Clustered CpG Sequences to Enhance Cytokine Secretion from Macrophages

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CG sequences (CpG ODNs) can activate innate immune and thus be potentially useful for immune stimulants in therapeutic use. By hybridizing a CpG ODN having a $(dA)_{40}$ tail with poly(dT), we prepared a DNA duplex that multiple CpG ODNs are hanging from the duplex. IL-12 cytokine secretion reached the maximum at the composition that two to four CpG ODNs in one duplex. These phenomena can be rationalized by an allosteric or cluster effect, and the present clustered CpG system is the simplest and most elucidating tool to investigate the cluster effect of CpG ODNs.

Unmethylated CG sequence is more prevalent in bacterial than in vertebrate genomic DNAs. Distinguishing this frequency difference, vertebrate antigen-presenting cells (APCs) can recognize microbial DNAs and eventually activate innate immune response.[1,2] The initial event of this process includes the uptake of microbial DNAs by a particular type of APCs and subsequent the fragmentation of microbial DNAs in endocytosis pathway. The fragmented CG sequences are recognized by a pattern recognition receptor called toll-like receptor 9 (TLR-9).[3] The similar immune responses can be induced by a variety of synthetic oligodeoxynucleotides containing CG sequences (CpG ODNs) and thus CpG ODNs are applicable as an immune-therapeutic adjuvant. Recent researches have focused on the relationship between CpG sequence and its immune response.

Marshall et al.[4] are the first to report that multiple CGs in one ODN sequence secrete more cytokine than single CG in one sequence when the total amount of CG is kept equivalent. When the multiple ones are ingested by APCs, the local concentration of CG in the endocytosis vesicles should be increased compared when single one is ingested. We presume that this increased CG concentration can allosterically induce the recognition of TLR-9. This effect can be termed the "cluster effect," which is normally described in the case that branched oligosaccharides bind much more tightly to receptors than monosaccharides or single chain oligosaccharides.

We have previously reported that the polysaccharide called schizophyllan can form a complex with CpG ODN and the more cytokine secretion is induced when the molecular weight of schizophyllan is increased.[5] Since the larger molecular weight of the polysaccharide has more CpG ODN within a single complex, our result can be rationalized in the same framework of the cluster effect of CpG ODN.

The aim of this paper is to clarify this cluster effect by use of a simple model. In this work, we used a phosphothioate ODN: 5′-TCC ATG ACG TTC CTG ATG $(dA)_{40}$ that consists of a CpG ODN (the key sequence is underlined) part and a $(dA)_{40}$ tail. We denote this sequence by CpG-A hereinafter, and we used a phosphothioate type for CpG-A because of its good cellular ingestion ability. When CpG-A is hybridized with poly(dT) (in our work, the base number is approximately 320), the $(dA)_{40}$ tail can bind to poly(dT) and the CpG ODN part should come out on the outer side of the DNA double helix. The length of the dA tail was chosen so that the (AT) duplex is stable at physiological condition or low salt conditions. By changing the CpG-A/poly(dT) mixing ratio, the number of CpG on one hybridized poly(dT) can be controlled from 1 to 8, as presented in Figure 1. Hereinafter, the CpG-A to poly(dT) molar ratio is denoted by 2/1, 4/1, and so on.

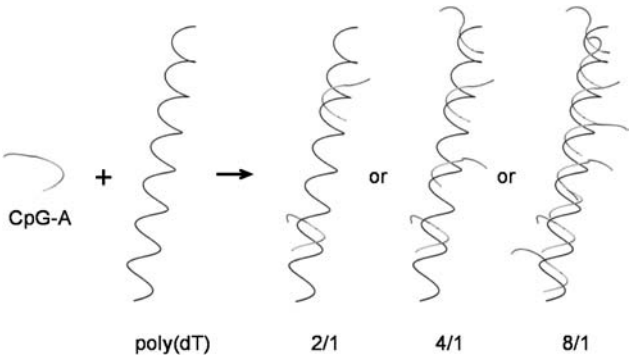

Figure 1. Schematic illustration of the clustered CpG ODNs along the poly(dT) chain. The fractions indicate the molar ratio of CpG-A to poly(dT).

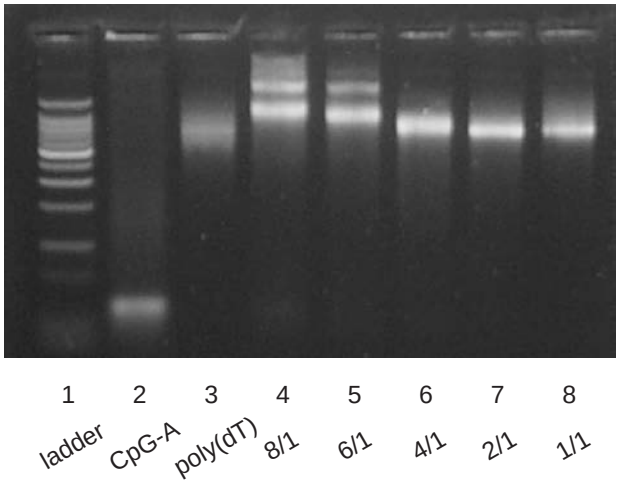

Figure 2. Gel electrophoresis patterns for 100 bp ladder (lane 1), CpG-A (lane 2), poly(dT) (lane 3), and five mixtures of CpG-A and poly(dT) (lanes 4–8). The mixtures were annealed for 2 days in 150 mM NaCl. 2 wt % NuSieve agarose gel (BMA) was used and the gel was stained with GelStar® (BMA).

Figure 2 shows the gel electrophoresis patterns for CpG-A (lane 2), poly(dT) (lane 3), and five CpG-A/poly(dT) mixtures (lanes 4–8). With increasing the CpG-A molar ratio, the bands

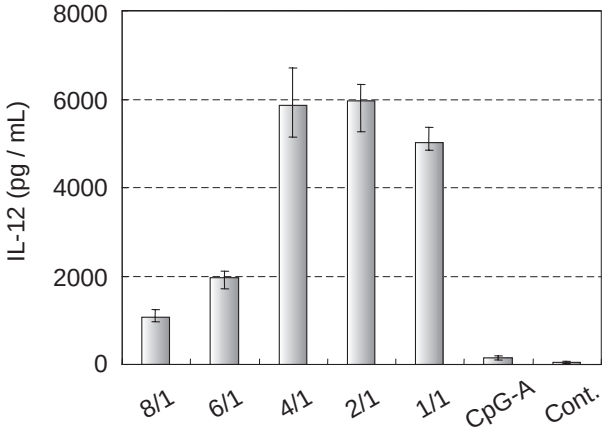

Figure 3. Comparison of IL-12 secretion from murine macrophage-like cells (J774.A1) induced by CpG-A/poly(dT) duplexes with different CpG contents. The cells were cultured in 96-well plates (1×10^5 cells/well). The CpG-A concentration was fixed at 1.5 μM for all samples. After the CpG-A/poly(dT) duplexes were exposed for 24 h, the supernatants were taken and the amount of IL-12 was determined with ELISA ($n = 3$).

are shifted to higher molecular weight side, confirming that the number of the bound CpG-A is increased as illustrated in Figure 1. In the lanes 4–8, no free CpG-A band is observed, indicating that all of the fed CpG-A molecules are bound with poly-(dT). At lanes 4 and 5 (CpG-A/poly(dT) = 8/1 and 6/1), additional bands are observed at the higher molecular weight side and this feature seems more enhanced at 8/1. This phenomena can be explained by dimerization and trimerization of poly(dT) due to connecting (or bridging) different poly(dT) molecules with the dA tail of CpG-A. The important conclusion of Figure 2 is that simple mixing of CpG-A and poly(dT) can provide a molecule with a different number of CpG portions.

Figure 3 represents the amount of the secreted IL-12 as determined by ELISA (Endogen), compared to the different CpG-A/poly(dT) ratios. Here, the CpG-A concentration is fixed at 1.5 μM for all samples and we confirmed that the IL-12 secretion ability from the cells did not reach the maximum. Since the cellular ingestion is presumably determined by the concentration of the phosphothioate CpG-A, we postulate that the average number of the ingested CpG-A molecules should be in the same range for all samples. This presumption is supported by the fact that the number of the ingested phosphothioate ODNs is generally increased in proportion to both the concentration and the ODN length. Nevertheless, we are currently examining the amount of the ingested CpG-A/poly(dT) as a function of the molar ratio with confocal laser scanning microscopy and fluorescence-activated cell sorters.

As presented in Figure 3, CpG-A itself secreted 141 pg/mL of IL-12 and this value was reproducible and consistent with our previous results.[5] Once CpG-A was hybridized with poly-(dT), the secretion level dramatically increased even at 1/1, reached the maximum at 2/1–4/1, and decreased with increasing the molar ratio. It is interesting that the secretion was magnified to about 42 fold of CpG-A alone at the maximum. Another interesting feature is that the secretion was reduced at the higher ratio.

The enhanced secretion can be mainly explained by two reasons; the first one is due to the cluster effect of CpG as mentioned above and the second is conformational effects of CpG DNAs. One might suppose that the duplexes can be more easily recognized with TLR-9 than the single ODNs. The duplex effect may be eliminated because when we carried out the similar experiment with non-CpG-ODN, no increased secretion was observed (data are not shown). We need more systematic experiments, possibly by changing the poly(dT) length and alternating the hybridized pairs to clarify the conformational effect.

One possible reason for the observed enhancement even at a ratio of 1/1 is that, since the 1/1 mixture has a large distribution and thus it contains 2/1 or 3/1 type molecules, these multiple CpG molecules play a significant role to induce the secretion. On the other hand, at the higher ratio, the secretion was reduced. These low secretions can be explained by; (1) too many CpG-As on one chain may induce steric hindrance for the TLR-9 recognition, or (2) the flexibility of the duplex was decreased and thus the recognition done by TLR-9 may be obstructed. These speculations need to be examined with further examination.

In conclusion, multiple CpG ODNs on the same chain can be produced by a simple model and they induced large amounts of cytokine secretion (IL-12) in murine macrophage (at most 42 fold of the control). This result can be rationalized by the cluster effect of CpG to induce TLR-9 recognition. Our finding should provide a new insight in design of an efficient artificial CpG ODN to control various cytokine secretions. Therefore, further studies should be needed to clarify the CpG cluster effect and other results presented in this paper.

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