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Mechanism and Function of a Newly Identified CpG DNA Motif in Human Primary B Cells

Gunther Hartmann*‡ and Arthur M. Krieg*†§¶

The vertebrate immune system recognizes bacterial DNA based on the presence of unmethylated CpG-dinucleotides in particular base contexts ("CpG motifs"). In contrast to mice, knowledge about CpG-mediated effects on human B cells is poor. In the present study we identify and determine an optimal human CpG motif. A phosphodiester oligonucleotide containing this motif strongly stimulated CD86, CD40, CD54, and MHC class II expression, IL-6 synthesis, and proliferation of primary human B cells. These effects required internalization of the oligonucleotide and endosomal maturation. The molecular mechanism of action of this CpG motif was associated with the sustained induction of the NF-κB p50/p65 heterodimer and of the transcription-factor complex AP-1. Transcription-factor activation by CpG DNA was preceded by increased phosphorylation of the stress kinases c-Jun N-terminal kinase and p38, and of activating transcription factor-2. In contrast to CpG, signaling through the B cell receptor led to activation of extracellular receptor kinase and to phosphorylation of a different isoform of c-Jun N-terminal kinase. These studies define the structure of a highly active human CpG motif and characterize its molecular mechanism of action in primary human B cells. The Journal of Immunology, 2000, 164: 944–952.

The CpG dinucleotides are underrepresented and selectively methylated in vertebrate DNA. In contrast, CpG dinucleotides are present at the expected frequency and are unmethylated in bacterial DNA (1). The recognition of unmethylated CpG dinucleotides within specific flanking bases is believed to be an ancestral nonself pattern recognition mechanism used by the innate immune system to detect DNA of microbes or viruses (2). In mice, optimal immune activation requires a CpG motif in which an unmethylated CpG dinucleotide is flanked by two 5′ purines and two 3′ pyrimidines (3, 4). DNA containing this CpG motif (CpG DNA) activates murine macrophages (5–8), murine dendritic cells (9, 10), murine NK cells (7, 11, 12), and murine B cells (13–15).

CpG DNA is known to be an excellent immune adjuvant in various murine disease models and to drive Th1 immune responses (16–21). Thus, CpG DNA might be useful for immunotherapy of allergy, infectious disease, and cancer (20, 22–24). The potent adjuvant activity of CpG DNA in mice is limited. Many CpG phosphorothioate oligodeoxynucleotides (ODNs) with strong stimulatory activity in the mouse system show only low activity on human immune cells (A. M. Krieg, unpublished observations). In human PBMCs, synthetic phosphodiester ODNs with hexamer palindromic sequences containing a central CpG dinucleotide have been described as inducing IFN-α synthesis (25). To date, the most active phosphorothioate oligonucleotide reported to stimulate human B cells is a 27-mer ODN (DSP30) originally designed as an antisense ODN to the rev gene of HIV (26–28). A 21 mer (27) and a 15 mer (29), both 3′-truncated forms of the original anti-rev 27 mer, were equally active as the complete 27 mer. DSP30 contains the sequence 5′-TCGTCG-3′ at its 5′ end, suggesting the hypothesis that repeating CGG motifs may activate human B cells. However, phosphorothioate ODNs containing (TCG)₃ showed a lower activity than DSP30 did (29). Furthermore, testing of phosphorothioate ODNs which were not related to DSP30 but contained multiple copies of various mouse 6-mer CpG motifs (GACGTT; TGACGTT; GACGTC; and TGACGTC) were also less active than DSP30 (29). A non-CpG-specific effect mediated by the phosphorothioate backbone was suggested to be mainly responsible for the proliferative response in these studies. Non-CpG-specific activation of human PBMCs by the phosphorothioate backbone has been described earlier (30). Thus, although human cells can be stimulated by a variety of ODNs, the requirement for CpG dinucleotides and the optimal flanking bases, if any, remain unclear.

The identification of a potent human CpG motif is critical for the transfer of therapeutic strategies derived from animal models to clinical settings. We recently demonstrated that CpG DNA induces activation of human monocytes (31) and dendritic cells (32), effects which are distinct from LPS-mediated effects. The goal of the present study is to systematically search for an optimal human CpG motif, to determine its functional effects on human B cells, and to elucidate its molecular mechanism of action.

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3 Abbreviations used in this paper: ODN, oligodeoxynucleotides; ERK, extracellular receptor kinase; JNK, c-Jun N-terminal kinase; ATF-2, activating transcription factor-2; MFI, mean fluorescence intensity; MHC-II, MHC class II; FSC, forward light scatter; CFSE, (5-(and-6-) carboxyfluorescein diacetate succinimidyl ester; SAPK, stress-activated protein kinase.
Materials and Methods

ODNs and DNA

Unmodified (phosphodiester) and modified nucleoside-resistant (phosphorothioate) ODNs were purchased from Operon Technologies (Alameda, CA) and Hybridon Specialty Products (Milford, MA). The sequences used are provided in Table I. *Escherichia coli* DNA and calf thymus DNA were purchased from Sigma (St. Louis, MO). Genomic DNA samples were purified by extraction with phenol-chloroform-isooamyl alcohol (25:24:1) and ethanol precipitation. DNA was purified from endotoxin by repeated extraction with Triton X-114 (Sigma) and tested for endotoxin using the LAL assay (BioWhittaker, Walkersville, MD; lower detection limit 0.1 EU/ml) or by extraction with phenol-chloroform-isooamyl alcohol (25:24:1) and tested for endotoxin using the high-sensitivity assay for endotoxin described earlier (lower detection limit 0.0014 U/ml). Cells were suspended in RPMI 1640 culture medium supplemented with 10% (v/v) heat-inactivated (56°C, 1 h) FCS (HyClone, Logan, UT); 1.5 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (all from Life Technologies, Grand Island, NY; complete medium). All compounds were purchased endotoxin tested. Viability was determined before and after incubation with ODN by trypan blue exclusion (conventional microscopy) or by propidium iodide exclusion (flow cytometry) or by trypan blue exclusion (conventional microscopy) or by propidium iodide exclusion (flow cytometry).

Cell preparation and cell culture

Human PBMCs were isolated from peripheral blood of healthy volunteers by Ficoll-Paque density gradient centrifugation (Histopaque-1077, Sigma) as described (33). Cells were suspended in RPMI 1640 culture medium supplemented with 10% (v/v) heat-inactivated (56°C, 1 h) FCS (HyClone, Logan, UT); 1.5 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (all from Life Technologies, Grand Island, NY; complete medium). All compounds were purchased endotoxin tested. Viability was determined before and after incubation with ODN by trypan blue exclusion (conventional microscopy) or by propidium iodide exclusion (flow cytometry) or by trypan blue exclusion (conventional microscopy) or by propidium iodide exclusion (flow cytometry).

For signal transduction studies, human primary B cells were isolated by immunomagnetic cell sorting using the VARIOMACS technique (Miltenyi Biotec, Auburn, CA) as described by the manufacturer. In brief, PBMCs obtained from buffy coats of healthy blood donors (Elmer L. DeGowin Blood Center, University of Iowa) were incubated with a microbeads-conjugated Ab to CD19 and passed over a positive selection column. Purity of B cells was higher than 95%. After stimulation, whole cellular extracts (Western blot) and nuclear extracts (EMSA) for signal-transduction studies were prepared at the indicated time points.

Flow cytometry

Staining of surface Abs was performed as previously described (34). mAbs to HLA-DR were purchased from Immunotech (Marseille, France). All other Abs (mAbs to CD19 (B43), CD40 (5C3), CD54 (HA58), and CD86 (2331 (FUN-1)) were purchased from PharMingen (San Diego, CA). IgGlκ (MOPC-21) and IgG2bκ (27–32, 35–37) were used to control for specific staining. Intracellular cytokine staining for IL-6 was performed as described (31). In brief, PBMCs (final concentration, 1 × 10^6 cells/ml) were incubated in the presence of brefeldin A (final concentration, 1 µg/ml; Sigma). After incubation, cells were harvested and stained using a FITC-labeled mAb to CD19 (B43), a PE-labeled rat anti-human IL-6 mAb (MQ2-6A3, PharMingen), and the Fix and Perm Kit (Caltag Laboratories, Burlingame, CA). Flow cytometric data of 5000 cells/sample were acquired on a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA). Nonviable cells were excluded from analysis by propidium iodide staining (2 µg/ml). Data were analyzed using the computer program FlowJo (version 2.5.1, Tree Star, Stanford, CA).

Proliferation assay

CFSE (5- (and-6-) carboxyfluorescein diacetate succinimidyl ester; Molecular Probes, Eugene, OR) is a fluorescein-derivied intracellular fluorescent label which is divided equally among daughter cells upon cell division. Staining of cells with CFSE allows both quantification and immunophenotyping (PE-labeled Abs) of proliferating cells in a mixed-cell suspension. Briefly, PBMCs were washed twice in PBS, resuspended in PBS containing CFSE at a final concentration of 5 µM, and incubated at 37°C for 10 min. Cells were washed three times with PBS and incubated for 5 days as indicated in the figure legends. Proliferating CD19-positive B cells were identified by decreased CFSE content using flow cytometry.

Preparation of whole-cell, nuclear, and cytosolic protein extracts

For Western blot analysis, whole-cell extracts were prepared. Primary B cells were treated with medium, the phosphodiester ODNs 2080 (CpG) or 2078 (non-CpG) at 30 µg/ml, or anti-IgM (10 µg/ml) as indicated. Cells

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### Table I. ODN panel used

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5’ to 3’</th>
</tr>
</thead>
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<tr>
<td>PE 2079</td>
<td>TCG ACG TTC CCC CCC CCC CC</td>
</tr>
<tr>
<td>PE 2082</td>
<td>TCG GCC TTC CCC CCC CCC CC</td>
</tr>
<tr>
<td>PE 2080</td>
<td>TCG TCG TTC CCC CCC CCC CC</td>
</tr>
<tr>
<td>PE 2100</td>
<td>TCG GCC TTC CCC CCC CCC CC</td>
</tr>
<tr>
<td>PE 2107</td>
<td>ACG TCG TTC CCC CCC CCC CC</td>
</tr>
<tr>
<td>PE 2104</td>
<td>CCG TCG TTC CCC CCC CCC CC</td>
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<tr>
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<td>GCG TCG TTC CCC CCC CCC CC</td>
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<td>PE 2108</td>
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<td>TCG TCG TTC CCC CCC CCC CC</td>
</tr>
<tr>
<td>PE 2101</td>
<td>GGC CTT TTC CCC CCC CCC CC</td>
</tr>
</tbody>
</table>

* PE, phosphodiester; PS, phosphorothioate; bold, base exchange; bold Z, methylated cytidine; underlined, CpG dinucleotides.
were harvested, washed twice with ice-cold PBS containing 1 mM Na$_2$VO$_4$, resuspended in lysis buffer (150 mM NaCl, 10 mM Tris (pH 7.4), 1% Nonidet P-40 (NP-40), 1 mM Na$_2$VO$_4$, 50 mM NaF, 30 mg/ml leupeptin, 5 mg/ml aprotinin, 5 mg/ml antipain, and 50 μg/ml PMSF), incubated for 15 min on ice, and spun at 14,000 rpm for 10 min. The supernatant was frozen at −80°C. For the preparation of nuclear extracts, primary B cells were resuspended in hypotonic buffer (10 mM HEPES/KOH (pH 7.9), 10 mM KCl, 0.05% NP-40, 0.05 mM MgCl$_2$, 0.5 mM DTT, 0.5 mM PMSF, 30 mg/ml leupeptin, 50 mg/ml aprotinin, 5 mg/ml antipain, and 5 mg/ml pepstatin) and incubated on ice for 1 h. The nuclear suspension was centrifuged for 10 min at 16,000 × g at 4°C. Supernatant was collected and stored at −80°C. Biotinylated probes were prepared as described for the preparation of the nuclear extract. After centrifugation, the supernatant was removed as cytoplasmic fraction and stored at −80°C. Protein concentrations were measured using a Bradford protein assay (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions.

**Western blot analysis**

Equal concentrations of whole-cell protein extracts (25 μg/lane) were boiled in SDS sample buffer (50 mM Tris-Cl (pH 6.8), 1% 2-ME, 2% SDS, 0.1% bromphenol blue, and 10% glycerol) for 4 min before being subjected to electrophoresis on a 10% polyacrylamide gel containing 0.1% SDS (SDS-PAGE). After electrophoresis, proteins were transferred to Immobilon-P transfer membranes (Millipore, Bedford, MA). Blots were blocked with 5% nonfat dry milk. Specific Abs against the phosphorylated form of extracellular receptor kinase (ERK), c-Jun N-terminal kinase (JNK), p38, with 5% nonfat dry milk. Specific Abs against the phosphorylated form of specific Abs against c-myc promotor region 5′-TGCAAGTCGGTTTCCCAACCACC-3′ as probes. ODNs were end labeled with [γ-32P]ATP (Amerham International, Aylesbury, U.K.) according to the manufacturer’s instructions.

**EMSA**

To detect the DNA-binding activity of the transcription factor AP-1 and NF-κB, nuclear extracts (1 μg/lane) were analyzed by EMSA using the hsODNs 5′-GAT CTA GTG ATG AGT CAG CCG GAT C-3′ as probes. ODNs were end labeled with T4-polynucleotide kinase (New England Biolabs) and [γ-32P]ATP (Amersham International, Aylesbury, U.K.) according to the manufacturer’s recommended procedure.

**Statistical analysis**

Data were expressed as means ± SEM. Statistical significance of differences was determined by the unpaired two-tailed Student t-test. Differences were considered statistically significant for p < 0.05. Statistical analyses were performed by using StatView 4.51 software (Abacus Concepts, Calabasas, CA).

**Results**

**Identification of a potent human Cpg motif for activating primary human B cells**

Phosphorothioate ODNs containing the murine Cpg motif GACGGTT (for example ODN 1826) and used at concentrations which are highly active on murine B cells (3) show little or no immunostimulatory activity on human immune cells (data not shown). Added at high concentrations, phosphorothioate ODNs stimulate human B cells in a Cpg-independent manner (29). This likely is due to the phosphorothioate modification of the ODNs. The use of bacterial DNA or unmodified phosphodiester ODNs avoids the sequence-independent background activity of the phosphorothioate backbone. We found that B cells express increased levels of the costimulatory molecule CD86 (B7-2) in response to Cpg DNA but not in response to non-Cpg control DNA (Fig. 1). Assuming that bacterial DNA contains human Cpg motifs, we tested the effect of highly purified E. coli DNA (31) on human primary B cells. Repeated addition (at 0, 4, and 8 h) of 30 μg/ml E. coli DNA resulted in maximal expression of CD86 on human B cells within 2 days. Higher concentrations showed no further increase. Calf thymus DNA (non-Cpg control DNA) did not activate human B cells. High concentrations of LPS (1 μg/ml, 1000-fold more than needed for maximal activation of human monocytes) only slightly activated B cells above background (mean fluorescence intensity (MFI) CD86: E. coli DNA, 3.4 ± 0.9; calf thymus DNA, 1.5 ± 0.4; LPS 1.6 ± 0.4; medium only, 0.9 ± 0.1; n = 4).

In earlier studies on B cell activation in mice, we found that a Cpg-dinucleotide flanked by two 5′ pyrimines and two 3′ pyrimidines is optimal for a phosphodiester ODN to be active, and that the 6-mer motif 5′-GACGGTT-3′ is best (2, 3). Furthermore, testing all 16 possible diners at the 5′ end of a Cpg ODN, we found that the dimer 5′-Tpc-3′ added to the activity of the ODN. The presence of 3′-polypyrimidine sequences after the 6-mer CpG motif further enhanced the effect of the ODN (3).

Using this information we designed a 20-mer phosphodiester ODN with a TpC dinucleotide at the 5′ end preceding the optimal murine Cpg motif 5′-GACGGTT-3′ and followed by a poly C tail (2079, 5′-TGC ACG TTC CCC CCC CCC-3′). This ODN, if added to human primary B cells under the same conditions found to be optimal for E. coli DNA (repeated addition at 0, 4, and 18 h; 30 μg/ml at each time point), stimulated high levels of CD86 expression on human primary B cells after 2 days (Fig. 2). To determine the structure-function relationship of the Cpg motifs, we replaced the bases adjacent to the Cpg dinucleotides while maintaining the two Cpg dinucleotides within the sequence. Exchange of the adenine located between both Cpg dinucleotides by thymidine (2080) resulted in slightly higher activity (Fig. 2). Replacement by guanosine (2100) or cytidine (2082) at this position showed no major changes compared with 2079. In contrast, replacement of the thymidine 3′ to the second Cpg dinucleotide by the purines guanosine (2099) or adenine (2083) resulted in a major drop in activity of the ODN, whereas the pyrimidine cytidine caused only a minor decrease (Fig. 2). The thymidine immediately
to the first CpG dinucleotide was also critical. Replacement of the thymidine by any other base (e.g., guanosine; adenine; or cytidine) led to a marked decrease in activity of the ODN. Elimination of the first (or the second) CpG dinucleotide also partially reduced the activity (Fig. 2).

Consistent with the B cell activation by bacterial but not by vertebrate DNA, an ODN in which the cytidines of the CpG dinucleotides were methylated (2095) was not stimulatory (Fig. 2). Methylation of an unrelated cytidine (2094) did not change the potency to activate B cells. An ODN with an inversion of both CpG dinucleotides to GpC (2078) and another non-CpG control ODN derived from 2080 (2101; Table I and data not shown) did not stimulate CD86 expression above background (Fig. 2).

The addition of more 5'-GT
CG
TT-3' CpG motifs to the phosphodiester ODN containing the 8-mer duplex CpG motif (5'-T
CG
T
CG
TT-3', 2080) did not further enhance CD86 expression on B cells (2059). An ODN with the same sequence as 2080 but with a phosphorothioate backbone showed no activity above background (2116). This was surprising because the phosphorothioate backbone has been reported to greatly stabilize ODNs and to enhance CpG-induced stimulation (2). Therefore, we performed further structure-function analyses of phosphorothioate ODNs containing the 5'-GTCGT-3' and 5'-TGCCTTT-3' motifs, which showed that an active phosphorothioate ODN required the presence of additional CpG motifs (2006) (Fig. 2, and Hartmann et al., manuscript in preparation). Although the maximal activities of phosphodiester and phosphorothioate ODNs were similar, much lower concentrations of the phosphorothioate ODN (0.6 \( \mu \)g/ml) were sufficient to produce maximal activity, most likely due to the higher nuclease stability of the phosphorothioate backbone. The effect of 2006 at this concentration was CpG-specific because a control ODN with the same sequence but with methylated cytidines (2117) did not induce CD86 expression on primary B cells.

5' to the first CpG dinucleotide was also critical. Replacement of the thymidine by any other base (e.g., guanosine; adenine; or cytidine) led to a marked decrease in activity of the ODN. Elimination of the first (2108) or the second (2106) CpG dinucleotide also partially reduced the activity (Fig. 2).

Consistent with the B cell activation by bacterial but not by vertebrate DNA, an ODN in which the cytidines of the CpG dinucleotides were methylated (2095) was not stimulatory (Fig. 2). Methylation of an unrelated cytidine (2094) did not change the potency to activate B cells. An ODN with an inversion of both CpG dinucleotides to GpC (2078) and another non-CpG control ODN derived from 2080 (2101; Table I and data not shown) did not stimulate CD86 expression above background (Fig. 2).

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FIGURE 2. Identification of a potent human CpG motif. PBMCs were incubated with a panel of phosphodiester ODNs (see Table I) or E. coli DNA (added to PBMCs at 0, 4, and 18 h; 30 \( \mu \)g/ml at each time point), with phosphorothioate ODNs (added to PBMCs at 0 h; 0.6 \( \mu \)g/ml) and LPS (1 \( \mu \)g/ml). After 2 days, induction of CD86 expression on human primary B cells (CD19-positive cells) was examined by flow cytometry. The phosphodiester ODNs without a CpG dinucleotide (2078) are inactive. The ODN with the 8-mer motif 5'-T
CG
T
CG
TT-3' (2080) containing two CpG dinucleotides shows the highest activity. Replacement of the bases flanking the two CpG dinucleotides (5' position, middle position, 3' position) reduces the activity of this sequence. Both CpG dinucleotides within the 8-mer CpG motif are required for optimal activity (2108 and 2106). Cytidine methylation of the CpG dinucleotides (2095) abolishes the activity of 2080, whereas methylation of an unrelated cytidine (2094) does not. The addition of two CpG motifs into the sequence of 2080 resulting in 2059 does not further increase the activity of the phosphodiester ODN. The sequence of 2080 with a phosphorothioate backbone (2116) is inactive. Additional CpG motifs are required for a potent phosphorothioate ODN (2006). Data are shown as means of four independent experiments using different donors. The values of 2080 (MFI) are set to 100% for each experiment. Error bars indicate the SEM. * \( p < 0.001 \) (comparison of the no addition sample to 2080; absolute values).

FIGURE 3. Up-regulation of CD40, CD54, and MHC-II expression on human primary B cells. PBMCs were incubated for 2 days with the CpG ODN 2080 and its control 2078 (added at 0, 4, and 24 h; 30 \( \mu \)g/ml at each time point). Expression of CD40, CD54, and MHC-II on CD19-positive B cells was examined by flow cytometry. Data are shown as means of three independent experiments with PBMCs from different donors. The values of 2080 (MFI) are set to 100% for each experiment. Error bars indicate the SEM. * \( p < 0.001 \) (comparison of the no addition sample to 2080; absolute values).
Purified B cells isolated from peripheral blood by immunomagnetic cell sorting were activated by CpG DNA to the same extent as unpurified B cells within PBMCs (data not shown). Thus, activation of B cells is a primary response and not a secondary effect caused by cytokines secreted by other cells.

Induction of CD40, CD54, and MHC class II (MHC-II) expression and IL-6 synthesis by CpG DNA

In addition to the costimulatory molecule CD86, the functional state of B cells is characterized by other surface markers. For example, activated T helper cells stimulate B cells by CD40 ligation, ICAM-1 (CD54) mediates binding to other immune cells, and MHC-II is responsible for Ag presentation. We found that B cell expression of CD40, CD54, and MHC-II was up-regulated by the CpG ODN 2080 (Fig. 3). The non-CpG control ODN 2078 (CpG inverted to GpC) showed no activity compared with medium alone.

IL-6 synthesis by human primary B cells in response to CpG DNA was examined by intracellular staining and flow cytometry. Within the first 6 h, 18.3% of B cells incubated in the presence of the CpG phosphorothioate ODN 2006 (4 μg/ml) accumulated IL-6 compared with 3.4% of cells incubated with medium only (data not shown).

Sequence-dependent induction of B cell proliferation by CpG DNA

When PBMCs were incubated for 5 days in the presence of 2080 (added at 0, 4, and 18 h and every subsequent morning), it was intriguing that a subpopulation of lymphocytes increased in cell size (forward light scatter (FSC)) and became more granular (side light scatter) (Fig. 4, upper panel). To examine whether this subpopulation represented proliferating B cells, we stained freshly isolated PBMCs with CFSE at day 0 and incubated them for 5 days with 2080 as above. CFSE is a fluorescent molecule that binds irreversibly to cell proteins. Each cell division decreases CFSE stain by 50% (35). Cells staining low with CFSE (proliferating cells) were found to be mainly CD19-positive B cells (Fig. 4, middle panel). The ODN 2080 induced 60–70% of CD19-positive B cells to proliferate within 5 days. The control ODN 2078 induced less than 5% of B cells to proliferate. Proliferating B cells (CFSE-low) showed a larger cell size (FSC) and higher granularity (Fig. 4, lower panel).

Proliferating B cells expressed higher levels of CD86 than non-proliferating cells (data not shown). In agreement with this finding,
the ODN panel tested above for induction of CD86 expression resulted in an almost identical pattern of B cell proliferation (Fig. 5). Replacement of the 3' thymidine reduced activity more than changing the thymidine in the middle position. Methylation of the CpG dinucleotides (2095) blocked activity, whereas methylation of an unrelated cytidine (2094) did not influence the capacity to induce B cell proliferation. Activity of E. coli DNA was lower than activity of 2080. LPS did not induce B cell proliferation.

B cell activation requires endosomal maturation/acidification of CpG DNA

It has been shown earlier that chloroquine, an inhibitor of endosomal acidification, blocks CpG-mediated stimulation of murine APCs and B cells, but does not influence LPS-mediated effects (8, 15, 36). We found that the addition of 5 μg/ml chloroquine completely blocked CpG DNA-mediated induction of CD86 expression on primary B cells (MFI CD86: 2006, 4.7 vs 1.4; E. coli DNA, 3.4 vs 1.4; medium only, 0.9; n = 4). Furthermore, chloroquine completely inhibited the induction of B cell proliferation by the phosphorothioate ODN 2006 measured with the CFSE proliferation assay as well as with the standard. These results suggest that, as with murine cells, activation of human B cells by CpG DNA requires the uptake of DNA in endosomes and subsequent endosomal acidification.

CpG DNA stimulates rapid and sustained NF-κB binding activity in human B cells

Because the CpG motif requirement for maximal B cell activation is substantially different between mice (GACGTT) and humans (TCGTCGT), we were interested in whether the basic intracellular signaling events are comparable. This information will help to extrapolate from mouse studies to responses of the human immune system. Rapid induction of NF-κB binding activity has been found earlier in murine B cells and macrophages (6, 15). To investigate the NF-κB response to CpG DNA in humans, human primary B cells were isolated from peripheral blood by immuno-magnetic cell sorting and incubated with the CpG ODN 2080, the non-CpG control ODN 2078, or medium. At the indicated time points, cells were harvested and nuclear extracts were prepared. In the presence of CpG ODN, NF-κB binding activity was increased within 1 h and maintained up to 18 h (latest time point examined) (Fig. 6). The non-CpG control ODN 2078 did not show enhanced NF-κB activity compared with cells incubated with medium only. The NF-κB band was identified by cold competition and shown to consist of p50 and p65 subunits by supershift assay (Fig. 6).

Rapid and sustained stimulation of DNA-binding activity of transcription factor AP-1

The AP-1 transcription factor is involved in the regulation of immediate early genes and cytokine expression (37). In murine B cells, AP-1-binding activity is induced in response to CpG DNA (13). To determine whether this transcription factor would also be induced by CpG DNA in humans, we examined AP-1 DNA-binding activity in human primary B cells. Cells were incubated with the CpG ODN 2080 or the control ODN 2078. At the indicated time points, nuclear extracts were prepared and the AP-1-binding activity was analyzed by EMSA. AP-1-binding activity was enhanced within 1 h (Fig. 7A) and increased up to 18 h (latest time point examined), showing a sustained response.
CpG DNA induces rapid phosphorylation of JNK, ATF-2, and p38, but not of ERK.

Because AP-1 activity is induced by many stimuli (38), we were interested in signal-transduction pathways upstream of AP-1. The AP-1 transcription-factor complex integrates different mitogen-activated protein kinase pathways (37). Western blots were performed using whole-cell extracts from primary B cells incubated with the CpG ODN 2080, the control 2078, or only medium. Specific Abs to phosphorylated (p) p38, ATF-2, JNK, and ERK were used. CpG DNA and anti-IgM show phosphorylation of different isoforms of JNK (arrows).

CpG DNA induces increased DNA-binding activity of transcription factor AP-1 and phosphorylation of p38, ATF-2, and JNK but not ERK. Primary B cells isolated from PBMCs by immunomagnetic beads were incubated in the presence of the CpG ODN 2080, its non-CpG control 2078, or anti-IgM (signaling through B cell receptor) as indicated. A. Equal amounts of nuclear extracts (1 μg protein/lane) were analyzed by EMSA (6% polyacrylamide minigel) for AP-1-binding activity. The experiment was repeated three times with similar results. B. Cells were harvested after 60 min and whole-cell extracts were loaded on 10% SDS-PAGE minigel (20 μg/lane) and analyzed by Western blot. Specific Abs to phosphorylated (p) p38, ATF-2, JNK, and ERK were used. CpG DNA and anti-IgM show phosphorylation of different isoforms of JNK (arrows).

**FIGURE 7.** CpG DNA induces increased DNA-binding activity of transcription factor AP-1 and phosphorylation of p38, ATF-2, and JNK but not ERK. Primary B cells isolated from PBMCs by immunomagnetic beads were incubated in the presence of the CpG ODN 2080, its non-CpG control 2078, or anti-IgM (signaling through B cell receptor) as indicated. A. Equal amounts of nuclear extracts (1 μg protein/ lane) were analyzed by EMSA (6% polyacrylamide minigel) for AP-1-binding activity. The experiment was repeated three times with similar results. B. Cells were harvested after 60 min and whole-cell extracts were loaded on 10% SDS-PAGE minigel (20 μg/lane) and analyzed by Western blot. Specific Abs to phosphorylated (p) p38, ATF-2, JNK, and ERK were used. CpG DNA and anti-IgM show phosphorylation of different isoforms of JNK (arrows).

**Discussion**

CpG DNA has developed into an exciting tool to activate and to steer immune responses toward Th1 in mice. However, most ODNs which activate mouse cells show only weak activation of human immune cells, and to date, an optimal human CpG motif has not been demonstrated. We now report the discovery of a potent human CpG motif and the characterization of its effects and mechanisms of action on human primary B cells. DNA containing this CpG motif strongly stimulated primary human B cells to proliferate, to produce IL-6, and to express increased levels of CD86, CD40, CD54, and MHC-II. It increased DNA-binding activity of the transcription factors NF-κB and AP-1, as well as phosphorylation of the stress-activated protein kinases JNK and p38 and the transcription factor ATF-2. B cell signaling pathways activated by CpG DNA were different from those activated by the B cell receptor, which activated ERK and a different isoform of JNK, but did not activate p38 and ATF-2. Blockade of endosomal maturation with chloroquine abolished these effects.

The use of phosphodiester ODNs facilitates the identification of an active CpG motif. Base exchanges within the most potent 8-mer CpG motif (5′-TCGTCGTTT-3′) diminished the activity of the ODN. The thymines at the 5′ and the 3′ positions of this motif were more critical than the thymidine at the middle position. An adenine or guanosine at the middle position only slightly decreased the activity but resulted in a CpG dinucleotide flanked by two 5′ purines and two 3′ pyrimidines, a 6-mer sequence which was previously identified to be the optimal murine CpG motif (2). The significant change compared with the mouse motif is the additional requirement of a 5′ TC adding a second CpG. Although the human CpG motif is still active in mice (data not shown), the murine 6-mer CpG motif alone is not sufficient to produce high activity in humans. This argues for a refinement of the CpG recognition mechanism in primates.

CpG effects on B cells were strictly CpG specific. Replacement of CpG dinucleotides with GpC dinucleotides as well as cytidine methylation of the CpG dinucleotides abolished B cell activation. The methylation of an unrelated cytidine did not change the activity of the CpG ODN. Due to degradation by nucleases, detection of immune stimulation by phosphodiester ODNs required that they be added several times at relatively high concentration (30 μg/ml). Even at these high concentrations, phosphodiester ODNs without CpG dinucleotides showed no background activity.

The development of CpG DNA as a practical drug requires nuclease resistance such as is conferred by the phosphorothioate backbone which protects DNA from rapid degradation in vivo (2). Of note, our studies demonstrate that one human CpG motif within a phosphodiester ODN (2080) is sufficient to produce the maximal effect and that additional CpG motifs (2059) did not further enhance the activity. Surprisingly, the phosphorothioate modification completely abolished the immunostimulatory activity of an ODN with only one human CpG motif (2116), and additional CpG motifs were required to regain activity (2006). Furthermore, phosphorothioate ODNs with the murine CpG motif highly stimulatory to murine immune cells showed only low activity in human B cells (our unpublished results). Thus, the use of the phosphorothioate backbone requires the optimal CpG motif, whereas phosphodiester ODNs still show some activity if the motif is slightly changed. This suggests that the specificity or affinity of the interaction between the CpG DNA and a putative CpG binding protein is reduced by the phosphorothioate backbone. This is supported by EMSA studies in which we have been unable to detect binding of phosphorothioate CpG ODNs to a putative CpG-binding protein (data not shown) under conditions where phosphodiester CpG DNA binds well.

The nuclease-resistant phosphorothioate backbone dramatically reduced the concentration of an ODN required for maximal activity (0.6 μg/ml). At this concentration, B cell stimulation was CpG-specific and little background stimulation by the phosphorothioate backbone was found. Others have reported that, in contrast to
phosphorothioate ODNs, both bacterial DNA and CpG phosphodiester ODNs (phosphodiester forms of active CpG phosphorothioate ODNs) failed to induce proliferation of human B cells even when added repeatedly and in concentrations up to 100 μM/ml (29). However, in that study proliferation was only measured by [3H]thymidine, which can give misleading results because degradation products of unmodified DNA inhibit incorporation of [3H]thymidine by proliferating cells (40). In the present study we used the CFSE proliferation assay which is not sensitive to this potential artifact and clearly demonstrated sequence-specific effects of both phosphodiester and phosphorothioate DNA on human B cells.

The molecular mechanism by which CpG DNA activates human B cells has been unclear. Murine B cells do not appear to have a CpG-specific membrane receptor because no difference in their binding of fluorescence-labeled CpG and non-CpG ODNs could be detected, and CpG ODNs linked to a solid support are nonstimulatory (2). In the present study chloroquine abolished CpG-mediated B cell activation, confirming that uptake of ODNs into the endosomal compartment and subsequent endosomal maturation is required in the human system. It has been hypothesized that a yet undefined CpG-binding protein downstream of endosomal maturation specifically recognizes and binds CpG motifs (13, 36). We hypothesize that upon binding to the putative binding protein, CpG DNA induces a rapid and sustained increase in NF-κB-binding activity in human primary B cells. NF-κB p50/p65 was found to be the major nuclear heterodimer in human primary B cells in response to CpG DNA. This is in agreement with earlier findings showing that inducible NF-κB in mature B cells consists mainly of the p50/Rel heterodimer, whereas p50 and p65 are the major components in immature B cells (41).

CpG DNA stimulated a rapid and sustained increase of AP-1-binding activity in primary human B cells. AP-1, a transcription-factor complex comprised of members of the Fos, Jun, and ATF families, is involved in the regulation of immediate early genes and the expression of cytokines. AP-1 integrates signals from several signal-transduction pathways (37). Different types of mitogen-activated protein kinases contribute to the activity of AP-1. Among those are the SAPKs and the ERKs. In the present study, the SAPKs JNK (42) and p38, another SAPK originally identified as a kinase activated by LPS (43), were rapidly phosphorylated in human primary B cells in response to CpG DNA. Analogous to murine B cells, CpG DNA induced different isoforms of JNK in human B cells (13). ATF-2 (44), a substrate of both p38 and JNK, was also phosphorylated in response to CpG DNA. Interestingly, the ERK was activated by ligation of the B cell receptor on human B cells but not in response to CpG DNA. Taken together, our data on CpG DNA-initiated signal transduction are consistent with those obtained in mice (8, 13).

In conclusion, the discovery and the characterization of a highly active human CpG motif allows an extrapolation from mouse disease models to human therapeutic settings using CpG DNA. The nuclease-resistant human CpG phosphorothioate ODN 2006 and related ODNs are candidates to be tested in clinical trials as adjuvants for vaccination strategies including those for cancer, allergy, and infectious diseases. The delineation of an optimal human CpG motif forms the basis for further studies on the identification of the putative CpG-binding protein and on how the recognition of CpG DNA by this protein is translated into downstream signaling pathways.

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References
