Requirement for DNA CpG Content in TLR9-Dependent Dendritic Cell Activation Induced by DNA-Containing Immune Complexes

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Requirement for DNA CpG Content in TLR9-Dependent Dendritic Cell Activation Induced by DNA-Containing Immune Complexes

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Although TLR9 was originally thought to specifically recognize microbial DNA, it is now evident that mammalian DNA can be an effective TLR9 ligand. However, the DNA sequence required for TLR9 activation is controversial, as studies have shown conflicting results depending on the nature of the DNA backbone, the route of DNA uptake, and the cell type being studied. In systemic lupus erythematosus, a major route whereby DNA gains access to intracellular TLR9, and thereby activates dendritic cells (DCs), is through uptake as a DNA-containing immune complex. In this report, we used defined dsDNA fragments with a natural (phosphodiester) backbone and show that unmethylated CpG dinucleotides within dsDNA are required for murine DC TLR9 activation induced by a DNA-containing immune complex. The strongest activation is seen with dsDNA fragments containing optimal CpG motifs (purine-purine-CpG-pyrimidine-pyrimidine) that are common in microbial DNA but rare in mammalian DNA. Importantly, however, activation can also be induced by CpG-rich DNA fragments that lack these optimal CpG motifs and that we show are plentiful in CpG islands within mammalian DNA. No activation is induced by DNA fragments lacking CpG dinucleotides, although this CpG-free DNA can induce DC activation if internalized by liposomal transfection instead of as an immune complex. Overall, the data suggest that the release of CpG-rich DNA from mammalian DNA may contribute to the pathogenesis of autoimmune diseases such as systemic lupus erythematosus and psoriasis in which activation of TLR9 in DCs by self DNA has been implicated in disease pathogenesis. The Journal of Immunology, 2009, 183: 3109–3117.

**TLR9**

 Toll-like receptor 9 was identified as the receptor for oligodeoxynucleotides (ODNs) containing unmethylated CpG dinucleotides and was originally thought to distinguish pathogen DNA from that of the mammalian host on this basis, as unmethylated CpG dinucleotides are relatively uncommon in mammalian DNA compared with pathogen DNA (1, 2). This discrimination could be further refined by the identification of so-called CpG motifs, where the particular pattern of bases flanking the CpG dinucleotide appeared to further distinguish microbial and mammalian DNA. However, subsequent studies revealed specific instances in which mammalian DNA proved to be a highly effective TLR9 ligand (3–7) and pointed to a critical role for mammalian DNA in the pathogenesis of certain autoimmune diseases, including systemic lupus erythematosus (SLE) and psoriasis (8, 9).

As TLR9 is located intracellularly, it is necessary for the DNA to be internalized before it can engage TLR9 (10, 11). This intracellular localization serves to facilitate TLR9 access to pathogen DNA and at the same time restrict TLR9 access to self DNA (6). However, in SLE, DNA-containing immune complexes (ICs) can activate plasmacytoid DCs (pDCs) to produce IFN-α, a cytokine thought to be important in disease pathogenesis (12). This occurs by uptake and internalization of the ICs by a cell surface Fcγ receptor on the DCs with subsequent delivery of the DNA to intracellular TLR9 (4, 13). Similarly, autoreactive B cells can be activated through TLR9 by BCR-mediated internalization of mammalian DNA (3, 14). In the autoimmune skin disease psoriasis, the antimicrobial peptide LL37 (also known as CAMP) is released from neutrophils and epithelial cells in response to bacterial infection (7). Self DNA from apoptotic or necrotic cells in damaged skin binds to the peptide, and the peptide-DNA complex can then be internalized and trigger IFN-α production by pDCs through TLR9.

The ability of mammalian DNA to activate TLR9 raises questions as to the nature of the DNA sequence involved in this activation. This is controversial, with conflicting results being reported depending on the nature of the DNA backbone, the cell type being studied, and the route of DNA uptake by the cell. The large majority of studies examining the structure-activity relationship of DNA sequence and TLR9 activation have been done using synthetic ODNs protected from DNase degradation by the use of phosphorothioate linkages (2, 15). These studies found that unmethylated CpG dinucleotides were important for activation, and
also demonstrated that different CpG motifs elicited varying degrees of TLR9 activation.

A CpG motif is defined as the hexamer comprising the two bases to the 5′ and 3′ sides of the unmethylated CpG dinucleotide as well as the CpG dinucleotide itself (16). The optimal CpG motif is species-specific, but for mouse TLR9 is purine-purine-CG-pyrimidine-pyrimidine (2). However, the phosphorothioate backbone itself results in a large number of sequence-independent effects (2, 15), and it is thus possible that the DNA sequence requirements for TLR9 activation determined using phosphorothioate ODN may not necessarily reflect the DNA sequences required for TLR9 activation by pathogen or mammalian DNA, which utilize natural phosphodiester-linked DNA fragments (21, 22). This specificity analysis was further extended to elegant binding studies dependent on recombinant proteins that incorporated the entire TLR9 ectodomain. On the basis of these results, it was proposed that the CpG motif dependency of TLR9 activation is restricted to phosphorothioate-modified synthetic DNA (21, 22).

This conclusion differs from that reached in murine B cell studies where TLR9 activation by phosphodiester-linked DNA internalized through the BCR showed a strong preference for unmethylated CpG dinucleotides (14, 23). It is not clear whether this difference reflects an intrinsic difference between TLR9 activation in DCs and B cells or whether it is due to the particular routes of DNA internalization. Moreover, the relevance of the binding studies is confounded by recent reports demonstrating that CpG ODNs bind better to a TLR9 cleavage product than to full-length TLR9 (24, 25). Therefore, the importance of CpG content in TLR9 activation of DCs when phosphodiester-linked dsDNA is internalized by physiological routes, such as IC uptake via the FcγR, remains controversial. This is an important issue to resolve, as it addresses the fundamental question of whether TLR9 detection of mammalian DNA depends on TLR9 location or DNA sequence. If the latter is the case, it then becomes necessary to understand how the particular conditions associated with autoimmune diseases such as SLE and psoriasis enrich for the availability of these stimulatory DNA sequences.

To address these questions in this study, we determined the ability of dsDNA fragments of defined sequence and methyl status to activate TLR9 in murine DC after internalization in the form of an IC. This activation was also compared with that elicited following DNA fragment uptake by liposomal transfection. Additionally, the stimulatory capacity of DNA fragments cloned from CpG islands within the mammalian genome was evaluated.

Materials and Methods

Mice
BALB/c wild-type mice were purchased from The Jackson Laboratory. TLR9-deficient mice were backcrossed 11 generations onto the BALB/c genetic background. All mice were maintained at the Boston University School of Medicine Laboratory Animal Sciences Center or at Charles River Laboratories in accordance with the regulations of the American Association for the Accreditation of Laboratory Animal Care. All experimental procedures were approved by the Institutional Animal Care and Use Committee at Boston University School of Medicine.

Reagents
CpG-A ODN 2336 (Ggac gac gac gtc gtc gtc gggggg) was purchased from Copley Pharmaceutical Group. Capital letters indicate a phosphorothioate backbone, and lowercase letters indicate a phosphodiester backbone. LPS was purchased from Invivogen.

DNA fragments
The CG50 fragment, CGSubOp fragment, and CGneg fragment (Table I) were purified by digestion with BamHI and EcoRI of pLIT-CG50.1 plasmid DNA, pLIT-HIV (CG50) plasmid DNA and pUC-HIV (CG50) plasmid DNA, respectively (14). For the methylation studies, DNA fragments were methylated at their CG residues using M.SssI methylase (CpG methylase), or at their AGCT residues using Alul methylase (New England Biolabs). To confirm the completeness of methylation induced by M.SssI methylase, the methylated DNA fragments were digested with the methylation-sensitive restriction enzyme HpyCH4IV (New England Biolabs) (14). For studies requiring biotinylated fragments, biotinylation of CG50, CGSubOp, and CGneg was performed by digestion of the fragments with EcoRI and BamHI, followed by filling in 5′ and 3′ overhangs with Klenow polymerase in the presence of biotin-16-2′ deoxy-uridine-5′-triphosphate (Roche). CpG-free pCpG-mcs DNA (Invivogen) was purified using the Qiagen EndoFree Plasmid kit (Qiagen). The pCpG-mcs DNA was further purified by equilibrium centrifugation in cesium chloride-ethidium bromide gradients. Preparation of the CpG island dsDNA fragments and the small ubiquitin-related modifier (SUMO) dsDNA fragment by PCR has been described in detail previously (23). Biotinylation of these fragments was performed by digestion of the fragments with EcoRI and BamHI, followed by filling in 5′ overhangs with Klenow polymerase in the presence of biotin-16-2′ deoxy-uridine-5′-triphosphate (Roche). Primers and enzymes were removed from all DNAs using the DNA Clean & Concentrator-25 kit (Zymo Research). Endotoxin levels in the DNA fragment preparations were measured using the Limulus amebocyte lysate assay (Cambrex) and were <0.1 endotoxin units/ml.

Antibodies
The anti-DNA mAb PA4 (26) and the anti-biotin mAb 1D4 (23) have been described previously. The mAbs were purified using a protein A (GE Healthcare) affinity column. Endotoxin levels in the mAb preparations were <0.1 endotoxin units/ml.

Formation of DNA immune complexes and DNA-DOTAP complexes
DNA ICs were formed by incubating various concentrations of the DNA fragments with the anti-DNA mAb PA4 in complete RPMI 1640 medium (10% FBS, 2 mM L-glutamine, 50 μM 2-ME, 100 μM penicillin, and 100 μg/ml streptomycin) for 1 h at 37°C before addition of the DNA ICs to the DC cultures. Biotinylated DNA ICs were formed by incubating various concentrations of the biotinylated DNA fragments with the anti-biotin mAb 1D4 in complete RPMI 1640 medium for 1 h at 37°C before addition of the biotinylated DNA ICs to the DC cultures.

Table I. DNA fragments used in this study

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<tr>
<th></th>
<th>Size (bp)</th>
<th>% GC</th>
<th>Nonoptimal CGp</th>
<th>Optimal CGp</th>
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</table>


b Nonoptimal CpG: unmethylated cytosine—guanine, with flanking bases different from optimal CpG motif.
DNA-DOTAP complexes were formed by incubating the DNA fragments with DOTAP (Karl-Roth) at a DNA-to-DOTAP weight ratio of 1:2 in 50 μl of 150 mM NaCl (pH 7.4) buffered with 20 mM HEPES. After 15 min, 100 μl of complete RPMI 1640 medium was added. One hundred microliters of this DNA-DOTAP mixture in a total well volume of 200 μl was used in the subsequent DC activation assays.

DNA competition ELISA

MaxiSorp Immuno plates (Nunc) were coated with the anti-DNA mAb PA4 (2 μg/ml) and incubated overnight at 4°C. Plates were washed three times with PBS/0.05% Tween, blocked with PBS/1% BSA for 2 h at room temperature, and then unlabeled DNA fragments at various concentrations were added to the plates. After 1 h, biotinylated CGSubOp (5 ng/ml) or biotinylated CG50 (5 ng/ml) was added and incubated for 4 h at room temperature. Plates were then washed three times, incubated with streptavidin-conjugated HRP (BD Biosciences) for 20 min, washed, and developed with tetramethylbenzidine substrate (BD Biosciences) for 20 min, washed, and developed with tetramethylbenzidine substrate (BD Biosciences) for 20 min, washed, and developed with tetramethylbenzidine substrate (BD Biosciences). The reaction was stopped by H2SO4, and absorbance was measured at 450 nm. The percentage binding was calculated as the absorbance in the presence of competitor DNA relative to the absorbance in the absence of competitor DNA.

DC preparation

Bone marrow cells were seeded at 1.5 × 10^6 cells/ml in complete RPMI 1640 supplemented with 5–10% conditioned medium from B16 cells transfected with fms-like tyrosine kinase 3 ligand (Fl3TL or FL). The FL-B16 cells were originally made by Dr. H. Chapman (27) and were provided by Dr. U. Von Andrian (Harvard Medical School). The cells were used for experiments after 8 days, at which time >90% were CD11c-positive, of which 15–40% displayed a plasmacytoid DC phenotype (CD11c+Cd45RA+/B220+/CD11blow) and the remainder displayed a conventional DC phenotype (CD11c+Cd45RA-/B220+/CD11bhigh).

Cell sorting

To obtain purified pDC (CD11c+Cd20hi/Cd11bhi) and conventional DC (cDC; CD11c–Cd20-lo/Cd11blow), bone marrow cells were cultured with FL, which leads to the development of a mixed population of pDCs and cDCs, collectively referred to as FL-DCs. These pDCs and cDCs show equivalent properties to pDCs and cDCs isolated directly from mouse spleen (30).

DC stimulation

Before setting up each assay, the FL-DCs were routinely checked by flow cytometry for the relative percentages of pDCs and cDCs and the respective DC activation status after staining with anti-CD11c-PE, biotinylated anti-CD45RA followed by streptavidin-PE-Cy5, anti-B220-FITC, anti-CD11b-FITC, and anti-CD20-FL-DCs were purified from FL-DC cultures by cell sorting and stimulated with CG50-PA4 ICs (Fig. 1, A and B). To determine the DC subset responsible for the cytokine production, pDCs and cDCs were purified from FL-DC cultures by cell sorting and stimulated with CG50-PA4 ICs (Fig. 1C). This revealed that pDCs were the source of IFN-α and cDCs the major source of IL-6.

CpG content is required for TLR9-dependent DC activation induced by DNA-containing ICs

To evaluate the role of CpG content, we compared the stimulatory capacity of ICs formed with three dsDNA fragments of similar size but different sequence (Table I) (14). These were (1) the 607-bp CG50 fragment that contains 50 optimal CpG motifs; (2) a 726-bp fragment derived from the promoter region of HIV-1 that contains 27 unmethylated CpG dinucleotides but no optimal CpG motifs (referred to as CGSubOp); and (3) a 629-bp fragment derived from the HIV-1 gag gene that contains no CpG dinucleotides (referred to as CGneg). Our initial plan was to compare the stimulatory capacity of the different fragments in ICs formed with the PA4 anti-DNA mAb. However, we first used a competition ELISA to evaluate the relative binding affinity of the fragments to PA4. The competition ELISA was performed by measuring the ability of the individual fragments to compete with biotin-labeled CGSubOp or with biotin-labeled CG50 for binding to plate-bound PA4. Unexpectedly, although the binding of CGSubOp and CGneg to PA4 was equivalent, CG50 bound to PA4 with greater affinity (Fig. 2, A and B). Given the increased affinity of CG50 for PA4, the DNA fragment-PA4 system could not be used for comparing the stimulatory capacity of CG50 to CGSubOp or CGneg because it would not be possible to determine with certainty whether any differences observed in activation induced by CG50-PA4 ICs were due to the differences in CpG content per se or simply to a greater extent of DNA internalization.

We therefore used an alternative method to form DNA fragment ICs. The individual DNA fragments were labeled at the 5' and 3' ends with a single biotin molecule at each end, and are referred to as CG50-bio, CGSubOp-bio, and CGneg-bio. ICs were formed by

Results

Defined ICs containing optimal CpG motifs activate DCs through TLR9 and induce different cytokine profiles from plasmacytoid and conventional DCs

Before directly comparing dsDNA fragments of different sequence, it was necessary to demonstrate in our system that DNA fragment-containing ICs could in fact activate DCs and that TLR9 was involved in this process. To do this, we isolated a 607-bp EcoRI/BamHI DNA restriction fragment containing an array of 50 CpG motifs from the plasmid pMCG50 (29), as previously described (14) (Table I). The CpG motifs in this fragment, referred to as CG50, are unmethylated and have the optimal flanking bases (GACGTT) to activate murine TLR9. Immune complexes were formed by incubating the CG50 fragment with the IgG2a anti-DNA mAb PA4 (26). DCs for use in these studies were generated by culturing mouse bone marrow cells in vitro with FL, which leads to the development of a mixed population of pDCs and cDCs, collectively referred to as FL-DCs. These pDCs and cDCs show equivalent properties to pDCs and cDCs isolated directly from mouse spleen (30).

The addition of the CG50-PA4 ICs to the FL-DC cultures induced the production of IFN-α and IL-6 (Fig. 1A), as well as up-regulation of the costimulatory molecule CD40 (Fig. 1B). CG50 alone (in the absence of PA4) induced CD40 up-regulation and a lower level of IL-6 production but did not induce IFN-α production. These effects were completely TLR9 dependent, as no activation was seen in FL-DCs derived from TLR9-deficient mice (Fig. 1, A and B). To determine the DC subset responsible for the cytokine production, pDCs and cDCs were purified from FL-DC cultures by cell sorting and stimulated with CG50-PA4 ICs (Fig. 1C). This revealed that pDCs were the source of IFN-α and cDCs the major source of IL-6.

Statistical analysis

Statistical analysis was performed using the Wilcoxon signed-rank test. Values of p < 0.05 were considered significant.
incubating the biotin-labeled DNA fragments with an IgG2a anti-biotin mAb, 1D4 (23). Formation of the immune complex therefore depends on the affinity of the anti-biotin mAb for the attached biotin, which is independent of the DNA fragment itself. The individual biotin-labeled DNA-fragment ICs elicited markedly different responses when added to FL-DC cultures (Fig. 2C). Even at the lowest DNA fragment concentration of 100 ng/ml, the CG50-bio-1D4 ICs induced IFN-α and high levels of IL-6 production. The CGSubOp-bio-1D4 ICs induced lower levels of IL-6 and only at the highest DNA fragment concentrations. The CGneg-bio-1D4 ICs failed to induce IL-6 production at any of the DNA fragment concentrations.

To confirm the observed differences between CGSubOp and CGneg, we also compared the stimulatory capacity of these DNA fragments when the ICs were formed with the anti-DNA mAb PA4 instead of with 1D4. This approach could be used because, as previously noted, the affinity of CGSubOp for PA4 was equivalent to that of CGneg (Fig. 2, A and B). When added to the FL-DC cultures, the CGSubOp-PA4 ICs induced IL-6 production, whereas the CGneg-PA4 ICs did not (Fig. 2D). Similarly, up-regulation of the costimulatory molecule CD40 was only induced by the CGSubOp-PA4 ICs (Fig. 2E). The induction of both cytokine production and costimulatory molecule expression was completely TLR9-dependent (Fig. 2, D and E).

Overall, these data demonstrate that CpG content is required for TLR9-dependent DC activation by DNA-containing ICs. Additionally, although optimal CpG motifs induce the strongest response, effective activation is also induced by DNA that contains unmethylated CpG dinucleotides but lacks the optimal motifs.

**Methylation of CpG dinucleotides abrogates the stimulatory capacity of DNA that contains unmethylated CpG dinucleotides but lacks optimal CpG motifs**

If the difference between the stimulatory capacity of the CGSubOp-PA4 ICs and the lack of stimulatory capacity of the CGneg-PA4 ICs resides in the unmethylated CpG dinucleotides within the CGSubOp DNA fragment, then methylation of the CpG dinucleotides in CGSubOp should eliminate its ability to activate FL-DCs. To initially confirm that the methylation procedure was working adequately, CG50 was treated with the CpG methylase M.SssI, which methylates all cytosine residues within the double-stranded dinucleotide recognition sequence 5′...CG...3′ (31). This procedure eliminated unmethylated CpGs, as shown by the complete resistance of CpG methylase-treated CG50 to the restriction endonuclease HpyCH4IV, which cuts DNA at unmethylated CpG sites flanked 5′ by an adenine base, but does not cut at methylated CpG sites (Fig. 3A). CGSubOp was then methylated with the CpG methylase, M.SssI, using the same procedure as was used for CG50. In a competition ELISA, CpG methylase-treated CGSubOp (referred to as Met-CGSubOp) bound to the anti-DNA mAb PA4 at least as well as untreated CGSubOp (Fig. 3B). However, whereas CGSubOp-PA4 ICs induced IL-6 production when added to FL-DC cultures, no IL-6 production was induced by Met-CGSubOp-PA4 ICs (Fig. 3C). As an additional control to demonstrate the specificity of the methylation effect, we also tested the stimulatory capacity of CGSubOp after treatment with AluI methyltransferase, which methylates the cytosine residue of AGCT sequences, but does not methylate cytosine residues in CpG dinucleotides. AluI methyltransferase treatment of CGSubOp, which contains 6 AluI methyltransferase sites (Table I), did not affect the ability of CGSubOp-PA4 ICs to induce IL-6 production (Fig. 3C). Overall, these data demonstrate that unmethylated CpG dinucleotides are critical determinants of the ability of CGSubOp-PA4 ICs to activate DCs through TLR9.

**CpG-free DNA internalized into DCs by liposomal transfection is stimulatory, in contrast to CpG-free DNA internalized in an IC that is not stimulatory**

Previous studies have shown that CpG-free phosphodiester ODNs can activate TLR9 when the ODNs are internalized into DCs by liposomal transfection with DOTAP or by the addition of 3′ poly G tails to the ODNs (20, 21). However, we found that the double-stranded CpG-free fragment CGneg could not activate when internalized into DCs as an IC (Fig. 2, C–E). To directly compare the stimulatory capacity of CpG-free DNA internalized by DCs through these different routes, we used two different CpG-free fragments. The one fragment was CGneg. The other was a 3070-bp CpG-free plasmid from Invivogen, termed pCG-mcs.
pCpG-mcs bound comparably to the CpG-containing DNA fragment CGSubOp in a competition ELISA measuring binding affinity to the anti-DNA mAb PA4, demonstrating that pCpG-mcs could form ICs with PA4 (Fig. 4A). The pCpG-mcs-PA4 ICs did not induce either IFN-α or IL-6 when added to FL-DC cultures, even at pCpG-mcs concentrations as high as 3 μg/ml (Fig. 4B). In contrast, pCpG-mcs internalized with DOTAP induced both IFN-α and IL-6, with the activation being mostly, but not completely, TLR9-dependent (Fig. 4B). The TLR9-independent component of activation might represent DOTAP-mediated transport of pCpG-mcs to other DNA recognition receptors, such as the cytosolic DNA-dependent activator of IFN-regulatory factors (DAI, also called Z-DNA-binding protein 1 and DLM-1) (32) or the cytosolic dsDNA receptor absent in melanoma 2 (AIM2) (33–36).

FIGURE 2. CpG content is required for TLR9-dependent DC activation induced by DNA-containing ICs. A and B, Increasing concentrations of unlabeled dsDNA fragments CG50, CGSubOp, and CGneg were added to plate-bound PA4. After 1 h, biotinylated CGSubOp (5 ng/ml) (A) or biotinylated CG50 (5 ng/ml) (B) was added. The binding of biotinylated CGSubOp (A) or biotinylated CG50 (B) to PA4 was detected with streptavidin-HRP and tetramethylbenzidine substrate. Data represent means ± SEM of three experiments for both A and B. C, Biotinylated DNA fragments (100, 300, or 1000 ng/ml) with or without the anti-biotin mAb 1D4 (3 μg/ml) were added to FL-DCs from wild-type BALB/c mice. IFN-α and IL-6 concentrations in supernatants collected after 24 h were measured by ELISA. Data represent means ± SEM of four experiments. *p < 0.05. D, Bone marrow-derived FL-DCs from wild-type (WT) BALB/c or TLR9-deficient (TLR9−/−) mice were incubated with indicated concentrations of the DNA fragments CGSubOp and CGneg in the presence or absence of the anti-DNA mAb PA4 (10 μg/ml). IL-6 concentrations in supernatants collected after 24 h were measured by ELISA. Data shown are the means ± SEM of five experiments (WT) and three experiments (TLR9−/−). **p < 0.01 vs WT; DNA; #, p < 0.05 vs TLR9−/−; DNA + PA4. E, After removal of supernatants for cytokine analysis, the cells were analyzed by flow cytometry for CD40 expression, with stimulus-induced staining intensity compared with staining intensity of the nonstimulated cultures.

FIGURE 3. Methylation of CpG dinucleotides abrogates the stimulatory capacity of DNA that contains unmethylated CpG dinucleotides but lacks optimal CpG motifs. A, The 607-bp DNA fragment CG50 was treated or not with M.SssI methylase (CpG methylase) and then digested or not with the methylation-sensitive restriction endonuclease HpyCH4 IV. B, Increasing concentrations of unlabeled CGSubOp and CGSubOp treated with M.SssI methylase (Met-CGSubOp) were added to plate-bound PA4. After 1 h, biotinylated CGSubOp (5 ng/ml) was added. The binding of biotinylated CGSubOp to PA4 was detected with streptavidin-HRP and tetramethylbenzidine substrate. Data represent means ± SEM of three experiments. C, CGSubOp fragments were treated with M.SssI methylase or AluI methylase. FL-DCs from wild-type BALB/c mice were then incubated with the variously treated CGSubOp fragments alone (300 ng/ml) or with CGSubOp-ICs (300 ng/ml of CGSubOp plus 10 μg/ml of PA4). IL-6 concentrations in supernatants collected after 24 h were measured by ELISA. Data represent means ± SEM of five experiments. **p < 0.01.
ICs (CGneg 1000 ng/ml) were added to FL-DCs for 2 h at 37°C. The extent of binding and/or uptake of CGneg was at least 100-fold greater with CGneg-DOTAP than with CGneg-PA4 ICs (Fig. 4C). At the same DNA concentration used in the binding studies (1000 ng/ml), CGneg-PA4 ICs did not induce cytokine production by FL-DCs, whereas CGneg internalized with DOTAP did induce activation (Fig. 4D).

**FIGURE 4.** CpG-free DNA internalized into DCs by liposomal transfection is stimulatory, in contrast to CpG-free DNA internalized in an IC, which is not stimulatory. A, Increasing concentrations of the unlabeled DNA fragments pCpG-mcs and CGSubOp were added to plate-bound PA4. After 1 h, biotinylated CGSubOp (5 ng/ml) was added. The binding of biotinylated CGSubOp to PA4 was detected with streptavidin-HRP and tetramethylbenzidine substrate. Data represent means ± SEM of two experiments. B, pCpG-mcs alone, pCpG-mcs plus PA4 (10 μg/ml), or pCpG-mcs plus DOTAP (at weight ratio of 1:2) were added to FL-DCs from wild-type (WT) BALB/c or TLR9-deficient (TLR9−/−) mice. Data represent means ± SEM of three experiments. C, After 1 h, biotinylated CGSubOp (5 ng/ml) was added. The binding of biotinylated CGSubOp to PA4 was detected with streptavidin-HRP and tetramethylbenzidine substrate. Data represent means ± SEM of three experiments. *p < 0.05 vs pCpG-mcs and pCpG-mcs + PA4. C, Biotinylated CGneg was labeled with streptavidin-coated fluorescent Qdots. Qdot-labeled CGneg-DOTAP complexes (CGneg 1000 ng/ml + DOTAP 2 μg/ml), Qdot-labeled CGneg ICs (CGneg 1000 ng/ml + PA4 10 μg/ml), and Qdot-labeled CGneg alone (1000 ng/ml) were added to FL-DCs for 2 h at 37°C. The extent of Qdot-labeled CGneg internalization or binding to pDCs and cDCs was determined using flow cytometry to measure fluorescence intensity. Data represent one of three representative experiments. D, CGneg alone (1000 ng/ml), CGneg-ICs (CGneg 1000 ng/ml + PA4 10 μg/ml), and CGneg-DOTAP complexes (CGneg 1000 ng/ml + DOTAP 2 μg/ml) were added to FL-DCs from wild-type BALB/c mice. IL-6 concentrations in supernatants collected after 24 h were measured by ELISA. Data represent means ± SEM of three experiments. *p < 0.05.

One possible explanation for why DOTAP-mediated internalization of CpG-free DNA is stimulatory, whereas IC-mediated internalization is not, is that the amount of DNA able to be internalized by ICs might be relatively limited as compared with DOTAP-mediated internalization. To evaluate this possibility, CGneg-bio was fluorescently labeled using Qdot 655 streptavidin conjugate, and CGneg alone, CGneg-PA4 ICs, and CGneg-DOTAP were added to FL-DC cultures. The extent of binding and/or uptake of CGneg was at least 100-fold greater with CGneg-DOTAP than with CGneg-PA4 ICs in both pDCs and cDCs (Fig. 4C). At the same DNA concentration used in the binding studies (1000 ng/ml), CGneg-PA4 ICs did not induce cytokine production by FL-DCs, whereas CGneg internalized with DOTAP did induce activation (Fig. 4D).

**Endogenous mammalian CpG-rich sequences activate DCs**

Our studies to this point had shown that unmethylated CpG dsDNA sequences were required for DC activation by DNA-containing ICs. However, to demonstrate the relevance of this finding to human autoimmune disease, it was important to show that similar stimulatory DNA sequences were present in mammalian DNA. Although mammalian DNA in general has far fewer unmethylated CpG dinucleotides than does bacterial DNA, the promoter regions of mammalian genes contain areas called CpG islands that are CpG rich and unmethylated (37). In the context of CpG islands, CpG-rich fragments are defined as DNA fragments having a GC content >50% and a ratio of observed-to-expected CpGs >0.5 (38). To determine whether CpG-rich dsDNA fragments derived from mammalian cells can activate DCs, we used dsDNA fragments isolated from a mouse CpG island library (Table I). These fragments, termed clones 11, 12, 14, 15, and 23, have recently been shown by our group to activate autoreactive B cells following internalization through the BCR (23). They contain plentiful CpG dinucleotides but few or no optimal CpG motifs. A dsDNA fragment similar in size but containing only a single CpG dinucleotide, termed SUMO, was used as a control (Table I). The fragments were biotinylated at the 5′ end with a single biotin molecule, and ICs were formed by incubating the biotin-labeled DNA fragments with the anti-biotin mAb 1D4. Addition of the biotinylated CpG island fragments alone (in the absence of 1D4) to FL-DC cultures induced no IFN-α production and very low level or no IL-6 production (Fig. 5). However, three of the five biotinylated CpG island fragments complexed with 1D4 induced substantial amounts of both IFN-α and IL-6. In contrast, the control SUMO fragment induced no cytokine production, either on its own or complexed with 1D4. These data demonstrate that immune complexes containing CpG-rich dsDNA fragments from mammalian DNA can induce DC activation.

**DC priming with type I IFN enhances responses to CpG-rich dsDNA but not to CpG-poor dsDNA**

Type I IFN (IFN-α or IFN-β) overproduction has been linked to the pathogenesis of SLE (39–41), and priming of human DCs with type I IFN enhances responses to DNA-containing ICs (13, 42). To determine whether type I IFN priming would modify DC responses to DNA-containing ICs in our system, FL-DCs were pretreated with IFN-β for 2 h and then stimulated with ICs containing the various biotinylated DNA fragments. ICs containing the CpG island fragments induced a higher level of IFN-α and IL-6 production from IFN-β pretreated FL-DCs than from FL-DCs not pretreated with IFN-β (Fig. 5). Notably, IFN-β pretreatment enabled the two CpG island fragment ICs (clone 12 and clone 15) that were not stimulatory in the absence of type I IFN pretreatment to induce effective DC activation. IFN-β pretreatment also enabled the CpG island fragments alone (in the absence of 1D4) to induce modest levels of IL-6 production. However, even with IFN-β...
Data represent the means ± SEM of three experiments. *p < 0.05.

Discussion

In the autoimmune disease SLE, DNA-containing ICs are thought to contribute to disease pathogenesis through the activation of intracellular TLR9 in pDCs. To determine the requirement for DNA sequence in the activation of TLR9 under these conditions, we used dsDNA fragments of defined sequence and methylation status to activate TLR9 in murine DCs after internalization in the form of immune complex. This result was anticipated, as unmethylated CpG dinucleotides but lacks these optimal motifs. This finding implies that before self DNA can induce DC activation, DNA containing unmethylated CpG dinucleotides first needs to be released into the extracellular environment where it can bind with autoantibody to form an IC. Indeed, there is some evidence that this may occur in patients with SLE (43–46). Although unmethylated CpG dinucleotides are much less common in mammalian DNA as compared with pathogen DNA, they are found at high concentration in regions of the mammalian genome referred to as CpG islands (37, 47). Mammalian CpG islands could thus represent one potential source of this G-C-enriched DNA, and our study shows that CpG-rich DNA obtained from CpG islands is capable of effective DC activation after internalization as an IC. Optimal CpG motifs are not required for this DC activation, as at least two CpG island clones (clones 12 and 23, Table I) that contain CpG dinucleotides but lack optimal motifs can induce IFN-α and IL-6 production. Our group has recently reported that this CpG island DNA is also able to activate autoreactive B cells (23), which could be the initiating event leading to the production of the autoantibodies required for immune complex formation.

It is not known how CpG island DNA might become accessible to the immune system in patients with SLE. However, CpG islands show the properties that coincide with enhanced sensitivity to nucleases relative to bulk DNA (47), and so could be preferentially released during apoptosis and accumulate as a result of the impaired apoptotic cell clearance that characterizes SLE (48). It is also conceivable that there might be an element of selectivity at the level of immune complex formation. Anti-DNA autoantibodies from lupus patients show preferential binding to GC-rich DNA fragments (49), and certain anti-dsDNA autoantibodies from mouse lupus models bind to G-C much more strongly than to other base combinations (50, 51). In the present study we found that the anti-DNA Ab PA4 has a greater affinity for the DNA fragment CG50 containing optimal CpG motifs than for the DNA fragments CGSubOp or CGneg. One possible explanation for why anti-DNA Abs might preferentially bind CpG-rich DNA is that B cells with this binding specificity might be preferentially activated due to synergy between the BCR and TLR9.

Our data demonstrate that there are differences in DNA sequence requirements for TLR9 activation in DCs depending on the route of DNA internalization. Unmethylated CpG dinucleotides are absolutely required for TLR9 activation if the DNA fragments are internalized in the form of an IC, whereas CpG-free DNA can activate TLR9 if the DNA fragments are internalized by liposomal transfection with DOTAP. Presumably TLR9 activation in this setting is being mediated through non-CG bases and/or direct recognition by TLR9 of the DNA sugar backbone 2’ deoxyribose (20, 21). One possible explanation for the difference between immune complex-mediated and DOTAP-mediated internalization might be the absolute concentration of DNA fragment delivered to intracellular TLR9. We found that DNA fragment binding and/or uptake was at least 100-fold greater with DOTAP-mediated internalization. This interpretation would be consistent with previous studies using phosphorothioate ODNs showing that much higher concentrations of CpG-free ODNs are required to induce TLR9 activation as compared with ODNs containing CpG motifs (52).

However, it is necessary to consider additional possibilities. The precise intracellular location at which TLR9 engages its ligand plays an important role in determining the functional outcome (53, 54), and there may well be differences in intracellular trafficking of the DNA fragments depending on whether they are internalized as an immune complex or with DOTAP. Also, cationic lipids such as DOTAP form a highly ordered multilamellar structure with DNA, which can fuse with endosomal vesicles to form large stable aggregates (53, 55–57). In this way, DOTAP might affect both the DNA structure and the kinetics of DNA longevity in endosomes (53). Additionally, DOTAP has been reported to possess adjuvant activity in its own right through induction of the ERK pathway (58), and it is conceivable that this could modulate the DC response to DNA fragments, particularly as ERK activation has been reported to up-regulate TLR9 expression and increase the response to CpG ODNs (59).

Although immune complex formation markedly enhanced the stimulatory capacity of the DNA fragments, certain of the CpG island fragments induced low-level, but consistent, IL-6 production even in the absence of Ab. This was enhanced by type I IFN pretreatment of the FL-DCs. Additional studies will be needed to determine whether there are other immunological consequences of this Ab-independent uptake of DNA in SLE. For example, a recent study has shown that DNA from gut flora can directly activate TLR9 in DCs in the lamina propria and thereby limit the production of T regulatory cells (60). This effect was present whether or not DOTAP was used to internalize the DNA. We found that pretreatment of FL-DCs with type I IFN also enhanced their response to DNA-containing ICs, and this may reflect the in vivo situation in many SLE patients where the DCs may be exposed to elevated levels of circulating type I IFN. This result was anticipated, as other studies examining DC activation by DNA-containing ICs...
had shown similar enhancement (13, 42). However, it is notable in our study that type I IFN only enhanced the response if the DNA within the DNA-ICs contained CpG dinucleotides. This effect was particularly remarkable for two of the CpG island clusters that did not induce cytokine production in the absence of type I IFN pre-treatment but induced both IFN-α and IL-6 with type I IFN pre-treatment. Importantly, however, type I IFN did not enhance the response to DNA-ICs that contained CpG-free DNA, in contrast to what was observed for TLR9 activation in type I IFN-pretreated B cells (23).

In summary, the data demonstrate that unmethylated CpG dinucleotides within phosphodiester dsDNA are required for TLR9 activation in DCs induced by DNA-containing ICs. The data also show that CpG islands within mammalian DNA represent one possible source of this stimulatory CpG-rich DNA. Our study provides new information on the DNA sequence requirements within natural phosphodiester dsDNA for the activation of TLR9 in DCs and is consistent with the concept that, in normal circumstances, the immune system is protected from inadvertent activation by self DNA both by the intracellular location of TLR9 and the DNA sequence required for TLR9 activation. It will be important to try to understand how different forms of cell death might contribute to the release of CpG-rich DNA in autoimmune diseases such as SLE and psoriasis.

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References


