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Effects of a Hexameric Deoxyriboguanosine Run Conjugation into CpG Oligodeoxynucleotides on Their Immunostimulatory Potentials

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CpG oligodeoxynucleotides (ODNs) are promising immunomodulatory agents for treating human diseases and vaccine development. Phosphodiester CpG ODNs were demonstrated to have poor immunostimulatory potentials for cytokine production. However, the conjugation of consecutive deoxyriboguanosine residues, called a dG run, at the 3’ terminus of phosphodiester CpG ODNs significantly enhanced TNF-α and IL-12 production from mouse splenic dendritic cells (DCs). The optimal induction of cytokine production was achieved by the addition of a hexameric dG (dG₆) run. In contrast, the existence of a dG₆ run either at the 5’ terminus of phosphodiester CpG ODNs or at the 3’ terminus of phosphorothioate CpG ODNs diminished CpG-mediated cytokine induction, suggesting that the effects of a dG run depend on its location and the chemical property of the ODN backbone, respectively. In addition, we provided the evidence that the conjugation of a dG₆ run caused the structural transformation of CpG ODNs, which facilitates their targeting into mouse APCs such as splenic DCs, B cells, and peritoneal macrophages with a scavenger receptor type A ligand specificity. Among primary APCs, DCs were the most potent for CpG ODN-mediated IL-12 production. Furthermore, we demonstrated that the conjugation of a dG₆ run into the 3’ terminus of phosphodiester CpG ODNs was crucial for their ability to generate Th1 immunity in vivo. Thus, the conjugation of a dG₆ run into phosphodiester CpG ODNs would be an alternative way to optimize their immunostimulatory potentials in vitro and in vivo.


In this regard the tagging of consecutive dG residues, called a dG run (16), to CpG ODNs may modulate their immunostimulatory potential, since the dG run is known to bind to the scavenger receptor (SR) (17, 18), which is mainly expressed in the cells of monocytic lineage (19). Kimura et al. demonstrated that IFN production and NK activity from mouse splenocytes were increased by the addition of dG₁₂ runs next to the CpG motif (AACGTT) (17). Also, the existence of poly(dG) at both ends of CpG ODN, which is partially phosphorothioate modified, could enhance NK cell activation in human PBMC, although this activity was significantly decreased when the ODN was entirely phosphorothioate modified (20). These results imply that the dG run may increase the uptake of CpG ODNs into target cells through SR and influence CpG-mediated cellular signaling. However, it is unclear which types of cells are direct targets of dG run-containing CpG ODNs. Moreover, it remains to be determined whether the dG run conjugation can modulate in vivo functions of CpG ODNs.

Here, we demonstrated that the conjugation of a dG₆ run into the 3’ terminus of phosphodiester CpG ODNs significantly augmented their immunostimulatory potentials in terms of IL-12 production from mouse primary APCs and the ability to generate Th1 immunity in mice. Of note, the effects of a dG₂ run were dependent upon its location and the chemical property of the ODN backbone, and the conjugation of a dG₆ run induced structural transformation of CpG ODNs, which facilitated their binding to DCs in an SR-A-dependent manner.

**Materials and Methods**

**ODNs, reagents, and mice**

All CpG ODNs with phosphodiester and phosphorothioate backbone were purchased from GenoTech (Taejon, Korea). Several ODNs were modified by tagging the fluorescein into the 3’ or 5’ terminus (f-ODNs). Endotoxin contamination measured by the Limulus assay (Sigma, St. Louis, MO) was.
negligible in all ODN preparations. Dextran sulfate, chondroitin sulfate, fucoidan, oleic acid, fibrinogen, and low density lipoprotein were purchased from Sigma, and anti-CD18 mAb was purchased from PharMingen (San Diego, CA). Specific pathogen-free conditioned 6-wk-old female C57BL/6 and BALB/c mice were purchased from Dai-En Laboratory Animal Center (Eumseong, Korea) and Japan SLC (Shizuoka, Japan).

**Isolation of primary APCs**

Splenic DCs were isolated from female C57BL/6 mice as previously shown with minor modifications (21, 22). Briefly, splenocytes were adhered to a tissue culture plate for 90 min, and then nonadherent cells were discarded. Adherent cells were cultured in the presence of 10 ng/ml murine recombinant GM-CSF (R&D Systems, Minneapolis, MN). The weakly adherent cells were loaded on a Percoll density gradient, and low density cells from the interface were used as splenic DC-enriched populations. For the direct purification of splenic DCs, splenocytes were incubated with anti-CD11c microbeads and then passed through a column of miniMACS system (Miltenyi Biotec, Auburn, CA), and the remaining CD11c- cells were used. Resting peritoneal macrophages were harvested as previously described (23) by washing the peritoneal cavity of C57BL/6 mice with the culture medium and were seeded in a tissue culture dish. After incubation for 9 h, nonadherent cells were removed by washing three times with medium, and remaining adherent cells were used in the following experiments. Splenic B cells were isolated from the nonadherent populations of splenocytes. Total splenocytes were seeded into a tissue culture plate for 90 min, and anti-CD19 microbeads were coincubated with a non-adherent population of splenocytes. By passing through columns of the miniMACS system, CD191 B cells were finally purified.

**Delivery of ODNs**

ODNs (1.5–3 μM) were incubated with various APCs for either 10 h (TNF-α or 30 h (IL-12) with RPMI complete medium (Life Technologies/BRL, Gaithersburg, MD), which were supplemented with 10% FBS, 2-ME (50 μM), and l-glutamine (1.5 mM). Also, ODNs (0.3 μM) were code-livered with 3 μg of cationic lipid complex, Lipogen (BodYtech, Chuncheon, Korea), to DCs and were incubated for 4 h in serum-free RPMI medium. After washing the liposome-ODN complexes, cells were further incubated for 24 h with RPMI complete medium.

**Cytokine detection**

Various APCs were stimulated by ODNs or LPS, and the culture supernatants were quantitated by commercial ELISA kits (Genzyme, Cambridge, MA) for detection of both IL-12 and TNF-α or by specific mAb pairs for detection of both IFN-γ and IL-4. For sandwich ELISAs, purified anti-mouse IL-4/IFN-γ mAbs (PharMingen) were adsorbed to capture cytokines on polystyrene base microtiter immunoplates (Dynex Technologies, Chantilly, VA). The culture supernatants diluted appropriately were incubated following biotinylated anti-mouse IL-4/IFN-γ mAbs. Streptavidin-peroxidase (Southern Biotechnology Associates, Birmingham, AL) was used for detection following H2O2 and tetramethylbenzidine substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) solutions. Reombinant IL-4/IFN-γ (PharMingen) were used as standards for calculating cytokine concentrations in the culture supernatants tested.

**Binding and internalization assay with fluoresceinated ODNs**

CD11c+ DCs (104), CD19+ B cells, and peritoneal macrophages were preincubated at 4°C for 15 min to suppress endocytosis and then incubated with various f-ODNs (5 μM) for 30 min at 4°C. After complete washing the cells with PBS, the mean fluorescent intensity (MFI) was measured from f-ODNs bound to the cells by FACScan (Becton Dickinson, Mountain View, CA). For detecting the internalization of ODNs, f-ODNs (1 or 5 μM) were treated for 30 min at 4°C and then further incubated for 1.5 or 6 h at 37°C. After treatment of phosphorothioate-modified poly(dC)20 to deprive cell-bound f-ODNs, the MFI from internalized f-ODNs was measured by FACScan. The MFI was measured from the live cells, extracting the dead population of cells with propidium iodide staining.

**Nuclear magnetic resonance (NMR) analysis for ODN structure**

ODNs were chemically synthesized on an Applied Biosystems 392 (Foster City, CA) and were purified by PAGE and size exclusion chromatography. The purified samples were dialyzed against PBS buffer. After lyophilization, the samples were dissolved in 400 μl of PBS. NMR experiments were performed on a Bruker DMX 600 spectrometer (Billerica, MA). Jump-and-return pulse was used to suppress water peak. The delay between the two 90°C pulses was set to optimize the imino proton peaks. The spectral width was 12,000 Hz (20 ppm), and 256 scans were averaged for each sample. The recycle delay was 1.8 s. The FIDs were apodized with 1 Hz exponential functions. The polynomial baseline correction was applied to the imino proton regions.

**Immunizations**

Six- to 8-wk-old female BALB/c mice were immunized with various Ags with or without ODNs. Briefly, 5 μg of heat-inactivated HIV type 1 (HIV-1) particles that had been formulated with aluminum hydroxide (Sigma) were s.c. immunized together with 50 μg of phosphodiester ODNs. We also used gD2E2t protein (24), which is a fusion protein of herpes simplex virus type 1 glycoprotein D (gD) and C-terminal truncated hepatitis C virus (HCV) envelope protein 2 (E2) for the immunization. Two micrograms of phosphodiester (1826D, M21) or phosphorothioate (1826T) ODNs were i.m. administered with 5 μg of gD2E2t protein. Four weeks after the first immunization, mice were given booster immunizations with same immunization regimens. Humoral and cellular immune responses were monitored 2 wk after booster immunization. HIV- or HCV E2t-specific IgG responses were determined by ELISA. For the detection of cytokine expression from CD4+ T cells from immunized mice, CD4+ T cells were finally purified from nonadherent population of splenocytes using anti-CD4 microbeads and the miniMACS system. CD4+ T cells (2 × 10^6) were stimulated with E2t proteins purified from Chinese hamster ovary (CHO) cells for 5 days in the presence of 10% syngeneic APCs. APCs were isolated from the adherent population of splenocytes from naive BALB/c mice and then treated with mitomycin C (Sigma).

**Results**

The existence of a dG run at the 3′ terminus, not at the 5′ terminus, of phosphodiester CpG ODNs is critical for cytokine induction from splenic DCs.

To investigate the effects of a dG run on the cytokine induction by phosphodiester CpG ODNs, we sought the bacterial DNA sequence containing a dG run as well as a CpG hexamer nucleotide motif. The 20-mer ODN derived from ampR gene within the pUC19 sequences (nucleotides 2293–2312) (25), designated p19, was tested for its ability to induce IL-12 and TNF-α from splenic DCs (Table I). The putative dG run (GGGGCG)-containing phosphodiester CpG ODN p19 elicited higher levels of cytokine production, comparable to those induced by LPS. However, deletion of the putative dG run from p19 (p19d) completely abrogated cytokine production. As expected, the induction of cytokine production by p19 was entirely dependent upon the presence of a CpG sequence (M1). In addition, changes in nucleotides within the putative dG run from dG to dC (M7, M13, M14, and M15) resulted in dramatic reduction of IL-12 and TNF-α production, but substitution of a dC with a dG (M16) caused a marked increase in the production of cytokines, implying that the dG run is critical for cytokine induction by p19. However, the introduction into the 5′ terminus of a dG run of various lengths (M19, M26, and M27) did not enhance cytokine induction. To explore the optimal length of a 3′-dG run, we tagged various numbers of dG residues to the 3′ terminus of p19d (Fig. 1). It is likely that the 3′-dG run should have at least four consecutive dG residues for cytokine induction. The maximum induction of IL-12 and TNF-α production was achieved by the addition of a dG run (p19d-dG6, M16). In addition, the ability to induce cytokines was retained, but slightly decreased, when the longer dG runs (dG8, dG10, and dG12) were added. The conversion of CpG dinucleotide to GpC (p19d-dG12/GC) completely abrogated cytokine production, and poly(dG)20 did not induce cytokine production, indicating that the dG run alone could not induce cytokine production in the absence of a CpG motif.

**Enhancement of cytokine induction by dGx run depends upon its location and the chemical property of the ODN backbone**

To further address the effect of a dGx run on different sequence contexts of phosphodiester CpG ODNs, we added a dGx run to the 5′ or the 3′ terminus of two different CpG ODNs, such as 1826D...
and M12, respectively (Table II). There was a significant increase in the activity of cytokine production when a dG 6 run was fused to the 3′ terminus (1826D vs M21, and M12 vs M22), but not to the 5′ terminus (1826D vs M20, and M12 vs M23), suggesting that the effect of a dG 6 run depends on its location within the phosphodiester CpG ODNs. To further determine the specificity of a dG run, we replaced a dG6 run with other nucleotide runs (dA 6 , dT 6 , and dC 6 ) and tested for cytokine induction (Table II). M16A, M16T, and M16C were completely deprived of the ability to induce cytokines, indicating the specificity of a dG run for this function. To assess the effect of the dG run on other mouse APCs, CpG ODNs conjugated with a dG6 run were treated with either peritoneal macrophages (pMac) or splenic B cells. Although pMac and B cells produced much lower amount of IL-12 than did splenic DCs, the addition of a dG6 run to the 3′ of CpG ODNs (M21 and M16), but not into the 5′ (M20 and M26), also enhanced IL-12 production (Fig. 2). As expected, the replacement of a dG 6 run with a dC 6 run totally abolished the induction of IL-12. These results suggest that the dG run is effective in a broad range of cells in a position-dependent manner.

A similar approach was taken to test whether the conjugation of a dG 6 run at the 3′ terminus is also effective in a phosphorothioate-modified CpG ODN. The phosphorothioate CpG ODN 1826T greatly augmented cytokine induction relative to the phosphodiester CpG ODN 1826D. Interestingly, the existence of a dG6 run at the 3′ terminus of a phosphorothioate CpG ODN (M21T) decreased both TNF-α and IL-12 production compared with 1826T (Fig. 3), suggesting that the conjugation of a dG 6 run at the 3′ terminus inhibits immunostimulatory potentials of phosphorothioate-modified CpG ODNs. In particular, M21T produced lesser

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**Table I. Effects of dG run on cytokine induction from splenic dendritic cells**

<table>
<thead>
<tr>
<th>ODNs</th>
<th>Sequences</th>
<th>IL-12 (ng/ml)</th>
<th>TNF-α (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td>GGAAACGTTCGTTTGGGGGCGG</td>
<td>1.59 ± 0.25</td>
<td>844 ± 154</td>
</tr>
<tr>
<td>Media</td>
<td>0.07 ± 0.01</td>
<td>27 ± 4</td>
<td></td>
</tr>
<tr>
<td>p19</td>
<td>GGAAACGTTCGTTTGGGGGCGG</td>
<td>1.32 ± 0.31</td>
<td>640 ± 96</td>
</tr>
<tr>
<td>p19d</td>
<td>GGAAACGTTCGTTTGGGGGCGG</td>
<td>0.07 ± 0.01</td>
<td>25 ± 3</td>
</tr>
<tr>
<td>M1</td>
<td>GGAAACGTTCGTTTGGGGGCGG</td>
<td>0.05 ± 0.01</td>
<td>27 ± 3</td>
</tr>
<tr>
<td>M7</td>
<td>GGAAACGTTCGTTTGGGGGCGG</td>
<td>0.09 ± 0.02</td>
<td>30 ± 3</td>
</tr>
<tr>
<td>M13</td>
<td>GGAAACGTTCGTTTGGGGGCGG</td>
<td>0.07 ± 0.01</td>
<td>20 ± 1</td>
</tr>
<tr>
<td>M14</td>
<td>GGAAACGTTCGTTTGGGGGCGG</td>
<td>0.05 ± 0.01</td>
<td>22 ± 2</td>
</tr>
<tr>
<td>M15</td>
<td>GGAAACGTTCGTTTGGGGGCGG</td>
<td>0.08 ± 0.02</td>
<td>36 ± 5</td>
</tr>
<tr>
<td>M16</td>
<td>GGAAACGTTCGTTTGGGGGCGG</td>
<td>2.40 ± 0.77</td>
<td>960 ± 166</td>
</tr>
<tr>
<td>M19</td>
<td>GGAAACGTTCGTTTGGGGGCGG</td>
<td>0.13 ± 0.05</td>
<td>39 ± 6</td>
</tr>
<tr>
<td>M26</td>
<td>GGAAACGTTCGTTTGGGGGCGG</td>
<td>0.13 ± 0.07</td>
<td>96 ± 26</td>
</tr>
<tr>
<td>M27</td>
<td>GGAAACGTTCGTTTGGGGGCGG</td>
<td>0.07 ± 0.03</td>
<td>64 ± 19</td>
</tr>
</tbody>
</table>

* C57BL/6 mouse splenic DCs (10⁵ cells/well) were stimulated by various ODNs (1.5 μM) or LPS (0.5 μg/ml) for either 10 h (TNF-α) or 30 h (IL-12).

* Mouse TNF-α and IL-12 were measured from culture supernatants by ELISA kits. Numerical values represent mean and SEM of quadruplicate. Consensus CpG motifs are underlined. The experiments were repeated five times with similar results.

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**FIGURE 1.** The optimal length of a 3′-dG run for cytokine induction. Splenic DCs (10⁵) were stimulated by ODNs (1.5 μM) for either 10 h (TNF-α) or 30 h (IL-12). Mouse cytokines were determined from cell-free culture supernatants by ELISA kits (Genzyme). Data are expressed as the relative percentage of cytokine induction, and each bar represents the mean and SEM of triplicate determinations. The experiments were reproduced with similar results.
amounts of IL-12 but higher amounts of TNF-α than did M21, a dG₆ run-containing phosphodiester CpG ODN. These results imply that the dG run differently affects CpG-mediated cytokine induction depending upon the chemical property of backbone structure of CpG ODNs.

The conjugation of a dG₆ run influences both the uptake and the cellular signaling of CpG ODNs

To determine the effect of a dG run conjugation on the cellular binding and the internalization of CpG ODNs in CD11c⁺ splenic DCs, we performed FACS analysis using fluoresceinated phosphodiester CpG ODNs. Fluoresceinated p19 (f-p19), but not f-p19d, was shown to efficiently bind to DCs (Fig. 4A). f-M16 and f-M26 that contain a 3' and a 5'-dG₆ run, respectively, bound more efficiently than did f-p19. The cellular binding of ODNs was also enhanced by the tagging of a dG₆ run to the 3' and 5' termini of f-1826D (f-M21 and f-M20, respectively). Interestingly, CpG ODNs with a dG₆ run at their 3' termini bound to DCs less efficiently than those with a dG₆ run at their 3' termini. However, the substitution of a dG₆ run on f-M21 with a dC₆ run (f-M21C) completely abolished its binding activity, suggesting that the cellular binding of ODN is dG run specific. Moreover, the capacity for internalization of ODNs was correlated with the binding activity (Fig. 4B), implying that the binding of the dG₆ run resulted in an increase in the intracellular concentration of CpG ODNs. It is worth noting that the apparent ODN uptake shown in Fig. 4B may be less than the actual uptake due to the rapid degradation of phosphodiester ODN (26). Similar results were observed in B cells and pMac (data not shown). In addition, pMac had approximately 4 times higher binding affinity for ODNs than B cells and DCs. Nevertheless, the level of IL-12 production from pMac and B cells by CpG ODNs conjugated with a dG₆ run was much lower than that from DCs (Fig. 2 and Table II). Therefore, these data suggest that among primary APCs, DCs are the most potent cells for CpG ODN-mediated IL-12 induction.

We next addressed why the 5' conjugation of a dG₆ run has little effect on cytokine production, although it increases the binding and the internalization of CpG ODNs. We hypothesized that the 5'-dG₆ run may inhibit CpG motif-mediated cellular signaling for cytokine induction. To test this hypothesis, we delivered various ODNs together with liposomes into DCs to normalize the intracellular concentration of ODNs (Table II), and it was shown that all ODNs, except M16GC, greatly increased IL-12 production. Interestingly, CpG ODNs conjugated with a dA₆ or a dT₆ or a dC₆ run at their 3' termini (M16A, M16T, and M16C) also induced high levels of IL-12 production comparable to M16, suggesting that the 3'-dG₆ run is only necessary for the binding and the subsequent internalization of ODNs. In contrast, there was a significant reduction in IL-12 production by the 5' addition of a dG₆ run to CpG ODN (M20 and M23) compared with 1826D and M12 that lack a dG₆.

### Table II. Effects of a dG run on cytokine induction depend on its location

<table>
<thead>
<tr>
<th>Sequences</th>
<th>Cytokine Production&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-12 (ng/ml)</td>
</tr>
<tr>
<td>Media</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>1826D</td>
<td>0.37 ± 0.07</td>
</tr>
<tr>
<td>M21</td>
<td>3.04 ± 0.57</td>
</tr>
<tr>
<td>M20</td>
<td>0.42 ± 0.11</td>
</tr>
<tr>
<td>M12</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>M22</td>
<td>0.88 ± 0.17</td>
</tr>
<tr>
<td>M23</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>M16</td>
<td>2.40 ± 0.77</td>
</tr>
<tr>
<td>M16A</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>M16T</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>M16C</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>M16GC</td>
<td>0.08 ± 0.02</td>
</tr>
</tbody>
</table>

<sup>a</sup> C57BL/6 mouse splenic DCs (10⁵ cells/well) were stimulated by various ODNs (1.5 μM) for either 10 h (TNF-α) or 30 h (IL-12).

<sup>b</sup> Mouse TNF-α and IL-12 were measured from supernatants by ELISA. The 24-h supernatants were tested for IL-12. Consensus CpG motifs are underlined and nucleotide runs are shown by bold letters. All the experiments were repeated three times with similar results.

FIGURE 2. The effect of a dG₆ run in peritoneal macrophages and B cells. ODNs (3 μM) were incubated for 30 h with 10⁵ peritoneal macrophages and 2 × 10⁵ CD11c⁺ splenic B cells. The nucleotide sequence of M21C is TCCATGACGTTCCTGACGTTC CCCCC. Each bar represents the mean and SEM of triplicate determinations. The experiments were repeated three times with similar results. UD, undetected.
run, indicating that the dG_6 run at the 5' terminus may inhibit the cytokine induction mediated by CpG ODNs, even if it could increase their cellular binding and internalization activities.

The dG run-containing CpG ODNs bind to the receptor with a SR-A ligand specificity on splenic DCs

It was previously suggested that ODNs containing a dG_12 run at their 5' and 3' termini could bind to SR on macrophages (17). In fact, the expression of the SR family in subsets of DCs has not been clearly identified. Singh et al. showed that maleyl-BSA which is the ligand of many SR families (SR-A I/II, SR-BI, CD36) could bind to murine splenic DCs (21). In addition, human DCs could express CD36 (27) and Cla-1 (28), the human homologue of the murine SR-BI. Thus, we performed the competitive binding assays using f-M21 for SR families in DCs. The binding of f-M21 to DCs was significantly impaired by treatment with the competitive ligands for SR-A, such as dextran sulfate, fucoidan, and poly(dG)_20 (19) (Fig. 5). In contrast, chondroitin sulfate and poly(dC)_20 did not compete with f-M21 for binding to DCs, indicating the specificity of competition. In addition, pretreatment with low density lipoprotein and oleic acid, which are ligands for SR-BI and CD36, respectively, did not diminish the binding of f-M21 even at a high concentration (0.5 mg/ml), suggesting that the receptor with a SR-A ligand specificity may confer the binding activity to dG run-containing ODNs. It was previously reported that phosphodiester or phosphorothioate ODNs could bind to Mac-1 proteins (CD11b/CD18) in human polymorphonuclear leukocytes and that fibrinogen and anti-CD18 mAb efficiently compete with the binding of ODNs to Mac-1 (29). However, pretreatment of fibrinogen and anti-CD18 mAb could not decrease the binding of f-M21 to DCs.

FIGURE 3. The effect of 3'-dG_6 run conjugation into phosphorothioate CpG ODN on cytokine induction. Splenic DCs (10^5) were stimulated by ODNs for either 10 h (TNF-α) or 30 h (IL-12). Mouse cytokines were determined from cell-free culture supernatants by ELISA. 1826D and M21 are phosphodiester CpG ODNs, and 1826T, M21T, and control ODN are phosphorothioate-modified ODNs. The nucleotide sequence of control ODN is TCCATGAGCTTCCTGAGCTT. The experiments were repeated three times with similar results.

FIGURE 4. Conjugation of a dG_6 run enhanced the binding and the internalization of CpG ODNs in DCs. CD11c^+ splenic DCs (10^5) were incubated with various ODNs tagged by fluorescein. The MFI was measured from quadruplicate DC cultures. Data are expressed as the relative index, defined as the fold increase in MFI in the presence of f-ODNs over MFI in the presence of medium alone. The f-ODNs (5 μM) were incubated for 30 min at 4°C. The MFI from f-ODNs bound to cell membrane was measured (A). f-ODNs (1 μM) were treated for 30 min at 4°C and then further incubated for either 1.5 or 6 h at 37°C. MFI from internalized f-ODNs was measured by removing membrane-bound f-ODNs (B).
and to pMac, suggesting that Mac-1 was not a target receptor for the binding of dG\textsubscript{6} run-containing phosphodiester ODNs, at least in the murine APCs.

The conjugation of a dG\textsubscript{6} run induces the structural transformation of CpG ODNs

It was previously reported that short oligo-dGs could be assembled into a four-stranded helix, called a tetraplex, that was stabilized by G quartets and inhibited SR activity (18). To investigate whether specific structural determinants of CpG ODNs are required for binding to SR, we determined the tetraplex formation of various CpG ODNs with or without a dG run by the presence of unusual imino proton NMR resonances in 10–12 ppm (30, 31). The population of tetraplex structures relative to that of Watson-Crick base-paired hairpin or duplex structures was estimated for various ODNs (Fig. 6). All ODNs with four or longer consecutive dG residues formed tetraplex structures regardless of their location. Longer dG runs increased the tetraplex population. ODNs containing a 3’-dG\textsubscript{6} run (M16 and M21) formed more clear tetraplex structures than did those containing a 5’-dG\textsubscript{6} run (M16’ and M20), which may explain why CpG ODNs with a 3’-dG\textsubscript{6} run were more efficient in the cellular binding. For the 3’-dG run-containing CpG ODNs, the ability to induce IL-12 appeared to correlate with the population of the tetraplex structure, suggesting that the structural transformation into a tetraplex was critical for cytokine induction by CpG ODNs with a 3’-dG run.

The conjugation of a dG\textsubscript{6} run into phosphodiester CpG ODNs is crucial for the generation of Th1 immunity in vivo

To determine whether the conjugation of a dG run also affected the immunostimulatory potentials of phosphodiester CpG ODNs in vivo, alum-absorbed, heat-inactivated HIV-1 particles with or without ODNs were s.c. immunized into mice. Without the coadministration of ODNs, mice raised moderate levels of IgG, which were mainly composed of IgG1 isotype (Fig. 7A). The coadministration of a dG\textsubscript{6} run-containing CpG ODN, M16, increased total IgG production \((p < 0.05)\), especially the IgG2a isotype \((p < 0.03)\), which is one of the representatives of Th1 immune responses (32). In contrast, the coadministration of either M16C or M16GC did not significantly increase the levels of total IgG and IgG2a. These results suggest that the presence of both a CpG motif and a dG run are critical for the adjuvanticity of phosphodiester CpG ODN, whereby either a dG run or a CpG motif alone did not confer an immunostimulatory effect in vivo. Since only phosphorothioate-modified CpG ODNs have been used as adjuvants to date (33–35), we next compared the adjuvanticity of dG\textsubscript{6} run-tagged phosphodiester CpG ODN (M21) with phosphorothioate CpG ODN (1826T) for the generation of Th1 immunity. We also used the phosphodiester CpG ODN (1826D) as a control CpG ODN for these experiments. To clearly observe the adjuvanticity, we immunized gDE2t fusion proteins (24) in the absence of alum together with relatively low dose of ODNs \((2 \mu \text{g})\) into mice. The coimmunization of gDE2t proteins with 1826D elicited a low level of HCV E2-specific IgG2a production (Fig. 7B). In contrast, coadministration of M21 elicited IgG2a production approximately 50-fold higher than did 1826D, but 2-fold lower than that induced by guest on April 16, 2017 http://www.jimmunol.org/ Downloaded from

FIGURE 5. The d\textsubscript{G}\textsubscript{6} run-containing CpG ODNs bind to a receptor with an SR-A ligand specificity in splenic DCs. CD11c\textsuperscript{+} splenic DCs (10\textsuperscript{5}) were incubated with f-M21 (1 \(\mu \text{M}\)) in the presence of various ligands (0.25 mg/ml; 10 \(\mu \text{M}\) poly(dG)\textsubscript{20} and poly(dC)\textsubscript{20}), and MFI from bound f-M21 was measured. Data are expressed as the relative percentage of binding when the MFI from f-M21 alone was regarded as 100%. All experiments were repeated five times with similar results.

FIGURE 6. Imino proton NMR spectra and the population ratios of tetraplex structure for various ODNs. The population ratios of tetraplex structure to hairpin or duplex structure were estimated by calculating the peak integral ratios of G quartet imino proton resonances and Watson-Crick base-paired imino proton resonances at 10–12 and 12–14.5 ppm, respectively. The relative percentages of IL-12 induction from splenic DCs were also reported by setting the IL-12 induction by p19 as 100%. The nucleotide sequence of M16’ is GGGGGGAAAACGT TCGTC.
by 1826T, indicating that the tagging of a dG₆ run into 1826D enhances its adjuvanticity to elicit IgG₂a production. In concordance with IgG subtype responses, CD4⁺ T cells from mice that received either M21 or 1826T had a higher ratio of IFN-γ to IL-4 secretion than did mice that received 1826D by the stimulation with recombinant HCV E₂t proteins (Fig. 7C). Overall, our results suggest that the conjugation of a dG run is critical for induction of Th1 immunity to protein Ags when phosphodiester CpG ODNs are used as adjuvants.

Discussion
In this report we clearly demonstrated that the conjugation of a dG₆ run into phosphodiester CpG ODNs augmented cytokine induction from various mouse APCs, especially splenic DCs, only when a dG₆ run was located at the 3’ terminus. Tagging of a dG₆ run to phosphodiester CpG ODNs enabled them to efficiently target various APCs, including DCs, through SR-mediated endocytosis. It is of interest to note that DCs stimulated by CpG ODNs with a dG₆ run produced approximately 50-fold more IL-12 than did pMac, even though DCs bind to ODNs 4 times less efficiently than do pMac. Our competition analysis suggests that the potential receptor for the binding of dG₆ run-containing CpG ODNs is SR-A in CD1c⁺ splenic DCs, since only ligands for SR-A could inhibit the binding of CpG ODNs. However, we do not exclude the possibility that another receptor(s) sharing ligand specificity with SR-A confers the binding of dG₆ run-containing CpG ODNs. Receptor signaling by SR ligation has been reported to affect cytokine induction. The ligation of SR by maleyl-BSA could initiate transcription of the TNF-α gene on murine macrophages (36). In contrast, it is known that SR ligation by maley-BSA selectively suppresses LPS-induced IL-12 production in bone marrow-derived macrophages (37). However, it is unlikely that SR ligation through G quartets by itself affects intracellular signaling for cytokine induction in DCs, since neither TNF-α nor IL-12 is induced by either dG₆ run-containing ODNs that lacked a CpG motif or poly(dG)₂₀.

Formation of a tetraplex structure stabilized by G quartets dramatically affected both the efficiency of binding and subsequent internalization of dG run-containing CpG ODNs into APCs. It is unclear why a dG₆ run conjugated at the 5’ terminus is less efficient at inducing a tetraplex structure than a dG₆ run conjugated at
the 3′ terminus. Nevertheless, the location of a dG run within CpG ODNs appeared to be important for their functions, since a 5′-dG run inhibited cytokine induction elicited by CpG motifs when CpG ODNs were delivered with liposomes. Our results are partially consistent with a recent report by Krieg et al. (38) that demonstrated that some ODNs containing self-complementary and/or G-rich sequences (CpG-N) neutralized the ability of CpG motif-mediated cytokine induction in cis or trans. They also suggested that the higher ordered structure of CpG-N might exert neutralizing effects on cytokine induction. However, our structural data showed that a G quartet structure failed to induce IL-12 production only when it was located at the 5′ terminus of phosphodiester CpG ODNs (Fig. 6). The detailed mechanisms by which a 5′-dG run inhibits CpG-mediated cytokine induction should be explored.

Previous reports suggested that the sequence contexts flanking the CpG motif were important for the activity of CpG ODNs (10, 38, 39), but it was unclear how and why the flanking sequences influenced their functions. Even though 1826D induced a relatively low level of cytokine production from DCs, other phosphodiester CpG ODNs tested in this study had no activity in the absence of a dG run. However, these CpG ODNs elicited cytokine production similar to that by 1826D when they were delivered with liposomes (Table II), suggesting that the specificity of ODN sequences may be related to their internalization efficiency. In addition, it is likely that the length of the CpG ODN affects the efficiency of intracellular signaling for cytokine induction and/or the stability of the ODN, since 11-mer CpG ODN (M12) induced IL-12 much less efficiently than did longer CpG ODNs when delivered with liposomes (Table II). Taken together, our results suggest that both the sequence context and the length of phosphodiester CpG ODNs are important to modulate their immunostimulatory potentials. The chemical property of the backbone structure of CpG ODNs also affected their ability to induce cytokine. Of note, the conjugation of a dG run at the 3′ terminus of a phosphorothioate CpG ODN decreased its ability for both TNF-α and IL-12 induction from splenic DCs. Ballas et al. also demonstrated that the poly(dG) ends completely abrogated the enhancement of NK activity by CpG motifs when the entire backbone was phosphorothioate modified (20). Although additional experiments are required to clarify whether the backbone structure influences CpG-mediated cytokine induction, the phosphorothioate modification is suggested to reduce the binding affinity of the CpG ODN to a putative cellular target protein(s) (40).

The intrinsic immunostimulatory potential of the phosphorothioate backbone can generate Janus-faced consequences, since it strongly boosts cytokine induction elicited by CpG motifs and induces detrimental immunostimulation and toxicity (1, 41–43). In fact, we observed that a single s.c. administration of phosphorothioate, but not phosphodiester, CpG ODNs (30–50 μg) with 5 μg of dE21 protein absorbed in alum elicited granuloma formation at the injection sites in mice (data not shown). In contrast to phosphorothioate CpG ODNs, phosphodiester CpG ODNs without a dG run were poorly immunostimulatory by themselves in vitro and in vivo, possibly due to the low efficiency of nonspecific endocytosis and vulnerability to nucleases. However, the conjugation of a dG run may surmount the defects of phosphodiester CpG ODNs due to the following reasons. First, since the tetraplex structure was reported to confer serum nucleic resistance (44), it is possible that the conjugation of a dG run may extend a half-life of phosphodiester CpG ODNs. Second, the dG run efficiently targets CpG ODNs into various APCs via the SR. Our data demonstrated that the conjugation of a dG run into phosphodiester CpG ODNs can generate Th1 immunity in vivo. In addition, we observed that the binding and internalization of phosphodiester ODNs into HepG2 cells that express SRs were significantly increased in the presence of a dG run (unpublished observations), suggesting that the conjugation of a dG run has a great potential for delivery of ODNs into cells expressing SRs, such as hepatocytes (45, 46). In conclusion, it is likely that the conjugation of a dG run into phosphodiester CpG ODNs would be a promising modification for therapeutic purposes of CpG ODNs.

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References


