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The production and immunostimulatory activity of double-stranded CpG-DNA

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CpG-DNA, which contains unmethylated CpG dinucleotides in the context of specific sequences, has remarkable and diverse immunological effects, including induction of proinflammatory cytokine expression and regulation of the Th1/Th2 immune response. Here, we examined the immunostimulatory activities of double-stranded (ds) CpG-DNA in the human B cell line RPMI8226. To investigate whether dsCpG-DNA stimulates immune cells, we constructed a plasmid containing repeated dsCpG-DNA and produced dsCpG-DNA by PCR amplification and *EcoR* I digestion. PCR-amplified dsCpG-DNA alone did not have immmunostimulatory activity. However, dsCpG-DNA encapsulated with lipofectin induced IL-8 promoter activation, HLA-DRA expression, and IL-8 expression in a CG sequence-independent manner. The effects of encapsulated dsCpG-DNA were independent of minor endotoxin contamination. These findings suggest the potential use of dsCpG-DNA as a therapy for immune response regulation. [BMB reports 2010; 43(3): 164-169]

INTRODUCTION

Bacterial DNA and synthetic oligodeoxynucleotides (ODNs) that contain unmethylated CpG dinucleotides in the context of particular sequences are known as CpG-DNA and act as pathogen-associated molecular patterns (PAMPs) of bacterial components (1). Toll-like receptor 9 (TLR9) of the innate immune system recognizes CpG-DNA, based on the observation that TLR9-/- mice do not respond to immunomodulatory CpG-DNA (2, 3). CpG-DNA activates multiple cell types such as macrophages, dendritic cells, B cells and NK cells. Thus, CpG-DNA has remarkable and diverse immunological activities, including the upregulation of proinflammatory cytokine expression (e.g. IL-6, IL-12, IFN-α, IFN-γ) as well as regulation of the Th1/Th2 immune response (4-7). Accordingly, CpG-DNA is considered a useful therapeutic agent for infectious diseases, allergic diseases and immune adjuvants (3, 6).

Natural phosphodiester bond CpG-DNA (PO-ODN) may be susceptible to nuclease degradation in cells (8). Therefore, in order to provide resistance to nuclease activity and ensure efficient uptake of CpG-DNA into cells, phosphorothioate backbone-modified CpG-DNA (PS-ODN) containing sulfur substituted for the non-bridging oxygen atoms was developed (9, 10). Nuclease-resistant PS-ODN dramatically increased CpG-DNA-induced B cell activation (1). However, several laboratories have shown that PS-ODN induces non-specific DNA-protein interactions (11, 12). Furthermore, severe side effects occurred in PS-ODN-treated mice in a CG sequence- and backbone modification-dependent manner (13-16). For example, intra-articular injection of PS-ODN into the knee joints of mice can induce arthritis characterized by TNF-α expression and the accumulation of Mac-1^+ cells (15). In addition, it has been reported that intraperitoneal injection of PS-ODN causes transient splenomegaly, lymphoid follicle destruction, immunosuppression, and PS-ODN-specific IgM production (13, 14, 16). Therefore, production of a potent immunomodulatory CpG-DNA that does not cause side effects is highly desirable for inducing a well-controlled immune response.

Previously, we identified PO-ODN in the genomic DNA of *M. bovis* and *E. coli* through a computer-assisted analysis, and later screened the chromosomal DNA sequences that have immunomodulatory activity (17, 18). Our data show that PO-ODNs containing immunomodulatory CpG motifs, namely MB-ODN 4531(O) and DF-ODN 04(O), play functional roles in the induction of Th1 cytokine production and the Ag-driven Th1 response. We also found that MB-ODN 4531(O) may more effectively, compared to PS-ODN, enhance the innate immune response without producing side effects such as PS-ODN-specific IgM production or transient splenomegaly (16). Here, we explored the effects of double-stranded (ds) CpG-DNA on cytokine production. We constructed plasmids encoding repeated dsCpG-DNA (MB-ODN 4531(O)) sequences,

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produced dsCpG-DNA sequences, and analyzed their effects on cytokine production in immune cells.

RESULTS AND DISCUSSION

Construction of the plasmid expressing repeated dsCpG-DNA followed by the purification of dsCpG-DNA

We have shown previously that PO-ODNs induce a Th1 response, as evidenced by the CG sequence-dependent increase in IL-12 expression and Ag-driven Th1 responses in mice (17, 18). Here, we examined the effects of dsCpG-DNA on cytokine expression in human and mouse cells. We therefore produced dsCpG-DNA and observed whether dsCpG-DNA stimulates immune cells. First, we synthesized the MB-ODN 4531 (O) sequence along with its antisense orientation at the 5′- and 3′-ends, respectively, flanked by *EcoR* I sites. We also synthesized the MB-ODN 4531(O)GC sequence, a derivative of MB-ODN 4531(O), containing an *EcoR* I site along with three CG dinucleotides in reverse orientation as GC. Next, MB-ODN 4531(O) or MB-ODN 4531(O)GC sense and antisense ODNs were incubated at 90°C for 5 min followed by hybridization. The hybridized products containing repeated dsMB-ODN 4531 and dsMB-ODN 4531(O)GC sequences were observed by agarose gel electrophoresis (data not shown) and ligated into the *EcoR* I sites of pGEM-T vector, yielding pGEM-T-dsMB-ODN 4531(O) and pGEM-T-dsMB-ODN 4531(O)GC. The size of the insert produced by Not I digestion as well as DNA sequencing confirmed that pGEM-T-dsMB-ODN 4531(O) and pGEM-T-dsMB-ODN 4531(O)GC contained 8 and 20 repetitive MB-ODN 4531(O) sequences, respectively (Fig. 1A). The vectors containing repeated dsCpG-DNA were then digested with *EcoR* I in order to generate dsCpG-DNA consisting of 24 nucleotides (Fig. 1A). Digested dsCpG-DNA was then purified by ion exchange chromatography using a Q-sepharose column. Briefly, dsCpG-DNA and vector DNA bound to the Q-sepharose column were eluted with a 0.1-1 M NaCl gradient (Fig. 1B and C). Owing to its small size and negative charge, dsCpG-DNA was eluted at a lower NaCl concentration between 0.2-0.5 M, followed by confirmation of vector plasmids (about 0.7 M of NaCl).

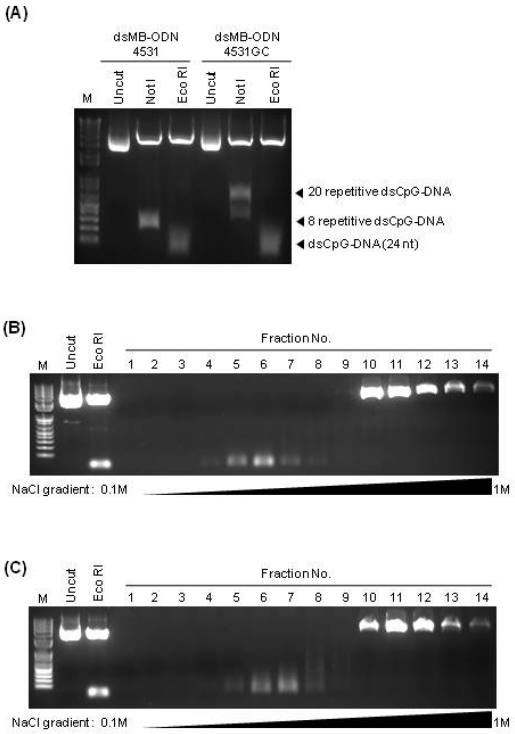

Fig. 1. Construction of the plasmid containing repeated dsCpG-DNA and purification of dsCpG-DNA. (A) Confirmation of the plasmid containing repeated dsCpG-DNA by restriction analysis. Purified plasmids containing repeated dsCpG-DNA were digested with *EcoR* I or *Not* I. Plasmids sequences were confirmed by DNA sequencing. (B) and (C) Purification of dsCpG- DNA. pGEM-dsMB-ODN 4531(O) (B) and pGEM-dsMB-ODN 4531(O)GC (C) plasmids were isolated, digested with *EcoR* I, and extracted with phenol/chloroform and ethanol precipitation. The digested products were separated by Q-sepharose column chromatography using elution buffer with a 0.1-1 M NaCl gradient.

Effect of plasmid-derived dsCpG-DNA on IL-8 promoter activation

It is well known that CpG-DNA contributes to the activation of the IL-8 promoter (17, 18). Therefore, we explored whether dsCpG-DNA can activate the IL-8 promoter in human B cell line RPMI 8226. Briefly, RPMI 8226 cells were transiently transfected with the IL-8 promoter-reporter construct and then treated with purified dsCpG-DNA from the pGEM-T-dsMB-ODN 4531(O) and pGEM-T-dsMB-ODN 4531(O)GC constructs. As shown in Fig. 2, dsCpG-DNA prepared from the plasmid con-

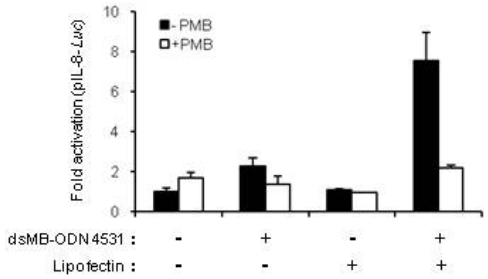

Fig. 2. Effects of plasmid-derived dsCpG-DNA on IL-8 promoter activation. RPMI 8226 cells were transiently transfected with an IL-8 promoter-reporter construct for 24 h. The cells were stimulated with dsCpG-DNA or lipofectin-encapsulated dsCpG-DNA for 12 h. To identify LPS contamination during dsCpG-DNA-induced IL-8 promoter activation, we preincubated the cells with polymyxin B (PMB, 100 ng/ml) for 1 h prior to dsCpG-DNA treatment. Cells were collected and cell lysates were obtained by freeze-thaw method. Luciferase activity was detected in relative light units (RLUs) and was normalized to *Renilla* activity. The results are presented as fold activation compared with control.

structs had no influence on IL-8 promoter activity. However, the IL-8 promoter was significantly activated when dsCpG-DNA was encapsulated with lipofectin prior to stimulation. This activation was inhibited when cells were pretreated with polymyxin B, which binds to LPS and inhibits the LPS-mediated response. This indicates the presence of LPS contamination while preparing dsCpG-DNA from the pGEM-T-dsMB-ODN 4531(O) and pGEM-T-dsMB-ODN 4531(O)GC constructs, and moreover that LPS synergized with lipofectin for activation of the IL-8 promoter.

Amplification of dsCpG-DNA by PCR

To avoid LPS contamination, we amplified repeated dsMB-ODN 4531(O) and dsMB-ODN 4531(O) GC sequences by PCR using the pGEM-T-dsMB-ODN 4531(O) and pGEM-T-dsMB-ODN 4531(O)GC constructs as templates. The PCR products containing repeated dsMB-ODN 4531(O) or dsMB-ODN 4531(O)GC sequences were digested using *EcoR* I, yielding 24 nucleotide-long dsCpG-DNA molecules (Fig. 3A). Digested dsCpG-DNA was extracted by phenol/chloroform and ethanol precipitation. The endotoxin content of dsCpG-DNA was less than 1 ng/mg.

Effect of PCR-amplified dsCpG-DNA on IL-8 promoter activation

PCR-amplified dsCpG-DNA alone did not influence IL-8 promoter activation. However, dsCpG-DNA encapsulated with lipofectin significantly activated the IL-8 promoter in RPMI 8226 cells (Fig. 3B). As dsMB-ODN-4531(O) and dsMB-ODN 4531(O)GC induced similar patterns of activation, IL-8 promoter activation by dsCpG-DNA appears to be CG sequence independent. In addition, IL-8 promoter activation was not affected by pretreatment of the cells with polymyxin B (Fig. 3B), which implies that LPS is not involved. We observed the same results when we performed experiments using the mouse cell line RAW 264.7 (Fig. 3C).

Effects of PCR-amplified dsCpG-DNA on IL-8 and HLA-DRA gene expression

Reportedly, inflammatory cytokines (e.g. TNF-α, IL-6, IL-12), chemokines (e.g. MIP-2, MCP-1), and major histocompatibility (MHC) class II molecules are induced by CpG-DNA stimulation (3-6). Therefore, we evaluated IL-8 and HLA-DRA expression in dsCpG-DNA-treated RPMI 8226 cells by RT-PCR analysis. Treatment of PCR-amplified dsCpG-DNA alone did not influence IL-8 and HLA-DRA expression (Fig. 4A). However, the expression levels of IL-8 and HLA-DRA were increased by dsCpG-DNA encapsulated with lipofectin in a CG sequence-independent manner (Fig. 4B).

The innate immune system can discriminate between self and foreign microbial components, including LPS, bacterial DNA and lipoteichoic acid (19, 20). Bacterial DNA containing unmethylated CpG motifs are known as CpG-DNA and act as PAMPs of bacterial components. TLR9 recognizes CpG-DNA, whereupon proinflammatory cytokine production and other inflammatory responses are induced via activation of the MyD88/IRAK pathway (21, 22). Synthetic single-stranded (ss)

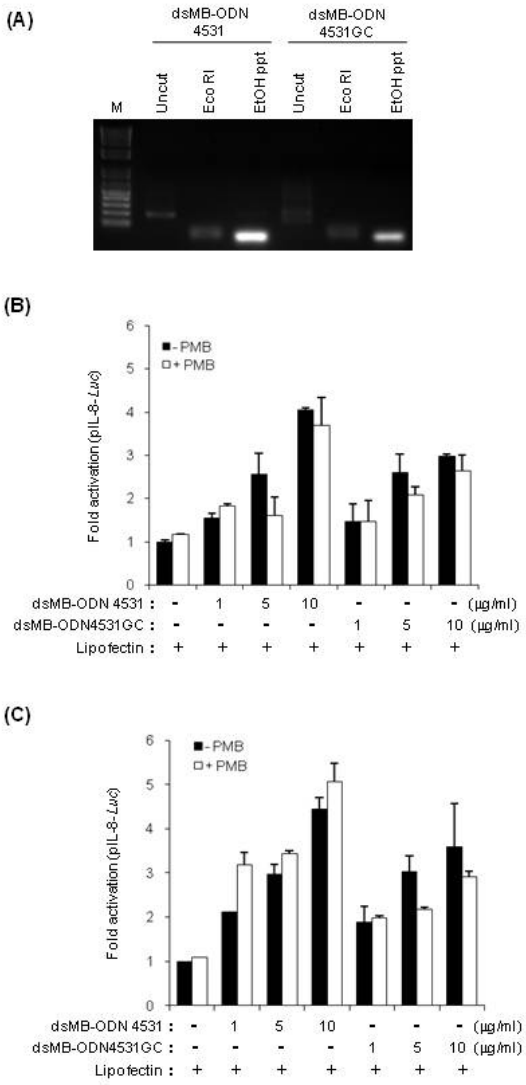

Fig. 3. Production of dsCpG-DNA by PCR amplification and the effects on IL-8 promoter activation. (A) Preparation of PCR-amplified dsCpG-DNA. Repeated dsCpG-DNA inserts in the pGEM-T-dsMB-ODN 4531(O) or pGEM-T-dsMB-ODN 4531(O)GC plasmids were amplified by PCR. PCR products containing repeated dsCpG-DNA were then digested with *EcoR* I. Digested dsCpG-DNA was extracted with phenol/chloroform and ethanol precipitation. (B and C) Effect of PCR-amplified dsCpG-DNA on IL-8 promoter activation. RPMI 8226 cells (B) and RAW 264.7 cells (C) were transiently transfected with an IL-8 promoter-reporter construct for 24 h. The cells were stimulated with dsCpG-DNA or lipofectin-encapsulated dsCpG-DNA for 12 h with or without pretreatment with polymyxin B (PMB, 100 ng/ml). Luciferase activity was obtained as described in Fig. 2.

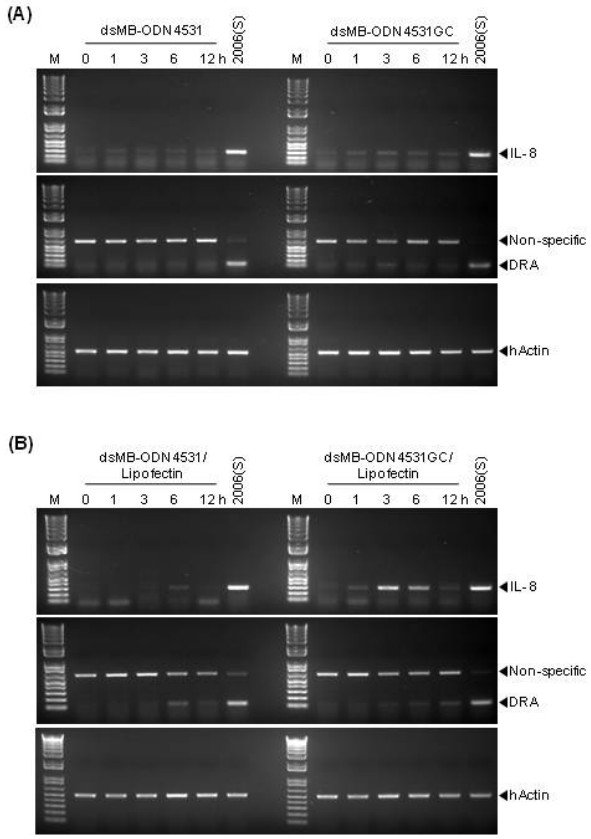

Fig. 4. Effect of PCR-amplified dsCpG-DNA on IL-8 and HLA-DRA gene expression. RPMI 8226 cells were treated with dsCpG-DNA (A) or lipofectin-encapsulated dsCpG-DNA (B) for the indicated periods and analyzed by RT-PCR. CpG-DNA 2006(S) was used as a positive control. The β-actin expression level was used as an internal loading control. The letter M denotes a standard DNA marker (A and B).

PS-ODNs are known to bind TLR9 and induce B cell activation in a CpG motif-dependent manner (23). In contrast, Yasuda *et al*., showed that ssPO-ODN activates TLR9 and induces activation of Flt3 ligand-induced dendritic cells in a CpG motif-independent manner. The binding ability of ssPO-ODN to TLR9 was lower than that of ssPS-ODN (24). In addition, Takaoka *et al*. reported that DLM1/Z-DNA binding protein 1/ZBP1 recognizes bacterial DNA in mouse fibroblasts and stimulates the DNA-mediated induction of genes involved in the innate immune response, such as type 1 IFN (25). Nevertheless, the dsCpG-DNA-induced activation of immune cells has not been elucidated in detail.

Here, we successfully produced dsCpG-DNA free of LPS contamination by cloning repeated CpG-DNA sequences and PCR amplification (Fig. 1 and 3). Assessment of dsCpG-DNA immunostimulatory activity revealed that dsCpG-DNA alone did not induce the expression of HLA-DRA and IL-8 in the human B cell line RPMI 8226. PO-ODN activates the innate immune system in mice, but triggers relatively low activity in human cells (2). However, it has been reported that activation of the innate immune response in human cells was induced with liposome-encapsulated PO-ODN (DOTAP and lipofectin) (26, 27). In testing whether encapsulated dsCpG-DNA has immunostimulatory activity, we found that IL-8 promoter activation, HLA-DRA expression, and IL-8 expression were significantly enhanced in a CG-sequence-independent manner (Figs. 3 and 4). Since dsCpG-DNA may be susceptible to nuclease activity, we exposed ssPO-ODN and dsCpG-DNA to DNase 1 and found that dsCpG-DNA was more rapidly degraded in RPMI 8226 cells (data not shown). Nonetheless liposome-encapsulated dsDNA showed potent immunostimmulatory effects, therefore encapsulation of dsCpG-DNA may protect against rapid degradation. Considering these findings, we suggest that dsCpG-DNA can potentially be used as an activator of the innate immune response and as a therapeutic agent for immune response regulation, particularly when its immunomodulatory functions are activated by lipofectin encapsulation, for example. Future studies will provide further insight into dsCpG-DNA, its immunomodulatory effects and its potential for developing a well-controlled immune response.

MATERIALS AND METHODS

ODNs and reagents

ODNs were purchased from GenoTech (Taejon, Korea). The CpG-DNA sequences used in this study were either phosphodiester (O) or phosphorothioate-modified (S). The phosphorothioate version of CpG-DNA 2006(O), CpG-DNA 2006(S), served as a positive control and consists of 24 bases with three CpG motifs (underlined): TCGTCGTTTTGTCGTTTTGTCGTT. To construct plasmids encoding repeated dsMB-ODN 4531(O) and dsMB-ODN 4531(O)GC sequences with *EcoR* I sites both at 5′ and 3′ ends, ODNs were synthesized with *EcoR* I sites at the 5′- and 3′-ends, along with ODNs that encode the following sequences in sense or antisense orientations: dsMB-ODN 4531(O) sense, 5′-AATTCAGCAGCGTTCGTGTCGGCG-3, antisense, 5′-AATTCGCCGACACGAACGCTGCTG-3′, dsMB-ODN 4531(O)GC sense, 5′-AATTCAGCAGGCTTGCTGTGCGCG-3, antisense, 5′-AATTCGCGCACAGCAAGCCTGCTG-3′. The *EcoR* I sites are underlined. The endotoxin content of the ODNs was less than 1 ng/mg, as measured by *Limulus amebocyte* assay (Whittaker Bioproducts, Walkersville, MD, USA). Minor LPS contamination in CpG-DNA and dsCpG-DNA was detected by pretreatment with polymyxin B (Fluka Chemie AG, Buchs, Switzerland).

Construction of the plasmid containing repeated dsCpG-DNA

dsMB-ODN 4531(O) sense and antisense ODNs, or dsMB-ODN 4531(O)GC sense and antisense ODNs, were hybridized in TE buffer (10 mM Tris-HCl, pH 7.5 and 1 mM EDTA)

after incubation at 90°C for 5 min. These annealed fragments were ligated into the *EcoR* I site of pGEM-T Easy vector (Promega, Madison, WI, USA), yielding pGEM-T-dsMB-ODN 4531(O) or pGEM-T-dsMB-ODN 4531(O)GC that contain repeated dsCpG-DNA. Plasmid sequences were confirmed by DNA sequencing.

Purification of dsCpG-DNA

pGEM-dsMB-ODN 4531(O) and pGEM-dsMB-ODN 4531(O)GC plasmids were isolated using an EndoFree plasmid purification kit (Qiagen, Valencia, CA, USA). Purified plasmids containing repeated dsCpG-DNA were digested with *EcoR* I, followed by extraction with phenol/chloroform and ethanol precipitation. To isolate digested dsCpG-DNA from the linear pGEM-T vector, the digested products were loaded onto a Q-sepharose column (Amersham Biosciences, Pittsburgh, PA. USA) pre-equilibriated in 25 mM Tris-HCl (pH8.4) buffer, sufficiently washed with buffer containing 0.1 M NaCl, and then eluted with buffer containing 0.1-1 M NaCl gradient. The eluted dsCpG-DNA was dialyzed in 10 mM Tris-HCl (pH7.5) buffer and analyzed for endotoxin contamination by *Limulus amebocyte* assay (Whittaker Bioproducts).

Production of dsCpG-DNA by PCR amplification

For amplification of the repeated dsCpG-DNA insert in pGEM-T-dsMB-ODN 4531(O) or pGEM-T-dsMB-ODN 4531(O)GC, the following PCR protocol was performed: 35 cycles for 1 min at 94°C, 30 seconds at 54°C, and 1 min at 72°C. The following oligonucleotides were used as primer sets: 5′ primer, 5′-GGCGGCCGCGAATTC-3′, and 3′ primer, 5′-GAATTCCCG CGGCCG-3′. The PCR products containing repeated dsCpG-DNA were digested with *EcoR* I, followed by extraction with phenol/chloroform and ethanol precipitation. The endotoxin content of dsCpG-DNA was less than 1 ng/mg as measured by *Limulus amebocyte* assay (Whittaker Bioproducts).

Cell culture

RPMI 8226 cells (human B cell line) and RAW 264.7 cells (mouse macrophage cell line) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). RPMI 8226 cells were grown in RPMI 1640 medium containing 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 100 U/ml penicillin and 100 μg/ml streptomycin. RAW 264.7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 100 U/ml penicillin and 100 μg/ml streptomycin.

Transfection and luciferase assay

For transfection, we employed a human IL-8 promoter-reporter construct, pIL-8-Luc, encoding the IL-8 promoter fragment -135 to +46 linked to the *luciferase* gene, as previously described (28). The transfection and luciferase assays were performed as previously reported (28). After transfection, cells were placed in complete medium for 24 h and then treated with dsCpG-DNA or lipofectin-dsCpG-DNA complex for an additional 24 h. dsCpG-DNA and lipofectin (Invitrogen, Carlsbad, CA, USA) complexes were prepared according to the manufacturer's specifications. To detect possible influence of LPS contamination in dsCpG-DNA-induced IL-8 promoter activation, we preincubated the cells with polymyxin B (100 ng/ml) for 1 h prior to dsCpG-DNA treatment. We verified comparable transfection efficiency by cotransfecting the promoterless Renilla luciferase vector pRL-null (Promega). The luciferase activities were evaluated by Dual-Luciferase Reporter Assay System (Promega) with a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA).

RT-PCR analysis

We performed RT-PCR analysis of total RNA isolated from dsCpG-DNA-treated cells using an RNeasy RNA isolation kit (Qiagen). Five micrograms of total RNA were reverse-transcribed in the first-strand synthesis buffer, which contained 6 μg/ml of oligo(dT) primer, 50 U of reverse transcriptase, 4 mM of dNTP and 40 U of RNase inhibitor. The standard PCR reaction for 25 cycles was performed using a cDNA mixture as a template with the following primer sets: HLA-DRA, 5′-CGAG TTCTATCTGAATCCTG-3′ (sense), 5′-GTTCTGCTGCATTGCTT TTGC-3′ (antisense); IL-8, 5′-ATGACTTCCAAGCTGGCCGTG GCT-3′ (sense), 5′-TCTCAGCCCTCTTCAAAAACTTCT-3′ (antisense); human actin, 5′-GGGTCAGAAGGATTCCTATG-3′ (sense), 5′-CCTTAATGTCACGCACGATTT-3′ (anti-sense). The PCR products were detected by UV light after being resolved on a 1% agarose gel.

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