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A Polysaccharide Carrier for Immunostimulatory CpG DNAs To Enhance Cytokine Secretion[||]

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Oligodeoxynucleotides containing unmethylated CpG sequences (CpG DNA) have been shown to stimulate a cell-mediated immune response for mammals.[1] This immune response is considered to be a defense system that mammals have evolved because unmethylated CpG sequence emerges more frequently in bacterial DNAs than in mammalian DNAs. Considerable attention is devoted to this response because CpG DNA can be extraordinarily effective adjuvants for many vaccines against infections agents, cancer antigens, and allergens.[1] Henmi et al.[2] demonstrated that CpG DNA can be recognized by Toll-like receptor 9 (TLR-9). The Toll-like receptor family is generally located on plasma membrane and can recognize bacterial cell wall components. However, Ahmad-Nejad et al.[3] demonstrated that TLR-9 is not localized on the cell surface, but intracellularily, predicting that if we can efficiently deliver CpG DNA to endosome and/or lysosome, the immune response can be enhanced and controlled artificially.

To deliver CpG DNA to intracellular endosome and/or lysosome, there are two major issues to overcome: instability of CpG DNA in biological fluids and low uptake efficiency into cells, similar issues for antisense DNA carriers.[5] Instability of CpG DNA is ascribed to hydrolysis mediated by deoxyribonuclease. The hydrolysis can be significantly suppressed by the use of phosphorothioates,[5] and thus many studies use phosphorothioate CpG DNAs. However, phosphorothioates can be bound to some plasma proteins in nonspecific manners, which can cause undesirable side effects. Therefore, CpG DNA carriers are required to prevent the phosphorothioates from forming such nonspecific interactions with proteins, as well as to protect them against hydrolysis mediated by deoxyribonuclease. Several materials have been studied as CpG DNA carriers. The liposomal delivery is one of the preferred methods;[6] however, there are some drawbacks in its use.[4]

Sakurai and Shinkai found that the β-(1 → 3)-D-glucan schizophyllan (SPG) forms a novel complex with some polynucleotides,[7] and the complex is applicable to an antisense DNA carrier.[8] Here, SPG is an extracellular polysaccharide produced by the fungus *Schizophyllan commune*, and the main chain consists of β-(1 → 3)-D-glucan and one β-(1 → 6)-D-glycosyl side chain linked to the main chain at every three glucose residues (Figure 1).[9] The complex is automatically dissociated when the pH becomes less than 6.0, because protonation of the nucleotide base induces conformational changes, which causes dissociation of the complex.[10] This pH response seems ideal for releasing the complexed CpG DNA in late endosome in which the pH is maintained about 5.0. The complexed oligonucleotides acquire stronger resistance to deoxyribonuclease-mediated hydrolysis and SPG can prevent the complexed phosphorothioate oligonucleotides from forming nonspecific interactions with plasma proteins.[11] These properties seem greatly advantageous as a CpG DNA carrier, and the main purpose of this communication is to present preliminary results to prove superiority of SPG for the carrier.

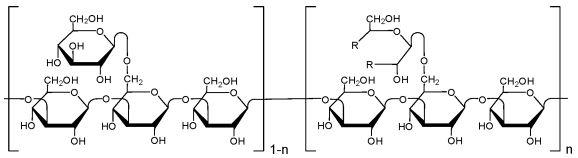
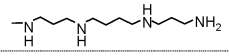
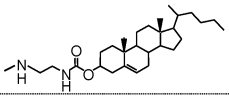
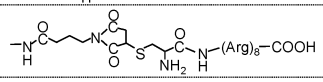
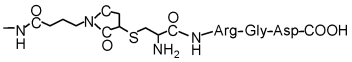


Figure 1. Chemical structure of the chemically modified schizophyllan (SPG) used in this study. The left-hand side shows the intact structure of SPG, and the right shows the modified one. The modification level in Table 1 is defined by "*n*" in the figure. The modification was made by the selective cleavage of 1,2-diol group of the glycosyl side chain with periodate, leading to formyl group formation and subsequently introducing a functional group (see Supporting Information).

Table 1. Sample Codes and the Introduced Chemical Groups

Sample code	R	Modification level [a]	N/P ratio[b]
SP-SPG		4.6 ± 0.3 mol%	0.27
Chol-SPG		6.9 ± 1.0 mol%	0.21
R8-SPG		0.5 ± 0.1 mol%	~ 0
RGD-SPG		1.3 ± 0.3 mol%	~ 0

The uptake efficiency of SPG itself is not so high; therefore, we modified SPG with a functional group that can induce passive cellular ingestion. In this work, we introduced spermine (SP), arginine-glycine-aspartic acid tripeptide (RGD), octaarginine (R8), and cholesterol (Chol) (see Table 1). As CpG DNA, we used phosphorothioate 5′-TCCAT*GACGTT*CCTGATG-$(dA)_{40}$-3′ (the immunostimulatory sequence; PuPuCGPyPy is italicized).[12] For a negative control, we used the sequence of 5′-TCCATGA*GC*TTC-CTGAGT-$(dA)_{40}$-3′, where only the CG sequence is reversed (italic) and denoted "non-CpG DNA". In both sequences, a poly$(dA)_{40}$ tail is attached at the 3′ end to increase the complex stability.[7,8] The complexation was carried out by the established method,[7] and the molar ratio (M_{s-SPG}/M_{ODN}) was fixed to 1.5, where M_{s-SPG} and M_{ODN} are the molar concentration of the repeating unit of SPG and

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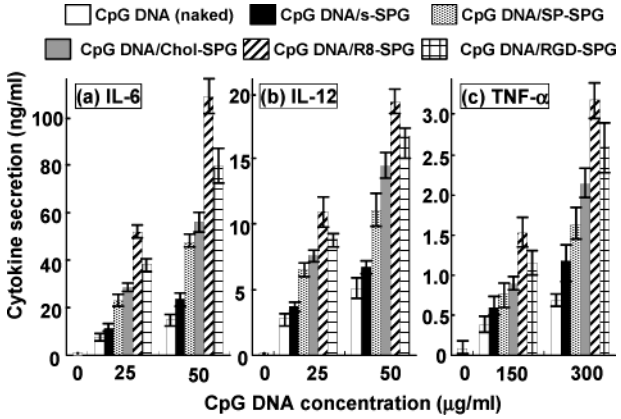

Figure 2. Effect of chemical modification of SPG on CpG DNA-mediated cytokine secretion. The murine macrophage-like cell J774.A1 (1×10^6 cells/mL, 100 μL/well) was stimulated with 25 and 50 μg/mL for IL-6 and IL-12 and with 150 and 300 μg/mL for TNF-α. The amount of secretions was determined with ELISA kits, after incubating the cells in the presence of CpG DNA or its complex for 24 h. The molar ratio (M_{s-SPG}/M_{ODN}) is fixed at 1.5.

CpG DNA, respectively. Although this molar ratio is in excess of SPG over the stoichiometric composition ($M_{s-SPG}/M_{ODN} = 2/3$),[7] the best antisense effect has been achieved at this composition.[8] Thus, we used this composition in the present work, and the total charge of the complex is maintained at negative at the composition.

When we exposed the murine macrophage-like cell J774.A1 to CpG DNA (complexed or naked), the cytokine secretion increased rapidly and reached a plateau after 9−15 h, being similar to the previous results.[12] After 24 h, all samples showed no increment and seemed to complete the secretion. Therefore, we evaluated the carrier performance from the amount of secreted cytokine after 24 h. Figure 2 plots the averaged amount of cytokine against the CpG DNA dose for three cytokines: IL-6 (a), IL-12 (b), and TNF-α (c), and the experimental variations are indicated by error bars.

When we exposed the cells to naked CpG DNA, the secretions of IL-6, IL-12, and TNF-α are 9.0 ± 1, 2.6 ± 1, and 0.75 ± 0.1 ng/mL, respectively. When the CpG DNA was added as a complex with nonmodified SPG (CpG DNA/s-SPG), the secretion is increased by about 20−40% from the naked assay. This difference should be ascribed to the facts that the complexed CpG DNA neither binds to serum proteins nor suffers hydrolysis from deoxyribonuclease,[11] although SPG itself does not have ability to enter cells. The modified SPG increases the secretion dramatically; it is 5−10-fold compared with the naked assays. Among them, R8−SPG shows the highest performance, RGD−SPG ranks the second, and Chol−SPG follows. The difference between RGD−SPG and Chol−SPG is prominent for IL-6; however, it is relatively small for IL-12 and TNF-α. As far as we know, this is the highest enhancement of the cytokine secretion by carriers.

The ingestion mechanism differs on introduced chemical moieties to the carrier. The cellular membrane is negatively charged, and therefore cations such as spermine can bind to the surface with the Coulombic forces and should be ingested by the regular pinocytosis cycle. This should be the case for SP−SPG; however, ingestion through the electrostatic interaction should not be effective for our case, because the complex is negatively charged in total (see Table 1). Therefore, it interferes with the spermine versus cell interaction. Generally, cholesterol-appended carriers are ingested through LDL receptor and RGD-appended ones, through integrins. Cellular ingestion for these is considered as receptor-mediated endocytosis. Therefore, after ingestion, the RGD−SPG or Chol−SPG/CpG DNA complex is eventually transported to endosome and finally to lysosome, where the compartment pH is kept to about 5 and digestive enzymes are highly activated. The complex releases the CpG DNA because of low pH,[10] and the naked CpG DNA is easily recognized by TLR-9 on the vesicular membrane. This may be a reason for the relatively high secretion for RGD−SPG and Chol−SPG complexes. In contrast with RGD, the arginine-rich peptides such as R8 are seemingly ingested by a different pathway from those of cations RGD and cholesterol, although there is still controversy and little agreement on the uptake mechanism.[13] Some data suggested that the pathway induced by R8 appears to deposit the R8-appended material directly into cytosol. If this were the only pathway to ingest the R8−SPG complex, the cytokine secretion would have been smaller than the others. The precise mechanism to uptake the R8−SPG complex and reason for such enhancement are not clear at this moment.

To sum up the present finding, when SPG is chemically modified with R8, RGD, and Chol and when the CpG DNA complex made therefrom is exposed to macrophages, dramatic enhancement in the cytokine secretion is observed. The secretion increased 5−10 times from the naked dose and 100 times from the background. This performance promises that SPG can be an excellent carrier for CpG DNA.

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Supporting Information Available: Experimental details, materials, SPG chemical modification procedures, cytotoxicity assay for the carriers, assay for non-CpG, confocal microscopy observation, and pH dependence of CD spectrum of the complex. This material is available free of charge via the Internet at http://pubs.acs.org.

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