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CpG DNA/zymosan complex to enhance cytokine secretion owing to the cocktail effect

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Abstract—Zymosan, classified among β-(1→3)-D-glucans, is produced from the cell wall of yeast and well known to induce proinflammatory cytokines when ingested by immune cells. We found that zymosan forms a complex with immunostimulatory CpG DNA, where both zymosan and CpG DNA can induce cytokine secretion according to the different mechanisms (i.e., recognized by different receptors). The complex activated macrophages and induced cytokine secretion, more efficiently than separate administration of zymosan or CpG DNA. Microscopic observation showed that this increment of the cytokine secretion can be explained by the fact that zymosan and zymosan/CpG DNA complex are up-taken more than naked CpG DNA. Additionally, existence of two different immunostimulants in the same cells may enhance the immunoresponse. This report presents a new strategy to construct a delivering vehicle for CpG DNA and to enhance its activity with the 'cocktail effect' of the two immunostimulants.

Many fungi produce β-(1→3)-D-glucans that can activate innate immune systems when they are administered into vertebrates. Among others, curdlan, lentinan, and schizophyllan (SPG) have already been used in tumor immunotherapy for more than decades. Curdlan is the simplest β-(1→3)-D-glucan among others and only consisting of a β-(1→3)-D-glucose main chain without any side chain. Other natural β-(1→3)-D-glucans have rather complicated structures: branching, long side chains, and β-(1→4) or β-(1→6) linkages in the main chain. For example, SPG has one β-(1→6)-D-glycosyl side chain that links to the main chain every three glucose residues. These tertiary structures are considered to provide significant effects on their biological activities as well as their physical properties.[1] In the case of SPG, the side chain provides water solubility. Zymosan is a cell wall component of yeast: *Saccharomyces cerevisiae* and classified among β-(1→3)-D-glucans; however, its tertiary structure is too complicated to assign the exact chemical structure. It is known that among the β-(1→3)-D-glucans, zymosan is the most effective immunostimulant.[2–4] Therefore, zymosan has been attracting attention in its clinical use.[5–7] Recent work in immunology identified that a pattern recognition receptor TLR-2 (Toll-like receptor 2) located on the cell surface is responsible for recognizing zymosan and other β-(1→3)-D-glucans. Pattern recognition receptors can recognize the targets from its tertiary structure. Therefore, the relationship between the tertiary structure of β-(1→3)-D-glucans and immunoresponse has become a hot research area to attract more attention.

There are other materials that can induce innate immune system. One of them is a DNA fragment that contains unmethylated CG-rich domains originated from bacterial genome.[8] Since bacterial DNA contains unmethylated CG sequences more frequently than those of vertebrates, vertebrate immune cells recognize these CG-rich sequences as a pathogen-associated molecule. Since artificial oligonucleotides containing a CG

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sequence (CpG DNA) induce the same immune response as bacterial DNA, CpG DNA can be an extraordinarily effective adjuvant. Recent breakthrough in immunology showed that TLR-9 can recognize the key sequence of PuPuCGPyPy in single-stranded DNA and it localizes in the late endosome or lysosome.[9]

Sakurai and Shinkai found that SPG can form a novel complex with polynucleotides[10] and demonstrated that the complex can be used as a CpG DNA carrier.[11] The major advantages of using SPG are: (1) the complex can protect the bound CpG DNA against enzymatic degradation during the cell culture or circulation of blood, (2) since SPG accumulates in macrophages, the complexed SPG can act as a targeting vehicle, and (3) since the complex becomes unstable under acidic conditions, CpG DNA can be easily recognized by TLR9 in the late endosome. Since CpG DNA is a much better immunostimulant than SPG, the complexed SPG seemed to act only as a delivery vehicle, although SPG itself is an immunostimulant to some extent. In this report, we use zymosan that is a better immunostimulant than SPG, and demonstrate whether zymosan can form a complex with CpG DNA, and how the complex induces cytokine secretion.

An immunostimulatory sequence containing PuPuCGPyPy[9] (italicized): phosphorothioate 5′-TCCAT*G ACGTT*CCTGATG-$(dA)_{40}$-3′ was prepared by Hokkaido System Science and denoted by CpG DNA. For a negative control, 5′-TCCATGA*GC*TTCCTGAGT-$(dA)_{40}$-3′ was used and denoted by non-CpG DNA, where only the CpG part is reversed. In both sequences, a dA_{40} tail was attached at the 3′ end to increase the complex stability.[10,12] Zymosan was purchased from Invivogen and used without further purification, and its molecular weight was more than 10^6 as determined by gel permeation chromatography. The complexation was carried out according to the same method as SPG.[10] Figure 1A confirms the complexation between CpG DNA and zymosan by gel electrophoresis. Naked CpG DNA migrated (lane 1), as expected. On the other hand, the complexed CpG DNA with zymosan or SPG had two bands: one stayed at the originally loaded hole and the other appeared as a broad smear band. These results indicate that molecular weight is increased with complexation, which becomes strong evidence for complexation with zymosan or SPG. Figure 1B compares the circular dichroic (CD) spectra between CpG DNA and its mixture with zymosan at 5 °C. The CD spectrum is drastically changed, when zymosan is mixed with the oligodeoxynucleotide in the same way that SPG was mixed with CpG DNA as described in our previous reports.[13] This feature also provides the evidence of the complex formation in the mixture. We measured the temperature dependence of CD spectrum for the CpG DNA/zymosan complex. The spectral change caused by the dissociation of the complex was observed over 37 °C (data not shown). The results indicate that the complex is stable at both room and cell-incubation temperatures.

The amounts of cytokines (IL-12, IL-6, and TNF-α) secreted from the murine macrophage-like cell J774.A1 were determined with ELISA kits (ENDOGEN), comparing among naked CpG DNA, complexed CpG DNAs with zymosan or SPG, and carriers themselves. Here, the molar ratio in the SPG/CpG DNA complex was fixed at $M_{SPG}/M_{DNA} = 1.5$, where M_{SPG} and M_{DNA} are the molar concentrations of the repeating unit of SPG and CpG DNA, respectively. The reason to use this composition was because $M_{SPG}/M_{DNA} = 1.5$ was the optimized condition for cytokine secretion.[11] The stoichiometric study showed that two main chain glucoses bind to one base, which corresponded to $M_{SPG}/M_{DNA} = 2/3$. Therefore, the optimized molar ratio is larger than the stoichiometric value, probably, owing to the possibility that an excess amount of SPG is more favorable to protect the bound DNA against enzymatic degradation. Since the exact chemical structure of zymosan is not known, we could not determine the molar concentration of zymosan. Thus, alternately, we prepared two zymosan complexes with the different compositions: one complex made from the same weights of zymosan and CpG DNA as the SPG/CpG complex (denoted by Zym in Fig. 2) and the other used the double amount of zymosan with the same amount of CpG DNA (denoted by 2 × Zym in Fig. 2).

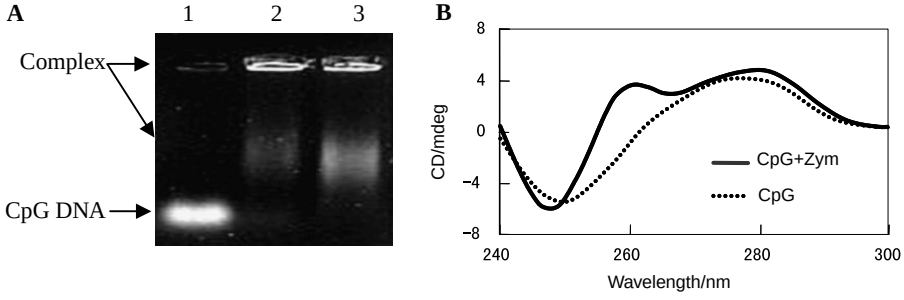

Figure 1. (A) Comparison of the gel electrophoresis migration patterns among naked CpG DNA (lane 1), CpG DNA/zymosan complex (lane 2), and DNA/SPG complex (lane 3). 2 wt % NuSieve agarose gel (BMA) was used and the gel was stained with GelStar® (BMA). Gel electrophoresis was carried out in TAE buffer containing 3% dimethylsulfoxide (20 V, 3 h). (B) CD spectra of CpG DNA and its mixture with zymosan. The dotted and solid lines indicate the CD spectra for CpG DNA and its mixture with zymosan, respectively. The mixture was kept at 5 °C for a night to lead to complexation. The CpG DNA and zymosan concentrations were 20 μM, 6.67 μg/ml, respectively. The water volume fraction of DMSO/water mixtures was 0.92, in 3 mM Tris–HCl (pH 8.0) containing 150 mM NaCl.

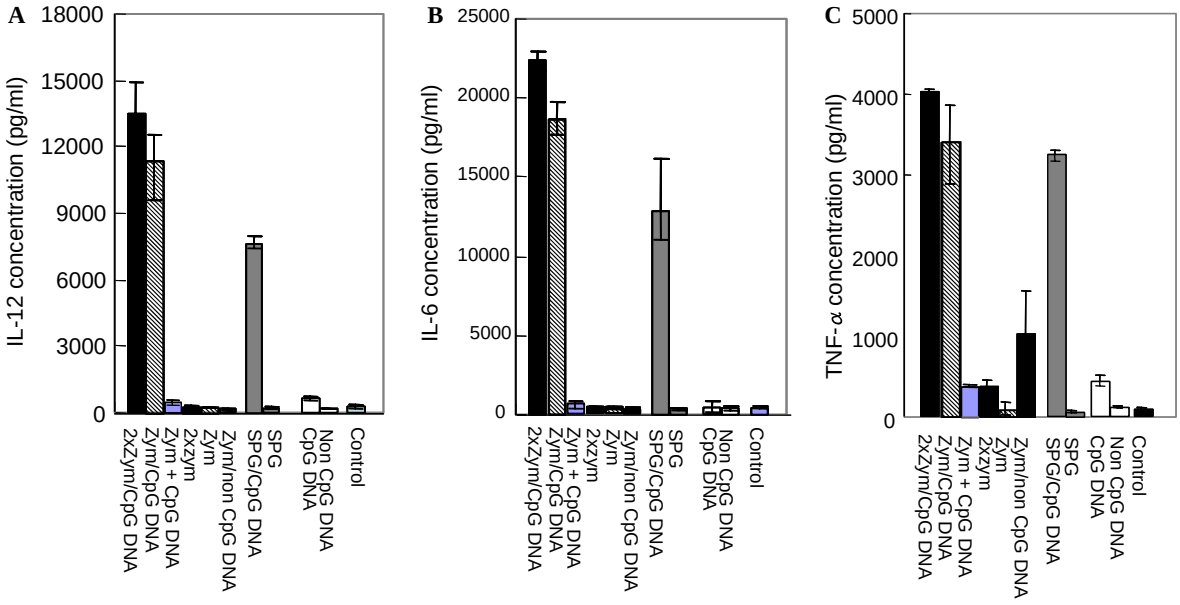

Figure 2. Comparison of the cytokine secretion (IL-12; A, IL-6; B, and TNF-α; C) among the complexed CpG DNA with zymosan or SPG, naked CpG DNA, non-CpG DNA, separately added CpG DNA and zymosan (zymosan + CpG DNA), and the carrier itself.

Figure 2 shows the results for IL-12 (A), IL-6 (B), and TNF-α (C), comparing with the control of saline solution dose. Zymosan itself enhanced TNF-α secretion, while no increments were observed for IL-12 and IL-6. SPG showed no change for all three cytokines. This difference between zymosan and SPG coincides with the previous reports.[2] The complexed SPG/CpG DNA showed more enhanced secretion than naked CpG DNA, by 12, 33, and 7 times for IL-12, IL-6, and TNF-α, respectively, confirming our previous results using SPG.[11] It is interesting that the complex with zymosan CpG DNA increased the secretion of IL-12, IL-6, and TNF-α by 21, 57, and 9 times of the naked doses, respectively. This enhancement level is clearly beyond experimental error and shows reliable reproducibility. All secretions were increased with the increase in zymosan concentration. When we added the same amount of zymosan and CpG DNA separately at the same time (i.e., without making the complex; denoted by Zym + CpG DNA in Fig. 2), the secretion level was much lower than the complex doses. These data show that the complexation of zymosan and CpG DNA is essentially important to stimulate an immune response.

The recognition of zymosan is achieved by a number of receptors, including CR3, scavenger receptors, Dectin-1, and TLR-2.[14,4] Binding Dectin-1 and zymosan induces phagocytosis and TLR-2 plays an important role in signaling the activation of cytokine secretion. On the other hand, CpG DNA can be recognized by intracellular TLR-9 in the late endosome.[15] Therefore, the enhanced effect with the zymosan complex can be rationalized by the hypothesis that zymosan enhances the cellular uptake of the CpG DNA complex. To examine this proposal, we exposed J774.A1 cells to the TAMRA-labeled complex made of dA_{40} oligonucleotide and zymosan, and subsequently observed the cell morphology with confocal microscopy (ECLIPSE TE2000-U, Nikon, Tokyo, Japan, attached to a confocal scan unit Radiance 2100, Bio-Rad, Tokyo, Japan). Figure 3 shows the results.

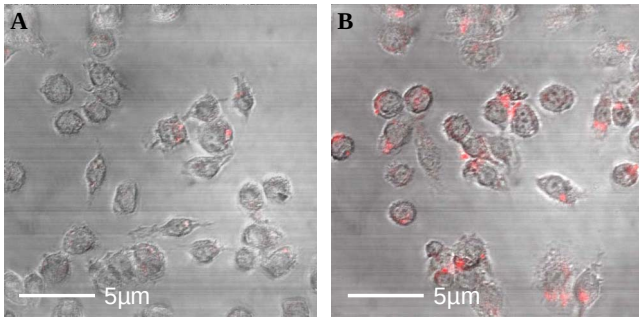

Figure 3. Confocal microscopy observation of TAMRA-labeled dA_{40} after the oligonucleotide was administered to J774.A1 at the naked (A) and zymosan-complexed states (B).

Photograph B (complex) gives many red spots, corresponding to the labeled oligonucleotide, in the cells or on the surface of the cells. On the other hand, such spots are scarcely observed in photograph A (naked DNA). Furthermore, the red spots seem to be located in vesicles more than in the other parts. These observations clearly confirm our foregoing proposal. All the results suggest that the zymosan/CpG DNA complex can be recognized by Dentin-1 and TLR-2 on the cellular surface and can induce phagocytosis leading to uptake of the complex. Subsequently, the complexed or released CpG DNA can be recognized by TLR-9 on endocytosis vesicles. When we simply increased the amount of CpG DNA, such a large increment in secretion level was not observed. This could suggest that the enhanced secretion can be ascribed not only to the increased amount of CpG DNA due to the zymosan-associated uptake, but also to the coexistence of two different immunostimulants in the same cell which may interfere in the up-regulation of cytokine secretion to enhance the signaling cascade for immune response. In this sense, we call the enhanced effect with zymosan the 'cocktail effect' of zymosan and CpG DNA.

Acknowledgments

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