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Novel Roles of CpG Oligodeoxynucleotides as a Leader for the Sampling and Presentation of CpG-Tagged Antigen by Dendritic Cells

Hidekazu Shirotai,* Kunio Sano,2,* Noriyasu Hirasawa,† Tadashi Terui, Kazuo Ohuchi,‡ Toshio Hattori,* Kunio Shirato,* and Gen Tamura*

Oligodeoxynucleotides containing CpG motifs have been highlighted as potent Th1 activators. We previously reported that Ag and CpG, when conjugated together, synergistically promoted the Ag-specific Th1 development and inhibited the Th2-mediated airway eosinophilia. In this study, we examined the mechanisms underlying the synergism of the covalent conjugation. The CpG-OVA conjugate enhanced the Th1 activation and development. These characteristic features of the conjugate could not be ascribed to the polymerization of OVA, but mirrored the augmented binding of the CpG-tagged Ag to dendritic cells (DCs) in a CpG-guided manner, because phycobiliprotein, R-PE, conjugated to CpG stained a higher proportion of DCs with higher intensity than the mixture. R-PE fluorescence was emitted from cytoplasmic portions of the DCs, which simultaneously expressed costimulatory molecules and IL-12. The CpG-conjugated R-PE trafficking described above actually served as a potent Ag. These results indicate that CpG conjugated to Ag exhibit novel joint properties as promoters of Ag uptake and DC activators, thereby potentiating the ability of DCs to generate Th1 cells. The DNA-mediated promotion of Ag uptake would be advantageous for evoking host immune responses against invading microorganisms. The Journal of Immunology, 2001, 167: 66–74.

The nature of DNAs as immune stimulators has recently been attracting much interest. The initial finding was made by Tokunaga and his colleagues (1, 2), who reported that DNA extracted from Mycobacterium bovis activated NK cells for IFN production and antitumor activities. They additionally discovered that the ability to stimulate NK cells was limited to DNAs from invertebrates but not vertebrates, and that particular sequences with a G-C motif(s) were required for the activity (3, 4). Then, Krieg et al. (5) reported that oligodeoxynucleotides (ODNs) containing CpG motifs with two 5’ purines and two 3’ pyrimidines triggered B cells to proliferate and differentiate into Ig-secreting cells. CpG were also found to activate monocytes and macrophages, which produced IL-12 and stimulated NK cells (6–9). Dendritic cells (DCs) were another target of CpG; CpG induced IL-12-secreting DCs, which in turn stimulated Th1 cells (10–14).

DCs are known as initiators of immune responses that present Ag in a form recognizable by T cells (15). The key steps include Ag uptake at an immature stage in the peripheral tissues and presentation of antigenic peptides along with the expression of costimulatory molecules after maturation in the lymphoid tissues (16–23). Two different processes, namely, Ag processing and maturation/activation, must be combined for full competence of DCs. Confrontation with microorganisms additionally endows DCs with the ability to secrete IL-12 (24–26), which skew immune responses toward the Th1-dominant phenotype (27, 28). Among microbial structures, CpG are reported to contribute to expelling the microbes by preferentially mounting Th1 responses as the result of the DC activation and IL-12 secretion (9, 13, 29, 30). As demonstrated by microbes, substances that possess both an antigenic component and a DC activation/maturation component are likely to propel DC-mediated Th1 cell stimulation.

Counterbalancing Th2-dominated allergic inflammation is one possible target for the control of allergy. We have previously reported that regulatory CD4+ T cells, such as TGF-β-producing T cells induced by oral or tracheal tolerance and Th1 cells induced upon exposure to Mycobacterium tuberculosis, inhibited Th2-mediated airway inflammation (31–33). More recently, we have reported that intratracheal coadministration of CpG and allergen inhibited airway eosinophilia and hyperresponsiveness in a synergistic manner and reasoned that the APC phagocytosing both CpG and Ag could target the CpG effects to Ag-specific T cells (34). This view was supported by subsequent experiments in which covalently linked conjugates of CpG with Ag inhibited airway eosinophilia and Ag-specific Th2 cells more efficiently than the unconjugated mixture (35). The efficacy of CpG-Ag conjugates was also reported in the other experimental systems (36–39).

In this study, we explored the mechanisms underlying the synergism of the covalent conjugation. We found an unexpected role of CpG as a leader of Ag uptake by DCs. Thus, CpG conjugated to Ag exhibit novel joint properties as promoters of Ag uptake and DC activators, thereby potentiating the ability of DCs to develop Th1 cells.

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3 Abbreviations used in this paper: ODN, oligodeoxynucleotide; SA, streptavidin; DC, dendritic cell; MFI, mean fluorescence intensity; Ig, transgenic; TLR9, Toll-like receptor 9; LN, lymph node.
Materials and Methods

Animals

BALB/c mice were bred in our animal facility and were used at 7–12 wk of age. BALB/c mice transgenic (tg) for TCR specific for OVA were 12A4 and I-A4 were established as described previously (40).

CpG and direct conjugation to Ags

The CpG ODNs (1826) used throughout this study consisted of 20 bases containing two CpG motifs (TTCCATGACGTTCCTGACGTT) (34, 35). The control ODN (1745) was identical except that the CpG motifs were rearranged (TCCATGAGCTTCGTAGCT). Phosphorothioate ODNs were synthesized by Nihon Gene Research Laboratories (Sendai, Japan) or Takara Shuzo (Osaka, Japan). The method for conjugating ODN to proteins was described previously (35). The CpG-OVA conjugate was prepared by Peptide Institute (Osaka, Japan). The LPS content of ODN was <6 pg of LPS per mg of DNA as measured by a Limulus HS-1 Single Test (Wako Pure Chemical, Osaka, Japan). Free ODNs were removed by extensive dialysis. The molar and weight ratio of ODN:OVA in the conjugate was calculated to be 8.3:1 and 1.1:1, respectively. CpG ODN or non-CpG ODN was also conjugated to R-PE (Molecular Probes, Eugene, OR) after R-PE was maleimide activated using sulfo-N-hydroxysuccinimide (NHS)-maleimidomethyl (NHS-ethyl)cyclohexane-1-carboxylate according to the manufacturer’s instructions (Pierce, Rockford, IL). The molar and weight ratio of ODN:R-PE conjugate was calculated to 3.7:1 and 0.092:1, respectively. The ratio of ODN:R-PE in the non-CpG-R-PE conjugate was equal to that in the CpG-R-PE conjugate.

In vitro stimulation of naive anti-OVA T cells with CpG and OVA

Spleen cells (5 × 10^6) from unimmunized anti-OVA TCR tg mice were cultured in 12-well plates with OVA (0.1 μg/ml) or CpG (0.11 μg/ml), either alone or in the mixed or conjugated form. After 6 days, viable lymphocytes (1 × 10^6) recovered by Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) density-gradient centrifugation were restimulated with 2 × 10^6 APCs in the presence or absence of OVA (100 μg/ml) in quadruplicate in 96-well plates. After 2 days of culture, the culture supernatants were assayed for IFN-γ and IL-4. APCs were prepared by treating spleen cells of unimmunized BALB/c mice with mitomycin C (50 μg/ml; Wako Pure Chemical) for 30 min at 37°C.

In vitro restimulation of anti-OVA Th1 or Th2 cells with CpG and OVA

Spleen cells (3 × 10^6) from unimmunized anti-OVA TCR tg mice were cultured in 12 ml of RPMI 1640 medium with OVA (100 μg/ml) in the presence of IL-12 (1 ng/ml; Genzyme, Cambridge, MA) for Th1 cells or IL-4 (10 ng/ml; Genzyme) plus anti-IL-12 mAb (0.1 μg/ml; Genzyme) for Th2 cells for 3 days. The cells were cultured in fresh medium for another 3 days. Viable lymphocytes were enriched for CD44 T cells by a panning method as described previously (32, 41). After coculture of 1 × 10^6 CD4+ T cells and 5 × 10^6 OVA-pulsed DC or APCs in the presence of OVA or CpG, either alone or in the mixed or conjugated form, for 2 days in quadruplicate in 96-well plates, the culture supernatants were assayed for IFN-γ and IL-4.

Fractionation of the CpG-OVA conjugate

Crude CpG-OVA conjugate was applied to a Sephacryl S-200 HR (Amersham Pharmacia Biotech, Piscataway, NJ) column, 1.8 × 73 cm, equilibrated in PBS (pH 7.4), and eluted with the same buffer. Aliquots of the fractions from the column were subjected to SDS-PAGE. Proteins and CpG were visualized with GelCode Blue Stain Reagent (Pierce) and SYBR Green II RNA Gel Stain (BioWhittaker, Rockland, ME), respectively. For in vitro experiments, each fraction was added to the in vitro cultures to induce or stimulate Th1 cells.

Fractionation of the CpG-R-PE conjugate

Crude CpG-R-PE was applied to a DE52 (Whatman, Tewksbury, MA) minicolumn, 5 × 5 mm, equilibrated in 5 mM potassium phosphate buffer (pH 7.0). Proteins were eluted with a stepwise gradient of 0.1–1.0 M NaCl in the same buffer (pH 7.0). Aliquots of fractions from the column were subjected to SDS-PAGE, and proteins and CpG were visualized as described above. For in vitro experiments, each fraction was incubated with splenic DCs as described below.

In vitro restimulation of R-PE-primed lymph node (LN) cells with CpG and R-PE

BALB/c mice were primed s.c. with 100 μg of R-PE emulsified in CFA in the hind footpads. After 7 days, popliteal LN cells (3 × 10^6) were cultured with CpG (1 μg/ml) or R-PE (11 μg/ml), either alone or in the mixed or conjugated form, in quadruplicate in 96-well plates. After 2 days, the culture supernatants were assayed for IFN-γ and IL-4.

Cytokine assay

Cytokine concentrations in the culture supernatants were determined using ELISA according to the manufacturer’s recommendations. Paired anti-IL-4, anti-IL-5, and anti-IFN-γ mAbs were purchased from Pharmingen (San Diego, CA). Tetramethylrhodamine reagent (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was used for color development, and ODs determined at 450 nm were converted to concentrations (ng/ml) according to a standard curve. Standard recombinant mouse IL-4, IL-5, and IFN-γ were purchased from Genzyme.

Enrichment for DCs from spleen

BALB/c spleen cells were cut into small fragments and incubated with RPMI 1640 supplemented with 1 mg/ml collagenase D (Boehringer Mannheim, Indianapolis, IN) for 30 min at 37°C. After washing, they were layered onto 50% Percoll and centrifuged for 20 min at 3000 rpm. The interface was recovered and used as the DC-enriched fraction for flow cytometry analysis. The average percentage of CD11c− cells was ~2% in the spleen before the enrichment, which was consistently increased to 35–40% after the enrichment.

Preparation of DCs from bone marrow

Bone marrow-derived DCs were prepared from BALB/c mice as described elsewhere (42). Briefly, bone marrow cells (2 × 10^6 cells) obtained from femurs were seeded into a 100-mm petri dish (Eiken Chemical, Tokyo, Japan) in 10 ml of RPMI 1640 supplemented with 10% FCS and GM-CSF (20 ng/ml; PeproTech, London, U.K.). At day 3, another 10 ml of medium containing 20 ng/ml GM-CSF was added to the plates. At days 6 and 8, half of the culture supernatants were replaced with the fresh medium containing 20 ng/ml GM-CSF. At day 10, the adherent cells were used as DCs. The cell numbers recovered from one plate were generally 3–5 × 10^6 cells.

Reagents used for flow cytometry

 Purified anti-CD11c mAb (N418) specific for DCs (43) was purchased from Serotec (Oxford, U.K.) and conjugated to FITC (Sigma, St. Louis, MO) in our laboratory. Biotinylated anti-CD40 and anti-CD86 mAbs were purchased from Caluag (Burlingame, CA). Allophycocyanin-conjugated streptavidin (SA), propidium iodide, and unconjugated and FITC-conjugated anti-IL-12 mAbs were purchased from Biomedia (Foster City, CA), Sigma, Pharmingen, and BioSource International (Camarillo, CA), respectively.

Staining of DC-enriched spleen cells and flow cytometry

The DC-enriched splenocytes were incubated with 1 μg/ml CpG or 11 μg/ml R-PE, either alone or mixed, or varying concentrations of CpG-conjugated R-PE for 1 h and then stained with FITC-conjugated anti-CD11c mAb. The R-PE staining of the gated CD11c+ cells was analyzed using a FACS Caliber (BD Biosciences, Mountain View, CA). Propidium iodide-stained dead cells were excluded from analyses.

Analyses of bone marrow-derived DCs by flow cytometry

The DC-enriched population derived from bone marrow was cultured overnight with 1 μg/ml CpG or 11 μg/ml R-PE, either alone or mixed, or varying concentrations of CpG-conjugated R-PE for 3 h and then stained with FITC-conjugated anti-CD11c mAb. The R-PE staining of the gated CD11c+ cells was analyzed using a FACS Caliber (BD Biosciences, Mountain View, CA). Propidium iodide-stained dead cells were excluded from analyses.

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Efficient induction of Th1 development by CpG-conjugated Ag

CpG ODN is known to induce potent Th1 responses (11–13). We first examined the efficacy of the conjugation between Ag and CpG to potentiate the antigenicity and Th1 inducibility. Spleen cells from anti-OVA TCR tg mice were cultured with OVA or CpG, either alone or in the mixed or conjugated form, and the induced Th cells were restimulated with OVA for IFN-γ and IL-4 production (Fig. 1A). OVA (0.1 μg/ml) alone or CpG (0.11 μg/ml) alone failed to manifest potent Th1-inducing ability. The mixture of OVA and CpG did not induce Th cells, whereas the conjugate between CpG and OVA induced T cells that secreted predominantly IFN-γ rather than IL-4 upon restimulation with OVA. The control non-CpG ODN conjugated with OVA did not induce Th1 development. These results indicate that low doses of Ag and CpG, which were below the threshold for T cell activation, acquired the ability to induce Th1 cells when covalently conjugated together.

**Preferential secretion of IFN-γ from Th cells by CpG-conjugated Ag**

We next examined the stimulation of Th1 and Th2 cells by the CpG-OVA conjugate. Th1- and Th2-enriched fractions were prepared by culturing anti-OVA TCR tg T cells in vitro under Th1 and Th2 skewing conditions, respectively. Th1 or Th2 population produced IFN-γ or IL-4 predominantly upon stimulation with 100 μg/ml OVA (Fig. 1B). One microgram of OVA/ml or 1.1 μg CpG/ml alone had no stimulatory activity on the Th1 or Th2 populations. The mixture of OVA and CpG still failed to stimulate Th cells, whereas the conjugate of CpG with OVA showed stimulatory antigenicity; the Th1-rich population produced higher amounts of IFN-γ by 1 μg OVA/ml conjugated to CpG than by 100-fold higher doses of OVA alone. A striking difference was noted in the activation of the Th2 population by the conjugate; although the levels of IL-4 production were comparable to those by 100 μg of OVA/ml alone, IFN-γ production was dramatically enhanced by the CpG-OVA conjugate. Thus, the conjugation of Ag to CpG enhanced the antigenicity, leading to the predominant production of IFN-γ from the Th cells, regardless of the conditions in which the Th cells had developed.

**Monomeric CpG-OVA as active Th1 stimulator**

The crude CpG-OVA conjugates we used in the above experiments comprised various molecular species (Fig. 2A, right lane).
In light of the nature of OVA to aggregate with each other, CpG-OVA was separated by gel filtration chromatography and used without concentration to minimize aggregation. When an equal volume of each fraction was examined for the ability to stimulate Th1 cells, the most potent stimulatory activity was observed in fraction 24 (Fig. 2C), where lower molecular species were stained intensely both for protein (Fig. 2A) and DNA (Fig. 2B), and corresponded to the monomeric CpG-OVA conjugate. Proteins with higher molecular mass in fraction 24 were stained faintly for DNA and might be a spontaneous aggregate after gel filtration. Fractions with higher molecular masses exhibited lower stimulatory activities (Fig. 2C). Fraction 28 was stained for protein but not DNA and lacked the stimulatory activity. The molecular size of fraction 28 was identical to that of OVA alone (data not shown). The monomeric CpG-OVA (fraction 24) possessed potent Th1-inducing (Fig. 2D) and -activating (Fig. 2E) ability, indicating that the activity of CpG-OVA can be ascribed to the monomeric CpG-conjugated OVA, but not to the aggregates.

CpG-guided augmentation of Ag binding to DCs

To examine the mechanisms underlying the enhanced immunogenicity of CpG-Ag conjugates, we first examined the binding/uptake of CpG-tagged protein to DCs, known as potent APCs to T cells. DCs were enriched from spleen cells as described in Materials and Methods, and the purity was always found to be 35–45%, as determined by anti-CD11c (N418) staining. We used phycobiliprotein, R-PE, to track the fate of the CpG-conjugated protein. DC-enriched spleen cells were incubated with R-PE, a mixture of R-PE and CpG, or R-PE-labeled CpG for 3 h, and R-PE staining in CD11c+ cells gated as shown in Fig. 3A was analyzed by flow cytometry. In Fig. 3C, the FL2 autofluorescence of the untreated DCs is shown. When DCs were incubated with R-PE alone, only minimal proportions of the CD11c+ DCs were positive for R-PE, with varying staining intensity (Fig. 3D). The R-PE staining was not amplified by the copresence of unconjugated CpG and R-PE (Fig. 3E), whereas when the same doses of R-PE and CpG were covalently conjugated, 88% of DCs were intensely stained with R-PE (Fig. 3F). The percentage of R-PE-positive cells and mean fluorescence intensity (MFI) of R-PE staining correlated with the doses of the CpG-R-PE conjugate added (Fig. 3, F–I). At CpG doses ranging from 0.01 to 1 µg/ml, the MFI of R-PE and CpG-R-PE concentration showed a nearly linear relationship (Fig. 3B). The staining intensity of DCs with a mixture of R-PE and CpG (Fig. 3E) was almost equivalent to that with 100-fold less R-PE conjugated to CpG (Fig. 3H), suggesting that CpG conjugation improved the protein binding/uptake to DCs by nearly 100-fold. Thus, the protein Ag is promoted to bind to DCs in a CpG-guided manner when the protein is conjugated to, but not when mixed with, CpG.

To ascertain that the phenomena observed above actually reflect the nature of CpG-conjugated R-PE, we purified the CpG-R-PE conjugate. When the crude CpG-R-PE was fractionated by ion exchange chromatography, the majority of CpG-R-PE was eluted at concentrations ranging from 0.3 to 1.0 M, whereas the purified R-PE was eluted at lower concentrations (Fig. 4A). After separation by SDS-PAGE, all fractions emitted R-PE fluorescence (Fig. 4B).
4B), whereas fluorescent emission from CpG was discernible only in fractions eluted by 0.6 or 0.8 M NaCl (Fig. 4C), indicating that CpG-conjugated R-PE is eluted at high buffer concentrations. The fractions that were eluted only at high, but not low, buffer concentrations bound to DCs (Fig. 4D), indicating that facilitated binding to DCs is a feature unique for CpG-conjugated R-PE, but cannot be ascribed to the contaminating free or aggregated PE. Because of these observations and the paucity of the purified materials, CpG-R-PE before fractionation was used in the following experiments.

Dose-dependent and coordinated increases in the Ag uptake and costimulatory molecule expression in DCs by CpG-conjugated Ag

We examined whether the binding of CpG to DCs resulted in the increased expression of costimulatory molecules. Bone marrow cells were cultured in the presence of GM-CSF, and the DC fraction was obtained by gating CD11c+ cells (Fig. 5A). The levels of CD40 expression were low in the DCs (Fig. 5B). When the bone marrow-derived DCs were incubated with 0.1 μg of R-PE-labeled CpG, slight increases in Ag uptake were observed, yet an induction of CD40+ DCs was not apparent (Fig. 5C). Increases in the doses of CpG-R-PE paralleled the increases in the proportion of CD40+ or R-PE+ DCs (Fig. 5D). At 1 μg/ml CpG, more than one-half of the DCs were activated and Ag bearing (Fig. 5E). The expression of CD86 (Fig. 5F-I) or MHC class II molecules (data not shown) was similarly affected by the incubation with CpG–R-PE. Thus, the increase in the Ag-laden, activated DCs was proportional to the dose of the CpG-R-PE added.

Next, we compared the effects of the conjugate with those of the mixture (Fig. 6, A–F). When the DCs were incubated with R-PE overnight, the R-PE-positive DCs comprised only minimal proportions and were confined mainly to CD40+ DCs (Fig. 6A). Incubation of the DCs with the mixture of CpG and R-PE promoted the activation of the majority of the DCs, whereas the accelerated
activation by CpG was not associated with the increase in the R-PE uptake (Fig. 6B). In sharp contrast, the DCs cultured with the CpG-R-PE conjugates exhibited a dramatic increase in the population double positive for R-PE and CD40 (Fig. 6C); the proportion of Ag-bearing cells in CD40+ DCs increased by >10-fold. The DCs that bound CpG-R-PE inevitably expressed CD40 molecules. The amounts of Ag uptake by each DC also increased; MFI of FL2 was 516 for the CpG-R-PE conjugate in comparison to 95 for the mixture of R-PE plus CpG. Similarly, enhanced Ag uptake correlated with the increased expression of CD86 (Fig. 6, D–F) and class II molecules (data not shown).

As controls, aliquots of the bone marrow-derived DCs were incubated with non-CpG coupled to R-PE. As shown in Fig. 6, G–J, the non-CpG-R-PE was phagocytosed by immature DCs to an extent similar to the CpG-R-PE, but failed to activate DCs to express CD40 or CD86. The DCs incubated with CpG-R-PE overnight were examined by confocal microscopy, and the experiments also verified that the majority of CD11c+ DCs emitted R-PE-derived red fluorescence from cytoplasmic portions (Fig. 7). These results indicate that conjugation of Ag with CpG promoted the Ag capture.

IL-12 expression in DCs that phagocytosed CpG-conjugated R-PE

We examined whether CpG-conjugated R-PE allowed the preferential expression of IL-12 in the DCs that phagocytosed the CpG-R-PE conjugates. After incubation of bone marrow-derived DCs with R-PE-CpG for 6 h, they were analyzed for IL-12 expression.

FIGURE 5. Dose-dependent increase in the Ag uptake and costimulatory molecule expression in DCs by CpG-conjugated Ag. Immature DCs were obtained from bone marrow cells after culture with GM-CSF for 10 days. After additional culture in the presence of graded doses of CpG-R-PE overnight, they were stained with FITC-conjugated anti-CD11c mAb in combination with biotinylated anti-CD40 (B–E) or anti-CD86 (F–I) mAb, followed by allophycocyanin-conjugated SA. CD11c+ cells, which generally comprised 60–70% of viable cells, were acquired (A), and the correlations between R-PE staining and the CD40 or CD86 expression are shown. Note that the percentages of activated DCs bearing Ag increased in proportion to the doses of CpG-R-PE. One representative of four independently performed experiments is shown.

FIGURE 6. Coordinated increase in the Ag uptake and costimulatory molecule expression in DCs by CpG-conjugated Ag. Experimental conditions are the same as those described in the legend of Fig. 5. The immature DCs were incubated with R-PE alone (11 μg/ml; A and D), the mixture of R-PE (11 μg/ml) with CpG (1 μg/ml; B and E), or CpG-conjugated R-PE (C and F) overnight, and then subjected to flow cytometry analysis. The correlations between the CD40 or CD86 expression and R-PE staining are shown. Note that the conjugation, but not the mixture, of CpG and R-PE increased the activated DCs that were also stained with Ag. In another experiment, the non-CpG was compared with CpG. The non-CpG-R-PE (H and J), which was phagocytosed by the immature DCs to a similar extent as the CpG-R-PE (G and I), failed to activate DCs for the expression of CD40 or CD86. Experiments were repeated at least twice independently with similar results.
by flow cytometry. When the DCs were cultured with R-PE alone, no induction of IL-12 expression was observed (Fig. 8A). The copresence of CpG with R-PE induced the expression of IL-12 in a significant proportion of the DCs, which was not, however, correlated with the increase in the R-PE uptake (Fig. 8B). In contrast, with the CpG-R-PE conjugates the IL-12-producing DCs were mainly confined to cells that were also strongly positive for R-PE (Fig. 8C). The IL-12 staining was specific, because the inclusion of free anti-IL-12 mAb inhibited IL-12 staining to levels comparable to that of the control that was not stained with FITC-conjugated anti-IL-12 mAb (Fig. 8, A–D). These results indicated the correlation between Ag uptake and IL-12 expression in DCs when the DCs encounter CpG-linked Ag.

Enhanced antigenicity of CpG-R-PE to R-PE-primed LN cells

To verify that the CpG-conjugated R-PE processed in the manner described above actually served as an Ag, we examined IFN-γ production from R-PE/CFA-primed LN cells. R-PE-primed LN cells produced minimal amounts of IFN-γ in response to R-PE or CpG alone (Fig. 9). The mixture of R-PE and CpG induced a slight but significant increase in IFN-γ production, while the conjugation between R-PE and CpG further enhanced IFN-γ production by 6.2 times. The observed increase in antigenicity and IFN-γ production by the CpG-conjugated protein Ag was consistent with the results in Figs. 1 and 2.

Discussion

The main purpose of the present study was to elucidate the mechanisms underlying the synergism of covalently conjugated CpG and protein Ag. We found that CpG-tagged Ag bound to and was phagocytosed by DCs in a CpG-guided manner (Figs. 3, 4, and 7). The DCs then induced expression of costimulatory molecules and IL-12 secretion. The activation of T cells by DCs is the result of sequential multistep processes, such as Ag uptake, peptide presentation, the expression of costimulatory molecules, and IL-12 secretion. The latter two processes, due to the “DC-activating” condition, were enhanced by CpG (10, 11), while the former two processes, i.e., the “Ag-processing” condition, require the copresence of Ag. It has already been stressed that the coinjection of both CpG and Ag is necessary for the manifestation of CpG activities (13, 34, 44–47). We reasoned that CpG and Ag were likely to be engulfed by the same APCs, which then secreted IL-12 and presented Ag, thereby fulfilling the requirements listed above (34). This notion...
was supported by the subsequent experiments with the CpG-OVA conjugates. The ability of the conjugates to induce Th1 cells was 100-fold higher than a mixture of equivalent amount of CpG and Ag (35). A similar efficacy of CpG-Ag conjugate was reported in the activation of CD8+ CTL, and it was reasoned that colocalization of CpG and Ag into the same APC could be achieved in a single step (36).

The present experiments were performed to provide further support for the idea described above. Our results revealed an additional mechanism underlying the synergism of CpG and Ag in the CpG-Ag conjugate. The striking observation was a CpG-guided increase of Ag uptake in DCs (Figs. 3 and 4). Although immature DCs are extremely well equipped to capture Ags (48), quantitative analyses revealed that only a minor fraction of the cells took up R-PE under the experimental conditions used (Figs. 6 and 8). It was found that the majority of DCs activated by CpG, as judged by the increased expression of costimulatory molecules, did not show the increased expression of Ag uptake (Figs. 6 and 8). In contrast, the CpG-conjugated R-PE bound to the majority of DCs with >100-fold higher intensity than the mixture (Figs. 3 and 5). It has been demonstrated that ODN bound to the cell surface is rapidly endocytosed and moves to the endosomal compartment (49–51). Experiments with confocal microscopy showed that the R-PE fluorescence was emitted from cytoplasmic portions of CD11c+ DCs 24 h after incubation (Fig. 7), indicating that the CpG-R-PE conjugates were endocytosed by the DCs. Thus, one of the features unique to the CpG-labeled Ag is the promotion of Ag uptake.

Immature DCs play a sentinel role in the peripheral tissues by sampling Ag (16–19). After homing to lymphoid tissues, they present the captured Ag and express high levels of MHC class II and costimulatory molecules as mature DCs (20–23). Mature DCs have weak phagocytic ability, and are poor at sampling new Ag. However, the experiments in Fig. 3 demonstrated that the CpG-R-PE conjugate stained the majority of splenic DCs, which are likely to be mature on the basis of the expression of MHC class II and costimulatory molecules (data not shown). Uptake of CpG–R-PE into mature DCs is probably guided by the CpG portion of the conjugate, and this process enables mature DCs to serve as APCs for the activation of R-PE-specific Th1 cells (Fig. 9).

Cellular internalization of phosphorothioate ODNs has been extensively studied from the standpoint of antisense treatment. ODNs first bind to the cell surface through adsorptive endocytosis and fluid-phase endocytosis (49, 50). Studies on the intracellular uptake of FITC-labeled ODNs indicated that cytoplasmatic accumulation started within 2–4 h after the application of ODNs in vitro (51). The endocytosed ODNs localized in the endosomal-lysosomal compartment, with little staining in the cytosol or nucleus (52). To express the effects of CpG, including activation of the transcription factors and secretion of cytokines by DCs, internalization of CpG and endosomal maturation/acidification are required (52). Despite close examinations about how ODNs are handled by the cells, little attention has been paid to the uptake and intracellular localization of CpG in the context of Ag presentation and DC activation.

CpG in the conjugate do not merely play a role of a guide leading Ags to DCs. Once the CpG is directly conjugated to Ag, the DC-activating and Ag-processing conditions are not independent. After incubation of DCs with the CpG-R-PE conjugate, high levels of IL-12 expression were observed in the DCs expressing the high levels of R-PE (Fig. 8), and these cells also expressed CD40 and CD86 (Figs. 5 and 6). This is in sharp contrast to the cells incubated with a mixture of CpG and Ag. In the DCs treated with the mixture, no relationship was observed between R-PE staining and IL-12 expression (Fig. 6). The results indicate that essentially all of the DCs, which incorporated the CpG-Ag conjugate, were activated by CpG and strongly suggest that these cells would present antigenic peptide for the preferential generation of Th1 cells.

The transduction of intracytoplasmic signals after CpG activation has been recently studied (53–55); however, it remains controversial whether CpG bind to a cell surface or intracytoplasmic receptor. Although a Toll-like receptor 9 (TLR9) is reported to mediate the cellular response to CpG, the expression of TLR9 on the cell surface has not been established (54). We demonstrated that CpG-tagged macromolecular R-PE, but not the mixture of CpG and R-PE, was endocytosed by and stimulated DCs, suggesting that CpG were endocytosed through some cell surface receptors (50). It would be intriguing to know whether binding/uptake of CpG-labeled R-PE is abolished in the absence of TLR9.

Then, what would be the physiological significance of the CpG-guided Ag uptake? When inflammation is evoked by an invasion of bacteria, DNA may be spilled from damaged microbes. Given that CpG-containing DNA works as a tag attached to the degraded microbes, DNA-mediated binding to phagocytic cells would promote the clearance of DNA-tagged bacteria, as in the case of Ig- or C-mediated opsonization. More importantly, efficient sampling by DCs of DNA-tagged bacterial Ags and subsequent Ag presentation to Th cells would facilitate the link between innate and acquired immunity against the microbes and promote the expulsion of the invading microbes.

In conclusion, we found that the increased activation of Th1 cells by CpG-Ag conjugates results from the enhanced Ag uptake and the coincorporation of both Ag and CpG by the same DCs. These novel features of CpG-conjugated Ag would be applicable to therapies for diseases in which Th1-dominant responses would be preferable, such as in allergies, infectious diseases, and malignant tumors.

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References


ENHANCED SAMPLING AND PRESENTATION OF CpG-TAGGED Ag BY DCs