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Plasmid DNA activates murine macrophages to induce inflammatory cytokines in a CpG motif-independent manner by complex formation with cationic liposomes

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Abstract

Plasmid DNA (pDNA) is very important in non-viral gene therapy and DNA vaccination. Unmethylated CpG motifs in bacterial DNA, but not in vertebrate DNA, are known to trigger an inflammatory response, which inhibits gene expression while improving immunological consequences. In this report, we investigated the cytokine secretion induced by pDNA/cationic liposome complexes using murine macrophages. Naked CpG DNA induced tumor necrosis factor-α (TNF-α) secretion from the macrophages, but DNA without CpG motif did not, demonstrating that the cytokine induction was mediated by CpG motifs. pDNA complexed with cationic liposomes, but not the cationic liposomes alone, produced a significant amount of TNF-α from the macrophages. Surprisingly, methylated pDNA and calf thymus DNA complexed with the cationic liposomes were also able to induce TNF-α production, indicating that these responses were not dependent on CpG motifs. Taken together, the present study demonstrated that for the first time DNA can stimulate murine macrophages in a CpG motif-independent manner when it is complexed with the cationic liposomes.

Keywords: Macrophages; Plasmid DNA; CpG motifs; Cationic liposomes; Tumor necrosis factor-α; Gene therapy; DNA vaccine

pDNA has become an important class of macromolecular agents suitable for non-viral gene therapy as well as DNA vaccination [1]. It is well known that unmethylated CpG sequences (CpG motifs) in bacterial DNA and pDNA, but not in vertebrate DNA, are recognized by the immune system as a danger signal [2]. Tokunaga et al. [3] were the first to report that DNA extracted from *Mycobacterium bovis* possesses a strong anti-tumor activity. Using synthetic oligodeoxynucleotide (ODN), Krieg et al. [4] defined unmethylated CpG motifs displaying the 5′-Pur–Pur–CpG–Pyr–Pyr-3′ nucleotide sequence as biologically active in terms of B-cell activation. There is a rapidly growing body of information on the biological effects of immune stimulatory CpG-motifs on a variety of immune cells including B-cells, NK-cells, monocytes, macrophages, and dendritic cells. For example, bacterial DNA and CpG ODN can activate cells of the innate immune system, such as macrophages and dendritic cells, to secrete pro-inflammatory cytokines including TNF-α, interleukins IL-1, IL-6, and IL-12 [5–9]. This phenomenon appears to be advantageous as far as DNA vaccination is concerned [10], since it is crucial in the subsequent development of T-helper 1 (Th1)-based T-cell lineages in response to CpG DNA [11,12]. On the other hand, recent reports have demonstrated that these inflammatory cytokines inhibit transgene expression with pDNA [13].

We have been studying the in vivo disposition characteristics of naked pDNA and found that liver non-parenchymal, probably Kupffer's (liver resident macrophages) cells play an important role [14,15].

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Further in vitro studies using cultured mouse peritoneal macrophages have demonstrated that a specific receptor like the class A scavenger receptor may be involved in the endocytic uptake of pDNA by macrophages [16,17]. In the light of these findings, the present study was undertaken to investigate the activation of cultured macrophages, one of the most important cell populations in the body for CpG motif-mediated immunostimulatory actions. The initial objective of this study was to compare the inflammatory responses induced by naked pDNA and its complex with cationic liposomes in relation to the uptake characteristics. Unexpectedly, we have found that pDNA can stimulate murine macrophages to secrete inflammatory cytokines in a CpG motif-independent manner when it is complexed with the cationic liposomes.

Materials and methods

Chemicals. RPMI 1640 medium and Hanks' balanced salt solution (HBSS) were obtained from Nissui Pharmaceutical (Tokyo, Japan). *Escherichia coli* DNA and calf thymus DNA were purchased from Sigma (St Louis, MO, USA). LipofectAMINE plus reagent and Opti-MEM were purchased from Lifetechnologies (Rockville, MD, USA). Triton X-114 was purchased from Nacalai Tesque (Kyoto, Japan).

Cell culture. Male ICR (5 weeks) or C3H/HeJ mice (LPS non-responder, 5 weeks) were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). Resident macrophages were collected from the peritoneal cavity of unstimulated mice with RPMI 1640 medium. Cells were washed, suspended in RPMI 1640 medium supplemented with 10% FBS, penicillin G (100 U/ml), streptomycin (100 μg/ml), and amphotericin G (1.2 μg/ml), and then plated on 24-well culture plates (Falcon, Becton Dickinson, Lincoln Park, NJ) at a density of 5×10^5 cells/well for the activation experiments. After a 2 h incubation at 37 °C in 5% CO_2–95% air, adherent macrophages were washed three times with RPMI 1640 medium to remove non-adherent cells and then cultured under the same conditions for 24 h. RAW264.7 cells were cultured with RPMI 1640 medium supplemented with 10% FBS, penicillin G (100 U/ml), and streptomycin (100 μg/ml). They were then plated on a 24-well culture plate at a density of 5×10^5 cells/ml and cultured for 24 h.

Plasmid DNA. pcDNA3 vector was purchased from Invitrogen (Carlsbad, CA, USA). pCMV-Luc encoding firefly luciferase gene was constructed, as described previously [18]. pcDNA3 has 26 5′-Pur–Pur–CpG–Pyr–Pyr-3′ sequences including two AACGTT sequences reported to be the most potent CpG motifs for mice [9]. pDNA was purified using an Endo-free plasmid Giga kit (Qiagen, Valencia, CA, USA). Methylated-CpG pDNA was synthesized by methylation of pDNA (pCMV-Luc) with 1 U CpG methylase (New England Biolabs, Beverly, MA, USA) per μg pDNA for 24 h at 37 °C. The methylated-CpG pDNA was tested for digestion with *Hpa*II (Takara, Kyoto, Japan) to confirm methylation. pDNA mobility was analyzed by 1% agarose gel electrophoresis.

Purification of DNA. To minimize the activation by contaminated LPS, we used DNA samples extensively purified with Triton X-114, a non-ionic detergent. Extraction of endotoxin from pDNA, methylated-CpG pDNA, *E. coli* DNA, and calf thymus DNA samples was performed according to previously published methods [19,20] with slight modifications. DNA samples were purified by extraction with phenol:chloroform:isoamyl alcohol (25:24:1) and ethanol precipitation. Ten mg DNA was diluted with 20 ml pyrogen-free water, then 200 μl Triton X-114 was added followed by mixing. The solution was placed on ice for 15 min and incubated for 15 min at 55 °C. Subsequently, the solution was centrifuged for 20 min at 25 °C, 600*g*. The upper phase was transferred to a new tube, 200 μl Triton X-114 was added, and the previous steps were repeated three or more times. The activity of LPS was measured by limulus amebocyte lysate (LAL) assay using the Limulus F Single Test kit (Wako, Tokyo, Japan). After purification using the Endo-free plasmid Giga kit, 1 μg/ml pDNA contained 0.01–0.05 EU/ml endotoxin. After Triton X-114 extraction, endotoxin levels of DNA samples could no longer be determined by LAL assay, i.e., 1 μg/ml DNA contained less than 0.001 EU/ml. Without extraction of endotoxin by Triton X-114, 100 μg/ml naked pDNA, which contains 1–5 EU/ml endotoxin, could release 521 ± 73 pg/ml TNF-α at 24 h.

Oligodeoxynucleotides. Phosphorothioate CpG ODN 1668 was purchased from GENSET (Paris, France). The sequences of CpG S-ODN 1668 are 5′-TCC ATG ACG TTC CTG ATG CT-3′, a proven activator of murine immune cells as previously described [4,6]. Phosphorothioate non-CpG ODN 1720 (5′-TCC ATG AGC TTC CTG ATG CT-3′) was used as a control.

Cationic liposome formulation. LipofectAMINE (LA) plus complexes were prepared according to manufacturer's instructions. In brief, DNA was diluted with 75 μl Opti-MEM and plus reagent was added at a concentration of 1.2 μl volume per 1 μg DNA. LA was diluted in 75 μl Opti-MEM. After a 15 min incubation, the LA solution was added to the mixture containing DNA and plus reagent. After a 15 min incubation, complex was added to the cells.

Cytokine secretion. Mouse macrophages, resident peritoneal macrophages from ICR and CH3/HeJ mice and RAW264.7, were washed three times with 0.5 ml RPMI 1640 before use. Naked DNA was diluted in 0.5 ml Opti-MEM. The cells were incubated with the naked DNA solution continuously for 8 h. In the case of DNA/LAplus complexes, cells were incubated for 2 h with 0.3 ml of the solutions containing the complexes. Then, the cells were washed with RPMI 1640 and incubated with RPMI 1640 with 10% FBS continuously for specified periods up to 48 h. At the indicated time-periods, the supernatants were collected for ELISA and kept at 80 °C and the cells were harvested with 100 μl PBS for the determination of reporter gene (luciferase) expression, as described previously [18]. The levels of TNF-α and IL-6 in the supernatants were determined by the AN'ALYSA Immunoassay System (Genzyme, Minneapolis, MN, USA).

Results and discussion

Although the important role of immunostimulatory effects mediated by CpG motif in gene therapy and DNA vaccination has been well defined, most in vitro studies focusing on the mechanisms of activation mediated by CpG DNA have been carried out using naked CpG ODN and naked bacterial genomic DNA. Many in vivo studies in mice have shown that pDNA/cationic liposome complexes can stimulate potent cytokine production [21–25]. It is generally believed that immune competent cells including macrophages are responsible for the in vivo cytokine production and the response is dependent on CpG motifs even when pDNA is complexed with cationic liposome.

At first, we examined cytokine production from macrophages by naked pDNA, which is taken up by the cells in a specific manner probably via receptor-mediated endocytosis [16,17]. Naked pDNA and *E. coli* genomic DNA containing unmethylated CpG motifs

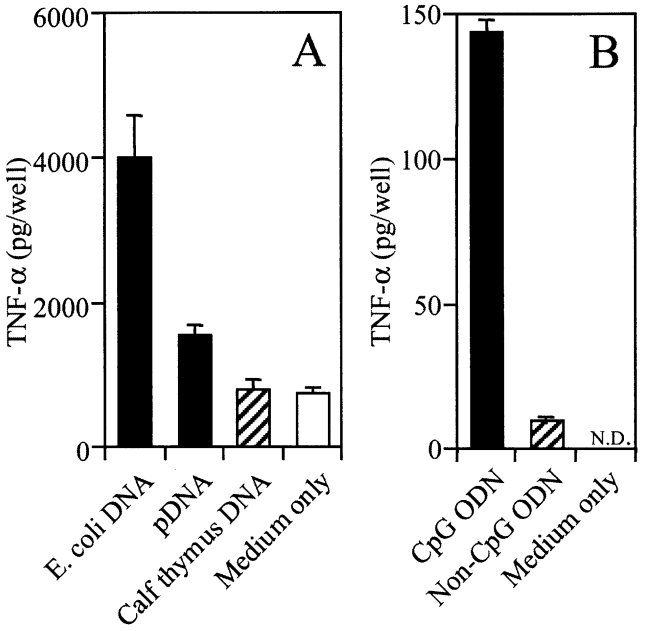

Fig. 1. TNF-α release induced by naked DNA from RAW264.7 cells (A) and naked ODN from peritoneal macrophages of ICR mice (B). (A) Cells were incubated with *E. coli* DNA, pDNA, and calf thymus DNA (10 μg/ml) for 8 h. (B) Cells were incubated with CpG ODN 1668 or non-CpG ODN 1720 for 8 h. The supernatants were collected at that time. The amount of TNF-α released from the macrophages was determined by ELISA. Each result represents the mean ± SD ($n = 3$). ND, not detected.

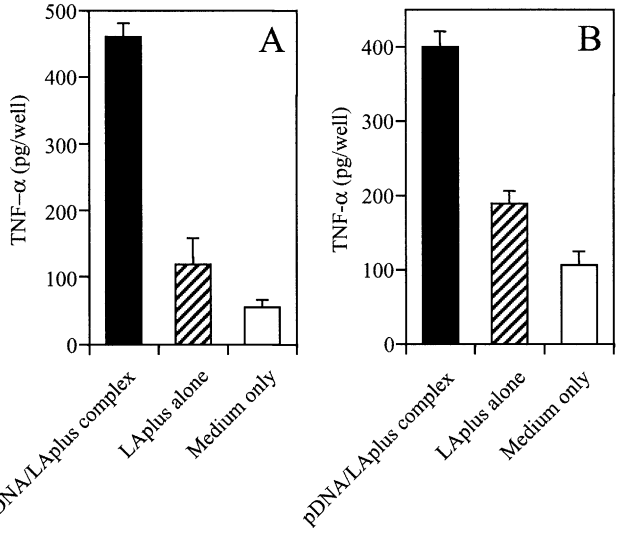

Fig. 2. TNF-α release by pDNA/LAplus complex from peritoneal macrophages of ICR mice (A) or RAW264.7 cells (B). Macrophages were incubated with pDNA/LAplus (2.5 μg:5 μg/well) or LAplus alone (5 μg/well). After a 2 h incubation, liposomes were removed and fresh growth medium was added to the macrophages. The supernatants were collected 24 h (A) or 8 h (B) after the incubation with liposomes. The amount of TNF-α released from the macrophages was determined by ELISA. Each result represents the mean ± SD ($n = 3$).

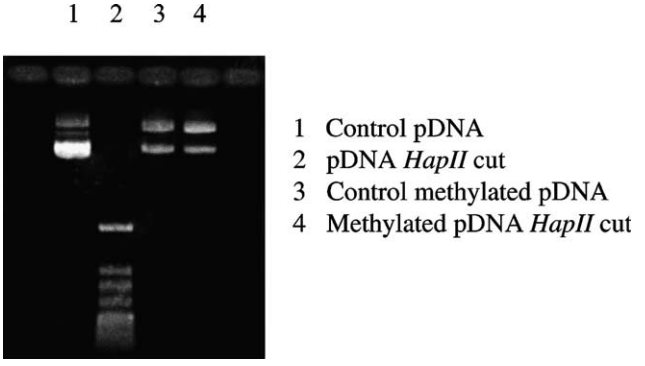

Fig. 3. The methylated-CpG pDNA was completely protected against digestion with *Hpa*II. Methylated-CpG pDNA was synthesized by methylation of pDNA with 1 U CpG methylase per μg pDNA for 24 h at 37 °C. Then pDNA was incubated with *Hpa*II. pDNA mobility was analyzed by 1% agarose gel electrophoresis. Lane 1, control pDNA (pCMV-Luc); Lane 2, pDNA digested with *Hpa*II; Lane 3, control methylated-CpG pDNA; Lane 4, methylated-CpG pDNA digested with *Hpa*II.

could stimulate a macrophage cell line, RAW264.7 cells, to produce a significant amount of TNF-α, however, mammalian calf thymus DNA without immunostimulatory CpG motifs could not (Fig. 1A). In addition, naked CpG ODN 1668 could induce the release of TNF-α from macrophages of ICR mice, while non-CpG ODN 1720 could not (Fig. 1B). These results confirmed that the TNF-α secretion from the macrophages was CpG motif-dependent as previously reported [6,26].

Next, we carried out the same experiments using pDNA complexed with the cationic liposomes LipofectAMINE (LA) plus, which should be taken up by the macrophages by non-specific mechanism based on electrostatic interaction. The pDNA/LAplus complex also stimulated macrophages to produce TNF-α (Fig. 2). Since liposomes alone were unable to stimulate the macrophages sufficiently to release TNF-α, these results show that pDNA is indispensable for TNF-α release by the liposome formulation.

It is well known that methylation of CpG motifs in DNA molecules almost completely abolishes the activation of immune responses induced by naked CpG DNA in vitro [4,7,26]. To explore whether unmethylated CpG motifs in pDNA complexed with LAplus are required for TNF-α induction from macrophages, we prepared methylated-CpG pDNA. We confirmed that restriction enzyme *Hap*II, which digests the unmethylated CG sequences, could not cut methylated pDNA (Fig. 3). Surprisingly, the methylated-CpG pDNA/LAplus complex induced a similar amount of TNF-α compared with the pDNA/LAplus complex from macrophages of ICR (Table 1). To confirm whether these results are not due to possible LPS contamination in DNA/LAplus complex, we used peritoneal macrophages isolated from C3H/HeJ mice, since they lack the Toll-like receptor-4 gene which transduces the LPS signaling and cannot respond to LPS [27]. Methylation of CpG pDNA could not

Table 1
TNF-α release by pDNA/LAplus complex or methylated-CpG pDNA/LAplus complex from macrophages of ICR or C3H/HeJ mice

Macrophages	Treatment	TNF-α (pg/well)
Peritoneal macrophages of ICR mice	CpG pDNA/LAplus complex (2.5 μg:5 μg/well)	518.9 ± 18.4
	Methylated CpG pDNA/LAplus complex (2.5 μg:5 μg/well)	547.9 ± 36.3
	LAplus alone (5 μg/well)	2.8 ± 4.4
	Medium only	33.8 ± 2.1
Peritoneal macrophages of C3H/HeJ mice (LPS non-responder)	CpG pDNA/LAplus complex	552.2 ± 9.6
	Methylated-CpG pDNA/LAplus complex (2.5 μg:5 μg/well)	358.8 ± 55.4
	LAplus alone (5 μg/well)	Not detected
	LPS (100 ng/ml)	2.4 ± 2.3
	LPS (10 ng/ml)	Not detected
	Medium only	Not detected

DNA/LAplus complex was added to the macrophages. After a 2 h incubation, the complex was removed and fresh growth medium was added to the macrophages. After a 6 h incubation, the supernatant was collected. Cytokine levels were determined by ELISA. Each result represents the mean ± SD ($n = 3$).

reduce TNF-α production from macrophages of C3H/HeJ mice. These results indicate that TNF-α induction from macrophages upon stimulation with pDNA/LAplus is a CpG motif-independent process.

Based on the finding that methylated pDNA can stimulate macrophages to produce TNF-α when complexed with LAplus, we performed further experiments using *E. coli* DNA and calf thymus DNA. When *E. coli* DNA, methylated pDNA, and calf thymus DNA were complexed with LAplus, a significant amount of TNF-α was released from the macrophage cell line RAW264.7, which was comparable with that induced by the pDNA/LA complex (Fig. 4). We confirmed that LAplus only could not induce TNF-α release (data not shown). Moreover, *E. coli* DNA or calf thymus DNA complexed with liposomes stimulated macrophages of C3H/HeJ mice to release the same level of TNF-α and IL-6 as pDNA complexed with liposomes. Similar results were obtained when pDNA, *E. coli* DNA or calf thymus DNA was complexed with Lipofectin, another type of cationic liposome (data not shown). These results imply that DNA complexed with cationic liposomes can induce the

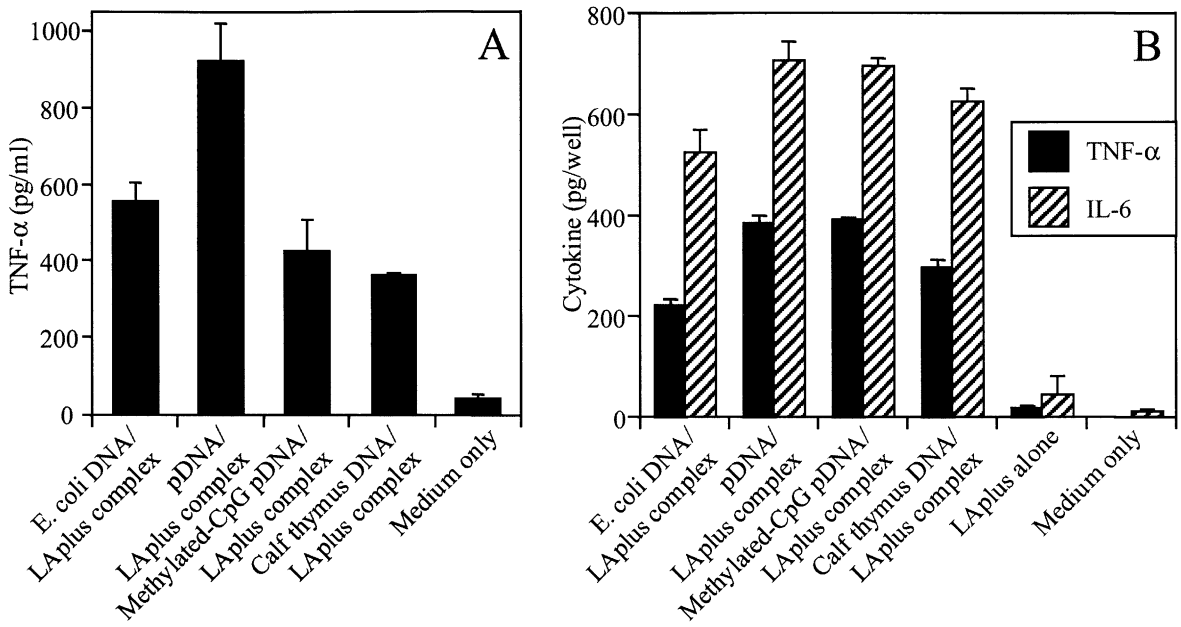

Fig. 4. TNF-α release by DNA/LAplus complex from macrophage cell line RAW264.7 (A) and peritoneal macrophages of C3H/HeJ mice (B). RAW264.7 cells (A) or peritoneal macrophages of C3H/HeJ (B) were incubated with pDNA, calf thymus DNA, *E. coli* DNA or methylated-CpG pDNA/LA complex (2.5 μg:5 μg/well). After a 2 h incubation, liposomes were removed and fresh growth medium was added to the macrophages. The supernatants were collected 8 h after the incubation with liposomes. TNF-α (closed bar) or IL-6 (hatched bar) levels were determined by ELISA. Each result represents the mean ± SD ($n = 3$).

same responses in cultured macrophages, whether they have CpG motifs or not. Some in vivo studies appear to support this speculation [28,29]. Although the immunostimulatory effect of CpG motifs in pDNA significantly contributed to the inflammatory cytokine secretion into bronchoalveolar lavage fluids after instillation of cationic lipid/pDNA complexes into mouse lung, inflammatory responses and severe pathology were observed even if unmethylated CpG motifs were methylated [29], or 14 of 17 5′-Pur–Pur–CpG–Pyr–Pyr-3′ motifs in pDNA were eliminated by deletion or selective mutagenesis [28]. On the other hand, the immune responses evoked by the DNA/liposome complexes at a cellular level are poorly understood, especially in macrophages. In cultured murine spleen cells, it has been demonstrated that CpG motifs are required to stimulate the cells. Sonehara et al. [30] reported that CpG ODN could induce IFN from the spleen cells in the presence of cationic liposomes, but ODN without CpG motifs could not. Pisetsky and Reich showed that the bacterial DNA and pDNA but not calf thymus DNA can stimulate murine spleen cells in vitro in the presence of Lipofectin [31]. In cultured human PBMC derived macrophage, cellular activation by pDNA in the presence of cationic lipid DOTAP was dependent on CpG motifs [12].

In conclusion, the present study has demonstrated for the first time that DNA complexed with cationic liposomes can induce CpG motif-independent activation to produce TNF-α and IL-6 in cultured resident peritoneal macrophages from mice or RAW264.7 cells. These findings will be an important basis for the DNA-based therapy including gene therapy and DNA vaccination.

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