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Short communication

Identification of Immunostimulatory Oligodeoxynucleotide from *Escherichia coli* Genomic DNA

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Bacterial DNA containing immunostimulatory CpG motifs can stimulate antigen-presenting cells to express co-stimulatory molecules and to produce various cytokines *in vivo* and *in vitro*. In this study, we fragmented macromolecular *E.coli* genomic DNA with DNase I, and analyzed the ability of the resulting DNA fragments to induce the NF-κB activation and humoral immune response. Furthermore, using computational analysis and luciferase assay for synthetic ODNs based on the sequence of the immunostimulatory DNA fragments (DF-ODNs), an active component of DF-ODNs sequences was investigated. Experimental results demonstrated that DF-ODN is optimal for the NF-κB-responsive promoter activation in the mouse macrophage cell line and the humoral immune response *in vivo*. In agreement with the activity of the DF-ODNs processed by DNase I, a synthetic ODN based on the DF-ODN sequences is potent at inducing IL-12 mRNA expression in primary dendritic cells. These results suggest that the discovery and characterization of a highly active natural CpG-ODN may be achieved by the analyses of bacterial DNA fragments generated by a nuclease activity.

Keywords: CpG-DNA, DNase I, *E. coli* chromosomal DNA, Humoral immune response, IL-12

Introduction

In order to elicit an immune response, the mammalian immune system must be able to recognize particular macromolecules of an infectious agent as nonself, generally either protein or lipid antigen (Beckman *et al.*, 1994). In addition to humoral immunity, microbial products such as formyl methionine, lipopolysaccharide (LPS), exopolysaccharride, peptidoglycan and teichoic acid are known as polyclonal activators of rapid immune activation, and induce the innate immune responses via the recognition of toll-like receptors (Janeway and Medzhitov, 1998; Aderem and Ulevitch, 2000). There is increasing evidence demonstrating that mammalian immune systems are able to distinguish bacterial DNA from self DNA, with bacterial DNA directly activating immune cells (Pisetsky, 1996A). *E. coli* DNA and a synthetic oligodeoxynucleotide (ODN) containing the palindromic sequence AACGTT identified from clones of mycobacterial DNA can induce IFN-γ production (Klinman *et al.*, 1996).

The general difference between bacterial and mammalian DNA lies in the frequency of CpG dinucleotides and the methylation status of cytosine in the CpG dinucleotides. CpG dinucleotides are under-represented and selectively methylated in vertebrate DNA. In contrast, CpG dinucleotides are present at the expected frequency (about 6.25%) with unmethylated cytosine in bacterial DNA. In B cells, activation by bacterial DNA requires unmethylated CpG motifs, and is mimicked by some CpG-containing ODNs. The most active ODNs contain CpG motif flanked by two 5'-purines and two 3'-pyrimidines (Krieg *et al.*, 1995). Synthetic oligodeoxynucleotides (ODNs) containing CpG motifs (CpG-ODNs) mimic the direct immunostimulatory effects of native bacterial DNA, and activate multiple cell types including macrophages, dendritic cells, NK cells, and B lymphocytes (Pisetsky, 1996B; Klinman *et al.*, 1996; Hartmann *et al.*, 1999; Krieg, 2002). Immunostimulatory activities of CpG-ODNs have gained attention as potentially useful therapeutics for immune adjuvant, inflammatory and allergic disease, and for immunoprotective agent. Recent studies from several laboratories have used phosphorothioate-modified oligodeoxynucleotides (PS-ODNs) for clinical applications of CpG-ODNs (Broide *et al.*, 1998; Krieg and Davis, 2001). The PS-ODN has a sulfur substitution for the nonbridging oxygens in the backbone,

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providing its nuclease resistance (Stein *et al*., 1998) and efficient uptake into the cells (Zhao *et al*., 1994). Although current studies of therapeutic CpG-ODN have focused on PS-ODN, the phosphorothioate backbone linkage is known to induce CpG-independent side effects (Monteith *et al.,* 1997; Liang and Lipsky, 2000). In previous study, we tried to identify potentially active natural CpG-DNA through the computer-assisted analysis of *M. bovis* genomic DNA and screened the genomic DNA sequences of *M. bovis* that have immunostimulatory activity. Our experimental analyses demonstrate that the potent CpG-DNA in the *M. bovis* genome has functional effects as a Th1-responsive adjuvant, and that it activates the transcription factor NF-kB (Lee *et al.,* 2006). Here, we investigated *E. coli* genomic DNA digested by DNase I and noticed that the nuclease activity is associated with immunological process by generating DNA fragments containing CpG motifs. In turn, *E. coli* DNA fragments processed by the DNase I provide a signal to immune activation. We have also identified a potent ODN sequence of *E. coli* DNA fragments (DF-ODN) having effective immunostimulatory function by the combination of computational analysis, luciferase assay, and IL-12 expression analysis.

Materials and Methods

Preparation of *E. coli* DNA fragments and oligodeoxynucleotides. 200 μg/ml of *E.coli* genomic DNA was incubated with 0.02 U of DNase I (Sigma) in 20 mM Tris pH 7.0, 10 mM $MgCl_2$, and 1 mM $CaCl_2$ at 37°C (Kwon and Kim, 2003). Reaction was stopped at indicated periods by extracting DNA with phenol/chloroform. To remove endotoxin, the reaction product was extracted with 0.5% Triton X-114, and then extracted with chloroform by three times. The DNA fragments were precipitated with ethanol, resuspended in distilled water, and measured observance at A_{260nm} to determine the concentration of DNA. The DNA fragments were resolved on a 15% TBE-polyacrylamide gel and visualized with UV light after stained with ethidium bromide. We purchased ODNs from GenoTech. The candidate of immunostimulatory DF-ODN 04(S) is the phosphorothioate version of DF-ODN 04(O), and DF-ODN 04(M) is consisted of upstream 16 bases of DF-ODN 04(O): CTCG CACGTTGCCGAA (Fig. 2A). A non-CpG-ODN 2041(S) served as a negative control (Lee *et al.*, 2004). The endotoxin content of the DNA fragments and the ODNs were less than 1 ng/ml of DNA as measured by a Limulus amebocyte assay (Whittaker Bioproducts).

Immunization and ELISA. We housed the BALB/c mice in specific pathogen-free conditions and injected them at six to eight weeks of age. Hen egg lysozyme (HEL, Sigma) and DNA fragments were dissolved in PBS, and groups of four mice were injected intraperitoneally with 50 μg of HEL supplemented with 50 μg of the DNA fragments. The mice were sacrificed two weeks after the injection and sera were collected from mice by a heart-punching method. To detect the HEL-specific IgG production, we performed an ELISA as previously described (Pang and Ru, 2005).

Sequence analysis of the DNA fragments. Based on the TBE-polyacrylamide gel analysis, DNA bands of 20-200 bp lengths (fragment 3, DNase I digestion for 40 min) were extracted, and end-filling reaction was performed by Klenow polymerase. The prepared DNA fragments were cloned into pGEM-T vector and the sequences are determined to gain totally 50,000 bp nucleotide sequence of the DF-ODNs. The sequences of *E. coli* K12 genome (GenBank accession no. NC000913) and the DF-ODNs were analyzed with the aid of a computer program. With an in-house PYTHON script, we counted the frequency of 256 hexameric sequences with a CG dinucleotide in the middle of the sequence (NNCGNN in which N is A, T, C or G, CpG hexamer), and then used the frequency as a scoring table (Table 1). We calculated the relative frequency of each CpG hexamer by dividing its frequency by the number of random hexamers in intact *E. coli* genome and DF-ODN sequence, respectively. To screen immunostimulatory DF-ODNs, we listed DF-ODN as a 20 nt long ODN with three CpG hexamers based on the DNA fragments sequence, in which we excluded the sequences containing sequential CG dinucleotides from the list (Fig. 2A).

Cell culture and luciferase assay. We obtained the mouse macrophage cell line RAW 264.7 from the American Type Culture Collection and maintained it in DMEM with 10% FBS. A day before the transfection, we placed the RAW 264.7 cells into 12-well plates at a concentration of 2×10^5 cells per well. We then transfected the cells with pIL-8-*Luc* using the FuGene 6 Transfection Reagent (Roche) in accordance with the manufacturer's instructions. After the transfection, we treated the cells for 6 h with DF-ODN (3 μM). We then determined the luciferase activities by using the Dual-Luciferase Reporter Assay System (Promega) with a TD-20/20 luminometer (Tuner Designs) according to the manufacturer's specifications. To confirm the equivalent transfection efficiency, we cotransfected the promoterless *Renilla* luciferase vector pRL-*null* (Promega) as an internal control (Lee *et al*., 2004; Jing *et al.,* 2004). The individual assays were normalized for *Renilla* luciferase activity, and the data are presented as a fold increase in activity relative to the unstimulated control.

Generation of bone marrow cells and RT-PCR analysis. Bone marrow cells were isolated by flushing off the femoral region of the BALB/c mice using a syringe with a 27-gauge needle. Cells were cultivated in RPMI 1640 medium supplemented with 10% FBS, 10 ng/ml GM-CSF and IL-4 (Biosource) for 6 days at 37°C in 5% CO_2. Non-adherent cells were removed on day 2 and 4 of culture, and the culture medium was changed with RPMI 1640 medium containing 10% FBS, 10 ng/ml GM-CSF and IL-4. After treating the cells with DF-ODNs (3 μM) or non-CpG-DNA 2041(S) for the indicated periods, we extracted the total RNA with a MicroRNA Isolation Kit (Stratagene) in accordance with the manufacturer's instructions. We then reverse-transcribed 7 μg of total RNA as described previously (Lee *et al.,* 2004). The primer pairs for IL-12, and GAPDH were as follows: IL-12, 5'-CGTGCTCATGGCTGGT GCAAAG-3' and 5'-CTTCATCTGCAAGTTCTTGGGC-3'; GAPDH, 5'-ATGGTGAAGGTCGGTGTGAACG-3' and 5'-GTTGTCATGG ATGATCTTGGCC-3'.

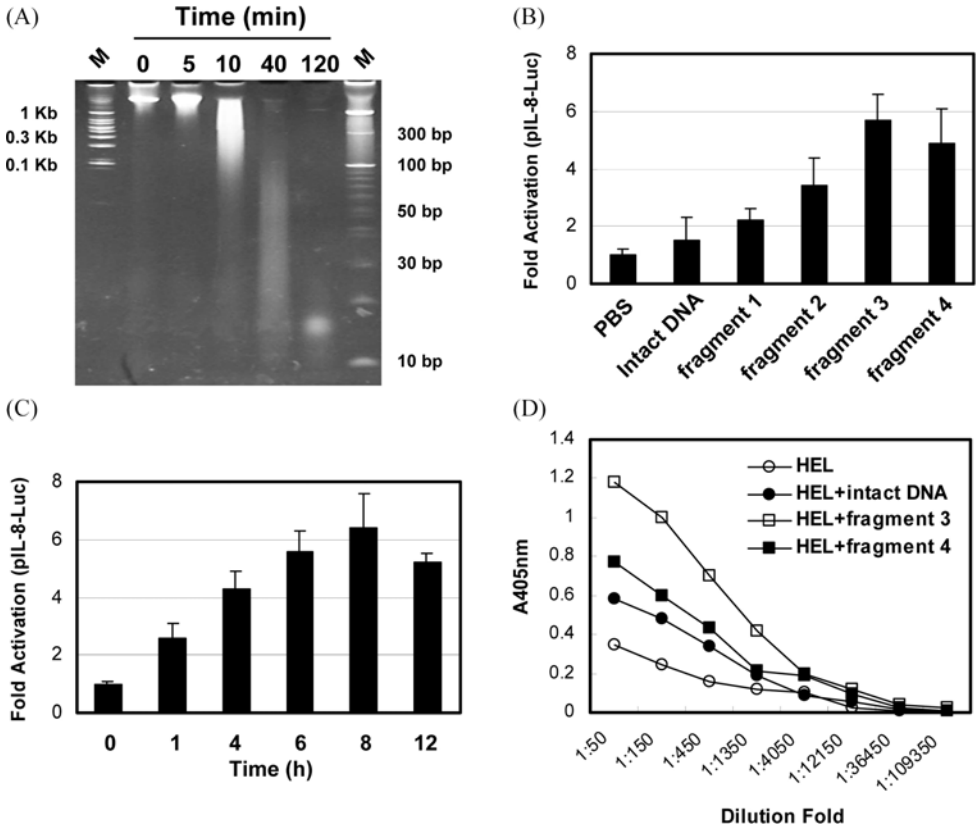

Fig. 1. Induction of the NF-κB-responsive promoter activation in RAW 264.7 cells and humoral immune response in mice by the *E. coli* DNA fragments processed with DNase I. (A) Time-dependent reaction products of *E. coli* genomic DNA digested with DNase I. *E.coli* genomic DNA (200 mg/ml) was incubated with 0.02 U of DNase I for the indicated periods, the DNA fragments were resolved on a 15% TBE-polyacrylamide gel and visualized with UV light after stained with ethidium bromide. M; DNA size marker. (B) The DNA fragments were extracted from each reaction product, and luciferase assay was performed with these fragments in RAW 264.7 cells as described under "Materials and Methods". Fragment 1, 5 min digestion; Fragment 2, 10 min digestion; Fragment 3, 40 min digestion, Fragment 4, 120 min digestion. (C) The cells were treated with the fragment 3 (DF-ODNs) during indicated periods, and the luciferase activity was measured. The results are represented as fold activation compared with the unstimulated control. D, BALB/c mice were injected IP with free HEL, HEL with 50 μg of intact *E. coli* DNA, or 50 μg of the fragment 3 and fragment 4. The sera were collected two weeks after injection, and production of HEL-specific antibody was assayed by an ELISA.

Results and Discussion

Preparation and analysis of immunostimulatory DF-ODNs. To gain an immunostimulatory sequence of bacterial genomic DNA, we first hypothesized that the macromolecular DNA needs to be processed by a nuclease activity prior to activation of immune cells. In this respect, we fragmented *E. coli* genomic DNA by DNase I, a nuclease that is secreted from mammalian cells. As shown in Fig. 1A, we observed time-dependent degradation of the *E. coli* genomic DNA by DNase I activity. The resulting DNA fragments were purified from each reaction, and then measured its potential of NF-κB-responsive promoter activation using IL-8 promoter-reporter construct in RAW 264.7 cells (Fig. 1B). Intact *E. coli* DNA has a very low activity of the promoter activation. However, when the cells were treated with the DNA fragments processed by DNase I, luciferase activity was increased significantly, which depends on the degree of fragmentation (fragment 1-4). Fig. 1C shows time-dependent activation of the promoter by the DNA fragments. We also determined a humoral immune response that is induced by immunizing HEL with the DNA fragments in mice. Consistent with the results of luciferase assay, production of HEL-specific IgG is enhanced with the fragment 3 significantly, which is more potential than the intact DNA or fragments 4 (Fig. 1D). Thus, the results revealed that 20-200 bp long DNA fragments processed by DNase I (DF-ODN) optimally induce the NF-kB-responsive promoter activation and humoral immune response.

Identification of an immunostimulatory DF-ODN sequence. To evaluate the DNA sequences of DF-ODNs in the context of immunostimulatory potential, we cloned the DNA fragments. Individual nucleotide sequence of the cloned fragments was determined by DNA sequencing to gain totally ~50,000 bp nucleotide sequence of DF-ODN. Using BLAST search

Table 1. Analysis of CpG hexamers in the DF-ODNs

No.	Sequences	Frequency in intact *E. coli* DNA (%)	Frequency in DF-ODNs (%)	Fold
1	AGCGGC	0.0494	0.0944	1.91
2	AACGGT	0.0377	0.0944	2.50
3	GCCGCC	0.0663	0.0944	1.42
4	GGCGAC	0.0365	0.0858	2.35
5	ATCGCC	0.0766	0.0858	1.12
6	ATCGGC	0.0479	0.0858	1.79
7	TTCGCC	0.0598	0.0858	1.43
8	TGCGCA	0.0457	0.0858	1.88
9	TTCGCT	0.0382	0.0858	2.25
10	CGCGGC	0.0461	0.0772	1.67
11	GGCGCA	0.0593	0.0772	1.30
12	GGCGAA	0.0596	0.0772	1.30
13	GGCGTT	0.0611	0.0772	1.26
14	GGCGCG	0.0606	0.0772	1.27
15	AGCGCG	0.0465	0.0772	1.66
16	CACGGG	0.0159	0.0772	4.86
17	GCCGGT	0.0408	0.0687	1.68
18	ACCGGC	0.0411	0.0687	1.67
19	GGCGGC	0.0671	0.0687	1.02
20	CGCGCC	0.0603	0.0687	1.14
21	AACGCT	0.0426	0.0687	1.61
22	GCCGCT	0.0496	0.0687	1.38
254	TCCGAC	0.0123	0.0000	0.000
255	GACGTA	0.0142	0.0000	0.000
256	CACGTG	0.0031	0.0000	0.000
Average frequency (%)		0.0292	0.0293	1.00
Total frequency (%)		7.472	7.502	1.00

The frequency of 256 hexamer sequences with a CG dinucleotide in the middle of the sequence (NNCGNN) were counted, and the relative frequency of the each CpG hexamer was calculated by dividing the hexamer's frequency by the number of the nucleotides. Fold column represents the relative ratio of the frequencies of each CpG hexamer in the DF-ODNs to intact *E. coli* genome.

program, we were able to confirm that the determined DNA sequences are indeed originated from *E. coli* genome. Based on the sequence determination of DF-ODNs, we then analyzed hexameric sequences (NNCGNN) that contain CG dinucleotide in the core (CpG hexamer). The relative frequency of the each CpG hexamer was calculated by dividing its frequency by the number of nucleotides. As shown in Table 1, we recognized that the overall CpG hexamers in the DF-ODN sequences appear at a frequency that is the same with that of the *E. coli* genome, but each frequency of the hexamer is different from that of the intact genomic DNA. These results imply that DF-ODNs have differential proportions of CpG motif from the intact *E. coli* genomic DNA, which may elicit an immunostimulatory function.

In order to further investigate the characteristics of individual DF-ODN, we screened the immunostimulatory DF-ODN comprising 20-nt long sequences with the three CpG hexamers as described in left panel of Fig. 2A. Using IL-8 promoter-reporter construct, we determined the luciferase activity when RAW 264.7 cells were treated with a synthetic ODN based on the sequences of the 34 kinds of DF-ODN. As shown in Fig. 2A, we found that most of DF-ODNs did not activate the NF-κB-responsive promoter even though they all have three CpG hexamers in their sequences. However, a candidate DF-ODN (DF-ODN 04) induced a significant increase of the luciferase activity. As indicated in Fig. 2B, DF-ODN 04 enhanced the promoter activation in a dose-dependent manner.

DF-ODN 04 consists of a 20 nt sequence with the three CpG motifs 'CTCGCA', 'CACGTT', 'GCCGAA', but whether these CpG motifs have an immunological effect was never considered before. Here, we show that DF-ODN 04, which has natural phosphodiester linkage, activates the NF-κB-responsive gene expression in RAW 264.7 cells. CpG-ODNs being used in several application studies were synthesized with phosphorothioate backbone modification to resist nuclease digestion (Broide *et al*., 1998; Krieg and Davis, 2001). However, we found that phosphothioate backbone modified-DF-ODN 04 (DF-ODN(S)) shows a slightly higher activity for the NF-κB-responsive promoter than the phosphodiester form of DF-ODN 04 (DF-ODN 04(O)) in the macrophage cell line (Fig. 2C). Furthermore, when we removed 4 nt of 3' region from DF-ODN 04(O), the derivative of DF-ODN 04(O) (DF-ODN 04(M)) was still potent at enhancing the NF-κB-responsive promoter. Taken together, these results confirm that DF-ODN 04 sequence only with the three CpG motifs can elicit an immunostimulatory function with regardless to the backbone modification.

Effect of DF-ODN on IL-12 gene expression in dendritic cells. It has been reported that NF-κB is associated with a CpG-ODN-mediated expression of IL-12 p40 mRNA (Takeshita and Klinman, 2000). As DF-ODN 04 enhances the NF-κB-responsive promoter activation, we tested the ODN for its ability to express endogenous IL-12 gene in primary cells. Time-course analysis was conducted to monitor the mRNA expression of IL-12 in bone marrow-derived dendritic cells. As shown in Fig. 3, expression of IL-12 mRNA in the cells treated with DF-ODN 04(O) was initiated with 4 h after the stimulation, and increased significantly in 8 h. When the cells were treated with a non-CpG-ODN 2041(S), expression of IL-12 gene was not detected. Based on this result, it is evident that DF-ODN 04(O) can also effectively stimulate an NF-κB-responsive proinflammatory cytokine gene expression in the mouse primary dendritic cells.

Identification of bacterial genomic DNA as foreign requires a sufficient process for exposing its structural determinants to a cognitive receptor such as TLR9 because the macromolecular

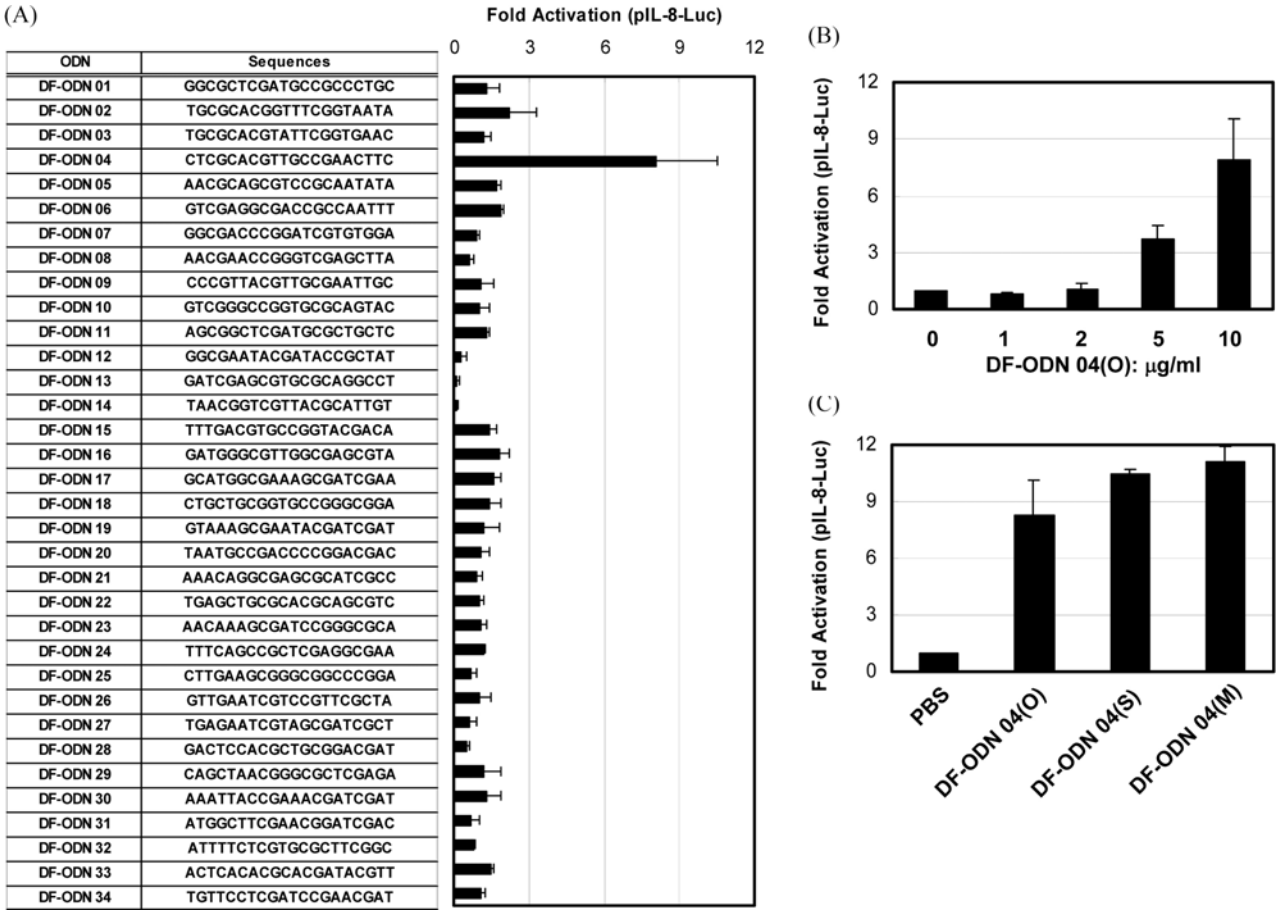

Fig. 2. Identification of an immunostimulatory DF-ODN. (A) Candidate DF-ODN sequences are listed (left panel) and the luciferase activity was measured with the synthetic ODNs based on sequences of the list (right panel). RAW 264.7 cells were transiently tansfected with an IL-8-*Luc*, and then stimulated with candidate DF-ODN for 6 h. (B) The cells were treated with increasing amount of DF-ODN 04, and assayed for luciferase activity. (C) The cells were cultured with 3 μM of DF-ODN 04(O), DF-ODN 04(S), and DF-ODN (M) for 6 h before the luciferase activity was measured. Data are from three independent experiments performed in duplicate with similar results.

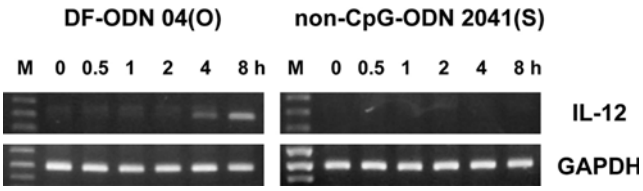

Fig. 3. DF-ODN-induced expression of IL-12 mRNA in primary dendritic cells. We isolated dendritic cells from mouse bone marrow, and treated the cells with DF-ODN or non-CpG-ODN 2041(S) for the indicated periods, and the total RNA was then extracted. After reverse transcription, expression of IL-12 gene was analyzed by PCR. Expression level of GAPDH mRNA was used as an internal control. M, DNA standard marker.

DNA cannot be endocytosed into the cells (Hemmi *et al.*, 2000; Ahmad-Nejad *et al.*, 2002). In this respect, we assumed the requirement of a nuclease activity for bacterial DNA to stimulate immune cells. However, a nuclease secreted from immune cells and responsible for this process has not been identified yet. As a model system, we used DNase I secreted from pancreas and analyzed the fragmentation of *E. coli* genomic DNA. As expected, the DNA fragments of 20-200 bp gain an immunostimulatory function by processing the genomic DNA with DNase I. Isolation and characterization of DNA processing enzyme activity *in vivo* will be an interesting project to be done in the future.

Using a computer-assisted program and luciferase assay, we analyzed the structure and sequence of DF-ODN responsible for its ability of activating immune cells. In this report, we show that DF-ODN 04, which has natural phosphodiester linkages, is highly active in induction of the NF-κB-responsive gene expression in a macrophage cell line and dendritic cells, and that it is potent regardless of the backbone modification. This report suggests a new strategy to identify another type of natural CpG-ODN that consists of only phosphodiester linkage, and that we may trigger optimal innate immune responses without severe side effects by finely tuning the CpG-ODN sequences originated from natural bacterial DNA.

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References

Aderem, A. and Ulevitch, R. J. (2000) Toll-like receptors in the induction of the innate immune response. *Nature* **406**, 782-787.

Ahmad-Nejad, P., Hacker, H., Rutz, M., Bauer, S., Vabulas, R. M. and Wagner, H. (2002) Bacterial CpG-DNA and lipopolysaccharides activate toll-like receptors at dictinct cellular compartments. *Eur. J. Immunol.* **32**, 1958-1968.

Beckman, E. M., Porcelli, S. A., Morita, C. T., Behar, S. M., Furlong, S. T. and Brenner, M. B. (1994) Recognition of a lipid antigen by CD1-restricted ab^+ T cells. *Nature* **372**, 691-694.

Broide, D., Schwarze, J., Tighe, H., Gifford, T., Nguyen, M., Malek, D. S., Van Uden, J., Martin-Orozco, E., Gelfand, E. W. and Raz, E. (1998) Immunostimulatory DNA sequences inhibit IL-5, eosinophilic inflammation, and airway hyperresponsiveness in mice. *J. Immunol.* **161**, 7054-7062.

Hartmann, G., Weiner, G. J. and Krieg, A. M. (1999) CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells. *Proc. Natl. Acad. Sci. USA* **96**, 9305-9310.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K. and Akira, S. (2000) A toll-like receptor recognizes bacterial DNA. *Nature*, **408**, 740-745.

Janeway, C.A. Jr. and Medzhitov, R. (1998) Introduction: the role of innate immunity in the adaptive immune response. *Semin. Immunol.* **10**, 349-350.

Jing, Z., Liu, Y., Dong, M., Hu, S. and Huang, S. (2004) Identification of the DNA binding element of the human ZNF333 protein. *J. Biochem. Mol. Biol.* **37**, 663-670.

Klinman, D. M., Yi, A. K., Beaucage, S. L., Conover, J. and Krieg, A. M. (1996) CpG motifs present in bacterial DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon g. *Proc. Natl. Acad. Sci. USA* **93**, 2879-2883.

Krieg, A. M. (2002) CpG motifs in bacterial DNA and their immune effects. *Annu. Rev. Immunol.* **20**, 709-760.

Krieg, A. M. and Davis, H. L. (2001) Enhancing vaccines with immune stimulatory CpG DNA. *Curr. Opin. Mol. Ther.* **3**, 15-24.

Krieg, A. M., Yi, A. K., Matson, S., Waldschmidt, T. J., Bishop, G. A., Teasdale, R., Koretzky, G. A. and Klinman, D. M. (1995) CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **374**, 546-549.

Kwon, H. J. and Kim, D. S. (2003) Production of nuclease activity in U937 cells by phorol 12-myristate 13-acetate and lipopolysaccharide. *J. Biochem. Mol. Biol.* **36**, 520-523.

Lee, K. W., Jung, J., Lee, Y., Kim, T. Y., Choi, S. Y., Park, J., Kim, D. S. and Kwon, H. J. (2006) Immunostimulatory oligodeoxynucleotide isolated from genome wide screening of *Mycobacterium bovis* chromosomal DNA. *Mol. Immunol.* **43**, 2107-2118.

Lee, K. W., Kim, D. S. and Kwon, H. J. (2004) CG sequence- and phosphorothioate backbone modification-dependent activation of the NF-kB-responsive gene expression by CpG-oligodeoxynucleotides in human RPMI 8226 B cells. *Mol. Immunol.* **41**, 955-964.

Liang, H. and Lipsky, P. E. (2000) Responses of human B cells to DNA and phosphorotioate oligodeoxynucleotides. *Curr. Top. Microbiol. Immunol.* **247**, 227-240.

Monteith, D. K., Henry, S. P., Howard, R. B., Flournoy, S., Levin, A. A., Bennet, C. F. and Crooke, S. T. (1997) Immune stimulation-a class effect of phosphorothioate oligonucleotides in rodents. *Anticancer Drug Des.* **12**, 421-432.

Pang L. Y. and Ru, B. G. (2005) Studies on the epitope of neuronal growth inhibitory factor (GIF) with using of the specific antibody. *J. Biochem. Mol. Biol.* **38**, 646-649.

Pisetsky, D. S. (1996A) Immune activation by bacterial DNA: a new genetic code. *Immunity* **5**, 303-310.

Pisetsky, D. S. (1996B) The immunologic properties of DNA. *J. Immunol.* **156**, 421-423.

Stein, C. A., Subasinghe, C., Shinozuka, K. and Cohen, J. S. (1988) Physicochemical properties of phosphorothioate oligodeoxynucleotides. *Nucleic Acids Res.* **16**, 3209-3221.

Takeshita, F. and Klinman, D. M. (2000) CpG ODN-mediated regulation of IL-12 p40 transcription. *Eur. J. Immunol.* **30**, 1967-1976.

Zhao, Q., Waldschmidt, T., Fisher, E., Herrera, C. J. and Krieg, A. M. (1994) Stage-specific oligonucleotide uptake in murine bone marrow B-cell precursors. *Blood* **84**, 3660-3666.