CpG DNA rescues B cells from apoptosis by activating NFκB and preventing mitochondrial membrane potential disruption via a chloroquine-sensitive pathway

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Abstract

Isolated murine splenic B cells gradually undergo spontaneous apoptosis while WEHI-231 B lymphoma cells undergo activation-induced apoptosis. Unmethylated CpG dinucleotides in a particular sequence context (CpG motif) in bacterial DNA or in synthetic oligodeoxynucleotides (CpG DNA) rescue both splenic B cells and WEHI-231 cells from apoptosis, an effect which could potentially contribute to autoimmune disease. Chloroquine has been used as an effective therapeutic agent for some autoimmune diseases, although the mechanism of action is not clearly understood. Low concentrations of chloroquine (<5 µM) selectively abolished CpG DNA-mediated protection against spontaneous apoptosis of splenic B cells and against anti-IgM-induced apoptosis of WEHI-231 cells without affecting anti-apoptotic activities of anti-CD40 or lipopolysaccharide. CpG DNA effectively prevented mitochondrial membrane potential disruption through a chloroquine-sensitive pathway in splenic B cells. Apoptosis protection by CpG DNA was also associated with increased expression of several proto-oncogenes and oncoproteins directly and/or indirectly through a rapid and sustained activation of NFκB in splenic B cells and WEHI-231 cells. These effects were also suppressed by chloroquine. Our results suggest that despite the difference in maturation phenotype of splenic B cells and WEHI-231 cells, CpG DNA rescues both from apoptosis by similar pathway, which is blocked at an early step by chloroquine.

Introduction

Cell death via apoptosis plays an important role in maintaining the size and composition of lymphocyte populations. Self-reactive B lymphocytes are eliminated by apoptosis in the bone marrow and the germinal centers of peripheral lymphoid organs (1–3). A breakdown of immune tolerance to self-antigen is thought to promote the development of autoimmune diseases such as systemic lupus erythematosus (SLE). WEHI-231 B lymphoma cells, which have some characteristics of immature B cells, undergo growth arrest and apoptosis in response to cross-linking of their antigen receptor by anti-IgM, while splenic mature B cells undergo spontaneous apoptosis and respond to antigen receptor cross-linking as a activation signal (4–9). These B cells are useful models for studying mechanisms of immune tolerance and the development of autoimmunity.

Unlike vertebrate DNA, bacterial DNA induces polyclonal B cell proliferation and Ig secretion (10). Recent studies have demonstrated that unmethylated CpG dinucleotides in particular sequence contexts (CpG motifs) in bacterial DNA are responsible for the bacterial DNA-mediated polyclonal B cell activation (11). In addition to their mitogenic effects on B cells, CpG motifs in bacterial DNA or synthetic oligo-
nucleotides (CpG DNA) induce secretion of pro-inflammatory cytokines such as tumor necrosis factor (TNF-α), IL-6, IL-10, and IL-12 from B cells and/or monocytes/macrophages (11–17). CpG DNA also synergizes with antigen receptor-mediated signals leading to TNF-α, IL-6 and Ig production, and B cell proliferation, indicating its function as a co-stimulatory factor in the presence of specific antigen (11,12, and A.-K. Yi and A. M. Krieg, unpublished data). CpG DNA activates NK cells to secrete IFN-γ, promotes cytotoxic activities of NK cells and enhances the efficacy of mAb therapy for lymphoma (15,16,18). CpG DNA protects mice from eosinophilic airways inflammation (asthma) and it is also a very effective vaccine adjuvant (19–24). While it has these very beneficial effects as a therapeutic agent, CpG DNA also can mediate some unwanted effects. In experimental models, CpG DNA contributes to septic shock-like syndrome (15,25). In addition, CpG DNA rescues mature splenic B cells from spontaneous apoptosis and WEHI-231 cells from antigen receptor-mediated apoptosis (9,26,27). The molecular mechanisms of these effects have been unclear, although we have recently demonstrated a possible role for reactive oxygen species (ROS) and NF-κB activation in mediating the anti-apoptotic effects of CpG DNA in WEHI-231 cells (27).

The antimalarial drug chloroquine is widely used in the treatment of SLE and rheumatoid arthritis. However, its mechanism of action is incompletely understood. We and others have recently reported that chloroquine inhibits certain of the immune stimulatory effects of CpG DNA, including the induction of cytokine expression and protection against activation-induced apoptosis in WEHI-231 cells (28,29). In this connection, we evaluated the mechanism through which chloroquine interferes with the anti-apoptotic effects of CpG DNA in WEHI-231 cells and primary splenic B cells. Here, we demonstrate that low concentrations of chloroquine (<5 µM) effectively and specifically inhibit the ability of CpG DNA to rescue WEHI-231 cells from activation-induced apoptosis and splenic mature B cells from spontaneous apoptosis. The protective effects of CpG DNA in these two systems are linked to the maintenance of the mitochondrial membrane potential (Ψm) and the nuclear translocation of NF-κB, and subsequent proto-oncogene and oncoprotein expression in these cells. Chloroquine specifically blocks all of these effects of CpG DNA, but does not block the effects of anti-CD40, anti-IgM or lipopolysaccharide (LPS). These chloroquine effects could potentially restore apoptotic susceptibility to self-reactive B cells protected by bacterial DNA.

Methods

Mice, cell lines and culture conditions

B6D2F1 specific pathogen-free mice at 8–18 weeks of age were obtained from the Jackson Laboratory (Bar Harbor, ME) and maintained under specific pathogen-free conditions in the University of Iowa animal care unit. Splenic B lymphocytes were obtained from B6D2F1 spleens by the BSA panning method as described previously (6). Cells obtained in this manner were 97% surface Ig+ B220+. Splenic B lymphocytes were either assayed directly (0 h) or placed in culture in RPMI 1640 (Gibco/BRL, Gaithersburg, MD) supplemented with 5% (v/v) heat-inactivated FCS (Sigma, St Louis, MO), 1 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM essential amino acids, 10 mM HEPEs, and 60 µM 2-mercaptopethanol. Murine B lymphoma WEHI-231 (clone 28) and CH31 cells were kindly provided by Dr David Scott (American Red Cross, Rockville, MD). WEHI-231 and CH31 cells were maintained in RPMI 1640 supplemented with 10% FCS, 1.5 mM L-glutamine, 50 µM 2-mercaptoethanol, 100 U/ml penicillin and 100 µg/ml streptomycin. All cells were cultured at 37°C in a 5% CO2 humidified incubator.

Oligodeoxynucleotides (ODN)

Nucleoside resistant phosphorothioate ODN (S-ODN) were purchased from Oligos Etc. (Wilsonville, OR). All ODN were purified by ethanol precipitation as described (12) using pyrogen-free solutions and had undetectable endotoxin by Limulus assay (QCL-1000; BioWhittaker, Walkersville, MD) following the manufacturer’s protocol. S-ODN 1826 (TCCAT-GAGCTTCGAGCCTT) was used as a CpG DNA, and S-ODN 1745 (TCCATGAGCTTCGAGCTT) was used as a control non-CpG DNA. Note 1 µM of CpG or non-CpG DNA equals 6 µg/ml.

Reagents

Goat polyclonal anti-mouse IgM (µ chain specific) was purchased from Sigma, and used at 1–10 µg/ml to induce growth inhibition and apoptosis in WEHI-231 cells. Anti-murine CD40 antibody was purchased from PharMingen (San Diego, CA) and used at 1–2 µg/ml. LPS and chloroquine were purchased from Sigma.

Cell proliferation assay

Mouse splenic B cells (5×10⁴ cells/100 µl/well) or WEHI-231 cells (2×10⁶ cells/100 µl/well) were treated with medium or chloroquine (0.5–2.5 µg/ml) for 1 h. Then cells were cultured in medium alone, anti-CD40 (1–2 µg/ml), LPS (10 µg/ml), CpG DNA (0.1–0.5 µM) or non-CpG DNA (0.1–0.5 µM) in the presence or absence of anti-IgM (2 µg/ml). Cells were incubated at 37°C for 24 h with a last 4 h pulse with 1 Ci/ml of [3H]thymidine. Cells were harvested using semi-automatic cell harvester (Skatron, Sterling, VA) and radioactivity was counted by scintillation counter (Packard, Meriden, CT).

Propidium iodide (PI) staining for hypodiploid nuclei and FACS analysis

For the induction of apoptosis, WEHI-231 B lymphoma cells (5×10⁶ cells/ml) were treated with medium or chloroquine (2.5 µg/ml) for 1 h. Then cells were stimulated with medium, anti-CD40 (1 µg/ml), CpG DNA (0.5 µM) or non-CpG DNA (0.5 µM) in the presence or absence of anti-IgM (1 µg/ml) for 48 h. The presence of apoptotic nuclei was analyzed after PI staining as previously described (26). The cells were analyzed by FACSScan (linear scale; Becton Dickinson, San Jose, CA). Apoptotic cells were identified by having less than diploid DNA content (hypodiploid nuclei). All FACSScan data analysis was performed using Lysys II software (Becton Dickinson).
Simultaneous analysis of cell cycle and apoptosis by acridine orange (AO)

Because the fluorescent dye AO (Polysciences, Warrington, PA) can stain double-stranded DNA and single-stranded RNA meta-chromatically, the percent of cells which are in G₀, G₁, S/G₂/M and apoptosis can be determined (30). Cell cycle and apoptosis were analyzed simultaneously by AO staining as previously described (8). Briefly, splenic B cells (1.15 × 10⁶/ml) were stimulated with medium, CpG DNA (0.05–0.1 µM), non-CpG DNA (0.05–0.1 µM), LPS (1 µg/ml) or anti-CD40 (1 µg/ml) in the presence or absence of various concentrations (0–1 µg/ml) of chloroquine. After 16 h incubation, cells were permeabilized in a buffer containing 0.1% sodium citrate, 0.02 M sodium phosphate, 0.1% Triton X-100, 0.2 M sucrose and 0.1 mM disodium EDTA at pH 3.5. After 1 min cells were stained by the addition of an equal amount of buffer containing 20 µg/ml of AO, 0.01 M sodium phosphate and 0.1 M NaCl at pH 3.5. Cells were analyzed on an Epics 753 Flow Cytometer (Coulter, Hialeah, FL) using a 488 nm argon laser excitation band, a 525 nm band-pass filter for DNA and 635 nm band-pass filter for RNA. Boundaries between G₀ and apoptosis and between G₀ and G₁ were determined on DNA versus RNA histograms of fresh cells (T₀) and cells cultured for 16 h in medium alone containing a substantial population of apoptotic cells. Hypodiploid cells (apoptotic cells) have less DNA fluorescence than G₀ cells. Events with <25% of the DNA fluorescence of G₀ cells were considered as debris and were excluded.

Annexin V staining for plasma membrane transition and 3,3′-dihexyloxacarbocyanine iodide [DiOC₆(3)] staining for mitochondrial membrane potential

Splenic B cells (1.15×10⁶ cells/ml) were stimulated with medium, CpG DNA (0.05 µM), non-CpG DNA (0.05 µM), LPS (2 µg/ml) or anti-CD40 (1 µg/ml) in the presence or absence of chloroquine (1 µg/ml for splenic B cells) for 4–40 h. Cells were washed twice with PBS, and then aliquoted for Annexin V staining and DiOC₆(3) staining. For Annexin V staining, cells were resuspended in 195 µl of Annexin V binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl and 2.5 mM CaCl₂), incubated at room temperature for 15 min with Annexin V–FITC (5 µl; Biosource, Camarillo, CA) and then resuspended further in 0.5 ml binding buffer prior to flow cytometric analysis for fluorescence at 525 nm. For DiOC₆(3) staining, cells were resuspended in 20 mM DiOC₆(3) (Molecular Probes, Eugene, OR) in PBS, incubated at 37°C for 20 min and then analyzed by flow cytometry for fluorescence at 525 nm. Decreased fluorescence indicates the loss of mitochondrial membrane potential.

Preparation of RNA and RNase protection assay (RPA)

WEHI-231 cells (2×10⁵/mкл) were pre-treated with medium or chloroquine (2.5 µg/ml) for 30 min. Cells were then treated with medium, CpG or non-CpG DNA (0.5 µM), or anti-CD40 (1 µg/ml) in the presence or absence of anti-IgM (10 µg/ml). Cells were harvested 9 h after DNA treatments and total RNA was isolated by using RNAzol B (Tel-Test, Friendswood, TX) following the manufacturer’s protocol. The c-myc, c-fos, c-myb, myn, egr-1, c-jun, c-Ki-ras, fas ligand, fas, bcl-xL, bcl-2, bax and L32 mRNA were detected using the RPA as previously described (31). Equivalent amounts of RNA were examined, as judged by the amount of L32, which encodes a ubiquitously expressed ribosome subunit protein (32), in each sample. The GenBank accession numbers and nucleotide sequences for those genes were previously reported (26).

Western blot analysis

Splenic B cells (1.15×10⁶ cells/ml) were stimulated for 6 h with medium, CpG DNA (0.05 µM), non-CpG DNA (0.05 µM) or anti-CD40 (1 µg/ml) in the presence or absence of chloroquine (1 µg/ml) and washed 3 times with ice-cold PBS. Whole-cell lysates were prepared as previously described (26). Equal concentrations of cell lysates (25 µg/lane) were boiled in SDS sample buffer 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% non-fat dry milk and murine c-Myc, Bcl-2, Bcl-xL/S or Bax protein was detected with specific antibodies. Antibodies against murine c-Myc and Bcl-xL/S were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and antibodies against Bcl-2 and Bax were purchased from PharMingen (San Diego, CA). Blots were developed in ECL reagent (Amersham, Arlington Heights, IL) according to the manufacturer’s recommended procedure.

Electrophoretic mobility shift assay (EMS)

WEHI-231 cells (2×10³/ml) or splenic B cells (1.15×10⁶/ml) were stimulated for 4 h (for splenic B cells) or 8 h (for WEHI-231 cells) with CpG DNA (0.05–0.5 µM), non-CpG DNA (0.05–0.5 µM), anti-CD40 (1 µg/ml) and/or anti-IgM (10 µg/ml) in the presence or absence of chloroquine (1–2.5 µg/ml). Cells were harvested and nuclear extracts were prepared as previously described (27). EMSA was done as described previously (27) using ³²P-labeled oligonucleotides containing the κB sequence from κ-intronic enhancer (5′-GTAGGGGACTT-TCCGAGTCGAGATCTTGG-3′) (33) as probe. [α-³²P]dATP was purchased from Amersham. Specificity of the NFκB bands was confirmed by competition studies with cold oligonucleotides with NFκB or other unrelated transcription factor binding sites and super-shift assay using specific antibodies for p50, p52, Rel A, Rel B and c-Rel (Santa Cruz Biotechnology) (data not shown).

Results

Chloroquine selectively inhibits CpG DNA rescue of WEHI-231 cells from anti-IgM-induced growth arrest and apoptosis

Anti-IgM-treated WEHI-231 cells were stimulated with CpG DNA in the presence or absence of low doses (~5 µM) of chloroquine, shown to be effective in our preliminary studies. As expected (26), cross-linking of the B cell receptor on WEHI-231 cells induced growth arrest (Fig. 1). Both CpG DNA and anti-CD40 protected WEHI-231 cells from anti-IgM-induced growth arrest but control non-CpG DNA did not (data not shown). Addition of chloroquine abolished CpG DNA-mediated protection against anti-IgM-induced growth arrest.
**CpG DNA protection of apoptosis and chloroquine**

**Table 1.** Chloroquine inhibits anti-apoptotic effects of CpG DNA in WEHI-231 cells.

<table>
<thead>
<tr>
<th></th>
<th>Medium</th>
<th>Anti-IgM</th>
<th>Anti-IgM + CpG</th>
<th>Anti-IgM + non-CpG</th>
<th>Anti-IgM + anti-CD40</th>
</tr>
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<tbody>
<tr>
<td><strong>Medium</strong></td>
<td>2.4</td>
<td>48.8</td>
<td>9.6</td>
<td>44.5</td>
<td>11.4</td>
</tr>
<tr>
<td><strong>Chloroquine</strong></td>
<td>2.7</td>
<td>50.3</td>
<td>56.1</td>
<td>ND</td>
<td>12.7</td>
</tr>
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WEHI-231 B cells (5 × 10^5 cells/ml) were pretreated for 30 min with chloroquine (2.5 µg/ml) or with regular medium. Cells were treated with medium, anti-IgM (10 µg/ml), or the combination of anti-IgM and CpG DNA 1826 (0.5 µM), non-CpG DNA 1911 (0.5 µM) or anti-CD40 (1 µg/ml) for 40 h. The presence of hypodiploid nuclei as an indication of apoptotic cell was analyzed by PI staining and flow cytometry as described (26). The percentage apoptotic cells is indicated as a number in the table. ND indicates not done. The experiment was repeated >3 times with WEHI-231 and CH31 cells with similar results.

**Fig. 1.** Chloroquine selectively blocks CpG DNA-mediated rescue from anti-IgM-induced growth arrest of WEHI-231 cells. WEHI-231 cells (1 × 10^6 cells/ml) were pretreated for 30 min with medium (empty bar) or chloroquine (2.5 µg/ml; solid bar). Cells were treated with medium, anti-IgM (2 µg/ml), or the combination of anti-IgM and CpG DNA 1826 (0.5 µM), non-CpG DNA 1911 (0.5 µM) or anti-CD40 (2 µg/ml) for 48 h with a final 4 h pulse with 1 µCi/well [3H] thymidine. Data represent the mean ± SD of triplicate determinations. The experiment was repeated at least 4 times with similar results.

**Fig. 2.** Chloroquine inhibits anti-apoptotic effects of CpG DNA in splenic B cells. Splenic B cells (1.15 × 10^6 cells/ml) were stimulated with medium, LPS (10 µg/ml), CpG DNA 1826 (0.05 µM), non-CpG DNA 1911 (0.05 µM) or anti-CD40 (XCD40; 1 µg/ml) for 16 h in the presence or absence of various concentrations of chloroquine (Chlor: 0–1 µg/ml). Cells were stained with AO, and then analyzed for apoptosis (A) and cell cycle entry (B) by flow cytometry. Fresh indicates freshly isolated splenic B cells at 0 h. Data points represent three to six experiments.
CpG DNA protection of apoptosis and chloroquine

Fig. 3. CpG DNA prevents plasma membrane transition and mitochondrial membrane potential disruption in splenic B cells via a chloroquine sensitive pathway. Splenic B cells (1.15x10^6 cells/ml) were treated with medium, LPS (2 µg/ml), CpG DNA 1826 (0.05 µM), non-CpG DNA 1911 (0.05 µM) or anti-CD40 (1 µg/ml) for 4 (open symbols) or 40 (black symbols) h in the presence or absence of chloroquine (1 µg/ml). Cells were analyzed for plasma membrane transition (percent Annexin V high; △) or mitochondrial membrane potential disruption (percent ψm low; ▽) by flow cytometry. Using forward scatter with either Annexin V or DiOC6(3), discrete high and low populations were easily distinguished. Data represents the mean with SE from five to six experiments.

Fig. 4. Effects of chloroquine on CpG DNA-induced oncoprotein production in mature spleen mature B cells. Splenic B cells (1.15x10^6 cells/ml) were treated with medium, CpG DNA (1826; 0.05 µM) or anti-CD40 (1 µg/ml) in the presence or absence of chloroquine (1 µg/ml) for 16 h. Cells were harvested and whole cell lysates (25 µg/ml) were analyzed by Western blots as described (26). The experiment was repeated 3 times with similar results.

Effects of chloroquine on CpG DNA-induced oncoprotein production

We evaluated whether chloroquine, at the concentrations that block CpG DNA-mediated B cell apoptosis protection, selectively inhibits CpG DNA-induced oncoprotein production. Freshly isolated splenic B cells were treated with medium, CpG DNA, control non-CpG DNA or anti-CD40 for 16 h in the presence or absence of chloroquine. As shown in Fig. 4, c-Myc and Bcl-2 were constitutively expressed in freshly isolated splenic B cells (T0 in Fig. 4). The levels of c-Myc protein gradually decreased after onset of the culture, but the levels of Bcl-2 protein were maintained throughout the culture period (T16 in Fig. 4). The expression of c-Myc protein increased and maintained for at least 16 h in the presence of CpG DNA or anti-CD40 in splenic B cells. Anti-CD40 induced expression of Bcl-xL and c-Jun proteins within 6 h, and these increased protein levels were maintained over 16 h (Fig. 4 and data not shown). CpG DNA induced expression of Bcl-xL and c-Jun proteins within 6 h, and these increased protein levels were easily distinguished. Data represents the mean with SE from five to six experiments.

Chloroquine inhibits CpG DNA-induced expression of proto-oncogenes

To determine whether the effects of chloroquine on the CpG DNA-induced oncoprotein production occurs at the RNA level,
the effects of chloroquine on the CpG DNA-mediated proto-oncogene mRNA expression were evaluated. As shown in Fig. 5, the mRNA expression for c-myc, myb, bcl-2 and bax was suppressed in WEHI-231 cells within 9 h of B cell receptor cross-linking (26). Addition of CpG DNA or anti-CD40 prevented anti-IgM-mediated down-regulation of these genes in WEHI-231 cells (Fig. 5A and B). CpG DNA-mediated preservation of these proto-oncogene mRNA levels was abolished by chloroquine, but anti-CD40-mediated expression of the same proto-oncogenes was not, illustrating the selective effect of this drug on the CpG DNA-mediated signaling pathway. Chloroquine alone, at the concentrations used in the experiment, did not affect these mRNAs in WEHI-231 cells (Fig. 5A and B).

**Chloroquine blocks CpG DNA-induced NFκB activation in WEHI-231 cells and primary splenic B cells**

The transcription factor NFκB has an important role in regulating several proto-oncogenes including c-myc. Because CpG DNA rapidly induces nuclear translocation of NFκB in WEHI-231 cells (27), we addressed the unanswered questions of whether CpG DNA can also induce nuclear translocation of NFκB in splenic B cells, and whether chloroquine has any effect on the CpG DNA-induced NFκB activation in either WEHI-231 cells or primary splenic B cells. As previously demonstrated (27,35), within 8 h after stimulation with anti-IgM, the levels of NFκB p50/c-Rel heterodimer declined while the levels of p50/p50 homodimer, which acts as a repressor, increased in WEHI-231 cells (Fig. 6A). Addition of CpG DNA or anti-CD40 maintained the levels of p50/c-Rel heterodimer in the presence of anti-IgM (Fig. 6A). Pre-treatment with chloroquine selectively inhibited CpG DNA-mediated maintenance of p50/c-Rel heterodimer formation without affecting anti-IgM-induced p50/p50 homodimer formation or anti-CD40-mediated NFκB activation in WEHI-231 cells (Fig. 6A).

A high level of baseline activation of the transcription factor NFκB was present in freshly prepared (T0) splenic B cells (Fig. 6B) (36-38). The levels of nuclear NFκB in splenic B cells gradually declined after initiation of culture (T4 in Fig. 6B and data not shown). CpG DNA induced further activation of NFκB in splenic B cells which was rapid (within 15 min after stimulation) and sustained (Fig. 6B and data not shown). Control non-CpG DNA failed to induce NFκB activation in splenic B cells (Fig. 6B). Like CpG DNA, anti-CD40 also induced further nuclear translocation of NFκB in primary splenic B cells (Fig. 6B). Addition of chloroquine completely abolished CpG DNA-mediated NFκB activation in splenic B cells. In contrast, neither the basal NFκB activation nor the anti-CD40-induced activation of NFκB in splenic B cells were affected by the same concentration of chloroquine (Fig. 6B).

**Discussion**

Recent studies have demonstrated that CpG motifs in bacterial DNA induce polyclonal B cell activation, and block apoptosis
in splenic B cells and the B cell line WEHI-231 (9,26,27). Since apoptosis is thought to be important in maintaining immune tolerance to self-antigens, these findings are compatible with the hypothesis that bacterial infection may potentiate autoimmune diseases such as SLE (39). Chloroquine has been used empirically as a therapeutic agent for autoimmune diseases such as lupus and rheumatoid arthritis without knowing its exact mechanisms of action. In the present study, we showed that chloroquine inhibits several of the B cell-activating effects of CpG DNA and examined candidate molecules likely to mediate CpG DNA action for sensitivity to chloroquine.

As previously reported, CpG DNA induced cell cycle entry and protected splenic B cells from spontaneous apoptosis (Fig. 2A and B) (9). It also rescued WEHI-231 cells from anti-IgM-induced growth arrest and apoptosis (Figs 1 and Table 1) (26,27). Very low concentrations of chloroquine (<5 μM) effectively abolished these CpG DNA-mediated effects in both splenic B cells and WEHI-231 cells (Figs 1 and 2, and Table 1). These results suggest that CpG DNA may induce growth in splenic B cells and WEHI-231 cells, and rescue them from apoptosis through pathways sharing a chloroquine-inhibitable step. The inhibitory effects of chloroquine at low concentrations were very selective for CpG DNA-mediated protection, failing to inhibit LPS- or CD40-mediated cell proliferation and apoptosis protection (Figs 1 and 2, Table 1, and data not shown). However, higher concentrations (>20 μM) of chloroquine not only inhibited the mitogenic and anti-apoptotic abilities of LPS or CD40 in splenic B cells and WEHI-231 cells, but also induced cell death (data not shown). A recent study showed that chloroquine and several of its chemical analogues, including some used as anti-malarial drugs, inhibited CpG DNA-mediated rescue of WEHI-231 cells from anti-IgM-induced apoptosis (29). However, the molecular mechanism by which these drugs inhibit CpG DNA-mediated protection against anti-IgM-induced apoptosis of WEHI-231 cells was not studied.

Reduction of the ψm, which involves the opening of permeability transition pores in the mitochondrial membrane, is thought to be a point-of-no-return in the apoptosis cascade (34). Whether this ψm loss takes place during the spontaneous apoptosis of splenic B cells had not been examined previously. Not only was ψm reduced during spontaneous apoptosis of splenic B cells, but all the anti-apoptotic agents we tested, including CpG DNA, LPS, and anti-CD40, effectively blocked loss of ψm in splenic B cells (Fig. 3 and data not shown). However, chloroquine only inhibited the mitochondrial protection provided by CpG DNA, and not that provided by LPS or anti-CD40 (Fig. 3). These results indicate that CpG DNA protects splenic B cells from apoptosis via a chloroquine-sensitive step preceding ψm reduction.

Previously, we have identified several proto-oncogenes and oncoproteins up-regulated by CpG DNA, which are potentially related to CpG DNA-mediated cell cycle entry and protection against spontaneous apoptosis in primary splenic B cells, and protection against anti-IgM-induced growth arrest and apoptosis of WEHI-231 cells (9,26). Our recent studies have also shown that chloroquine at concentrations <5 μM effectively inhibits CpG DNA-induced cytokine gene expression and protein production in murine B cell and monocytic cell lines (28). These results suggested that chloroquine might abolish CpG DNA-mediated cell growth and apoptosis protection by suppressing the CpG DNA-induced expression of this same set of proto-oncogenes in splenic B cells or WEHI-231 cells. Indeed, low concentrations of chloroquine selectively inhibited CpG DNA-induced expression of c-Myc, c-Jun, Bcl-xL and Bax mRNA and protein in both splenic B cells and WEHI-231 cells (Figs 4 and 5, and data not shown). However, even though other anti-apoptotic agents, such as anti-CD40 and LPS, closely resembled CpG DNA in their effects on oncogene and oncoprotein expression, low concentrations of chloroquine failed to block proto-oncogene expression or oncoprotein production induced by them in the same cells, indicating the specific action of this drug on the CpG DNA-mediated effects (Figs 4 and 5, and data not shown). These results indicate that CpG DNA induces expression of proto-oncogenes and oncoproteins, directly and/or indirectly involved in cell growth and/or survival, through a chloroquine-
sensitive pathway, which at some proximal point is distinct from the anti-CD40- or LPS-mediated signaling pathways.

CpG DNA rapidly induces degradation of IkBα and IkBβ and subsequent translocation of NFκB in B cell lines and macrophage/monocyte-like cell lines (27,28,40,41). Sustained NFκB activation in WEHI-231 cells has been proposed to play a key role in the CpG DNA-mediated protection against anti-IgM-induced growth arrest and apoptosis (27), but a similar role for NFκB in the CpG DNA-mediated effects on primary splenic B cells had not been investigated previously. We found NFκB to be highly activated in the freshly isolated primary splenic B cells, but DNA binding activity of NFκB gradually decreased after onset of culture (Fig. 6B and data not shown). These changes in the NFκB DNA binding activity in splenic B cells during in vitro culture correspond to the changes in the level of c-Myc mRNA and protein which might play an important role in splenic B cell growth and survival (Fig. 4 and data not shown) (9). In addition, recent studies have demonstrated that NFκB regulates the expression of the proto-oncogene c-myc, and that sustained NFκB activation and subsequent c-myc expression prevent WEHI-231 cells from undergoing apoptosis (27,37,42). Interestingly, all anti-apoptotic agents we tested including CpG DNA rapidly induced sustained NFκB activation and c-Myc expression in primary splenic B cells (Figs 4 and 6, and data not shown), suggesting the importance of sustained NFκB activation and c-Myc expression in the survival as well as the growth of splenic B cells. Indeed, blocking of NFκB activation by the inhibitors SN50 and gliotoxin abolished anti-apoptotic effects of CpG DNA or other anti-apoptotic agents in splenic B cells (Yi et al., manuscript in preparation). Moreover, chloroquine, which selectively inhibits CpG DNA-mediated apoptosis protection, blocked only CpG DNA-mediated NFκB activation, but failed to block LPS- or anti-CD40-induced NFκB activation, in both splenic B cells and WEHI-231 cells (Figs 6A and 6B).

Our results support the recent findings of other investigators that NFκB activation is a key factor for apoptosis protection in B and monocytic cells (27,37,42,43).

The earliest steps in the CpG DNA-mediated signaling pathway we have identified so far are intracellular ROS generation, NFκB and AP-1 activation, and c-Jun-N-terminal kinase and p38 kinase activation (12,28,40,44). Chloroquine inhibits activation of all of these CpG DNA-mediated intracellular signaling pathways in B lymphoma and monocytic cell lines (28,44), indicating that chloroquine acts at an even more proximal step in this CpG DNA-mediated signaling pathway, but its mechanism remains unclear. Because of its beneficial effects on treatments of rheumatoid arthritis and SLE, numerous efforts have been made to explore potential mechanisms of action of chloroquine (reviewed in 45). Since immune activation by CpG DNA requires cellular uptake of the DNA (11), it is possible that chloroquine might block the DNA uptake by the cell. However, we could not detect any inhibition of cellular uptake of DNA by chloroquine using flow cytometry analysis (data not shown). Chloroquine could intercalate into DNA (46) and block the function of CpG DNA. However, this also seems not to explain the inhibition of CpG DNA action based on a recent report by other investigators (29). Chloroquine also has been reported (47–49) to inhibit phospholipase A1 and phospholipase A2, but these effects require much higher doses of chloroquine (>1 mM) than those we used to inhibit CpG DNA function in this study (=5 µM). Chloroquine can modulate intracellular Ca2+ flux (50), but we failed to detect any increases in the intracellular Ca2+ level within 10 min after stimulation with CpG DNA in WEHI-231 or J774 cells (Krieg, et al., unpublished data). Chloroquine reportedly exerts an antioxidant effect and can influence the generation of ROS in leukocytes (51). CpG DNA induces intracellular generation of ROS, a critical mediator in CpG DNA-mediated signaling pathway (12,28). In theory, such an antioxidant effect of chloroquine could explain the inhibitory effects of chloroquine on CpG DNA-mediated immune activation. However, the concentrations of chloroquine that influence the ROS generation in leukocytes (20–100 µM) are markedly higher than those used in this study (=5 µM). Moreover, at this low concentration, chloroquine selectively inhibits CpG DNA-induced intracellular generation of ROS, but not that induced by other anti- or pro-apoptotic agents including LPS, anti-CD40, and anti-IgM (28). This study indicates that the inhibitory effects of chloroquine on CpG DNA-mediated apoptosis protection and other immune activation are not due to its antioxidant effect but may be due to inhibition of more proximal step(s) in the CpG DNA-mediated signaling pathway. Chloroquine increases pH within intracellular vacuoles, including endosomes, lysosomes and Golgi apparatus, and alters processes such as protein degradation, assembly of macromolecules and post-translational modification of proteins in those organelles (reviewed in 45). Indeed, high concentrations (100–250 µM) of chloroquine inhibit LPS-induced production of TNF-α by altering intracellular processing of TNF-α without affecting the LPS-mediated NFκB activation or mRNA expression (51). As shown in this and our previous studies, the inhibitory effects of low concentrations of chloroquine on CpG DNA-mediated apoptosis protection and other immune activation events take place at more proximal steps in the signaling pathway than intracellular processing of protein (28,44). Our recent data suggests that CpG DNA is taken up by cells via endocytosis (52), and that endosomal acidification of DNA is required for CpG DNA-mediated signaling (28). Therefore, it seems more probable that chloroquine blocks CpG DNA-mediated signaling by raising endosomal pH, resulting in inhibition of endosomal processing of DNA or of release of DNA from the endosome. CpG DNA does not appear to have any sequence-specific membrane receptor. However, there are sequence-specific CpG DNA receptor(s) (CpGBP) in both cytols and nuclei of B cells and monocytic cells (Tuetken et al., unpublished data). Chloroquine did not inhibit the binding activity of CpGBP to CpG DNA in vitro (data not shown). However, the impact of chloroquine on the binding of CpG DNA to CpGBP in vivo and downstream signaling cascades remain to be explored. Regardless of its exact mechanism of action, it is important to point out that all of the previously reported effects of chloroquine on the modulation of the immune system required higher drug levels (20–100 µM) than those found when chloroquine is used to treat arthritis or SLE patients (whole blood levels ~5 µM) (reviewed in 45). In contrast, concentrations of chloroquine which inhibit CpG DNA-mediated apoptosis protection and other immune activation including cytokine production in vitro...
were <5 μM (28,29,44). These observations suggest the interesting possibility that CpG motifs in bacterial DNA may contribute to the progression of arthritis and/or SLE, and that the remittive effects of chloroquine on arthritis and SLE may be at least partially due to its inhibitory action on the pathogenic effects of CpG motifs in bacterial DNA.

In summary, the present study demonstrates that low concentrations of chloroquine selectively abolish CpG DNA-mediated anti-apoptotic effects, including plasma membrane transition and 4μm loss, in WEHI-231 cells and primary splenic B cells, and cell cycle advancement in splenic B cells by inhibiting the CpG DNA-induced activation of NFκB and the subsequent expression of proto-oncogenes and oncoproteins involved in cell growth and apoptosis protection.

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Abbreviations
AO acridine orange
DiOC6(3) 3′,3′-dihexyloxacarbocyanine iodide
EMSA electrophoretic mobility shift assay
ODN oligodeoxynucleotide
PI propidium iodide
ROS reactive oxygen species
RPA RNase protection assay
SLE systemic lupus erythematosus
TNF tumor necrosis factor

References