucleases acting from the 3′ ends (45–47). Blocking groups, ligands or sequences forming secondary structures at the 3′ end of oligonucleotides increase metabolic stability (48–52). Ligation of two CpG DNAs through their 3′ ends could enhance nuclease stability and, thereby, immunostimulatory activity in the present study. If so, 5′–5′-linked immunomers should have similar or higher nuclease stability compared with CpG DNA **10** as oligonucleotides are also degraded from the 5′ end though to a lesser extent. However, 5′–5′-linked immunomer **38** failed to induce immunostimulatory activity despite expected equal, if not higher, nuclease stability compared with parent CpG DNA **10**. Therefore, the data strongly argue that the differences in immunostimulatory activity of 3′–3′- and 5′–5′-linked DNAs do not arise from differences in nuclease stability or their cellular uptake but from the accessibility to the 5′ end for receptor recognition and/or binding.

An immunomer with 11-nt segments shows increased immunostimulatory activity. Further reduction in length of immunomers lowered immunostimulatory activity compared with 11mer immunomer, but still maintained similar or higher activity than linear CpG DNA **10** in *in vitro* and *in vivo* assays. These results suggest that the receptor requires a certain length of nucleotides for recognition.

CpG DNA stimulates secretion of several cytokines by B cells, macrophages and dendritic cells (5–8). Recently it has been shown that CpG DNAs containing different backbone and sequences elicit different cytokine secretion properties, perhaps through targeting different immune cell populations (53–55). Although the mechanism is not clear, in the present study, we found altered cytokine secretion profiles by incorporating one or more copies of several linkers but without changing the backbone or sequence of immunomers. For example, incorporation of a single C3-linker (immunomer **29**) induced higher levels of IL-12 secretion, which is a Th1 type cytokine. Such an immunomer might be useful for cancer and asthma therapies or as an adjuvant with vaccines, antibodies and allergens. In contrast, when lower levels of IL-12 and higher levels of IL-6 are required, incorporation of two or more C3-linkers is appropriate.

Taken together, the present results suggest that 3′–3′-linked CpG DNAs are potent immunostimulators because of the presence of two 5′-accessible ends for receptor recognition. Additionally, the 3′–3′-linkage provides increased stability against nucleases (26,27,51). Linking of CpG DNAs through their 5′–5′ ends blocks the immunostimulatory activity, though they possess required CpG dinucleotides in appropriate sequence context, nuclease stability and cellular uptake

properties. Our preliminary results indicate potent immuno-stimulatory activity of immunomers containing synthetic YpG and CpR motifs instead of CpG. Additional immunomodulatory moieties (18–22) reported can also be incorporated in immunomers to modulate cytokine secretion profiles. We are currently examining the immunomers containing synthetic YpG and CpR motifs in human specific sequences for immunostimulatory responses.

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