CpG Motifs in Bacterial DNA Activate Leukocytes Through the pH-Dependent Generation of Reactive Oxygen Species

Ae-Kyung Yi, Rebecca Tuetken, Thomas Redford, Marianella Waldschmidt, Jeffrey Kirsch and Arthur M. Krieg

*J Immunol* 1998; 160:4755-4761;
http://www.jimmunol.org/content/160/10/4755
CpG Motifs in Bacterial DNA Activate Leukocytes Through the pH-Dependent Generation of Reactive Oxygen Species

Ae-Kyung Yi,* Rebecca Tuetken,* Thomas Redford,† Marianella Waldschmidt,* Jeffrey Kirsch,* and Arthur M. Krieg²*‡

B cells and monocytes endocytose DNA into an acidified intracellular compartment. If this DNA contains unmethylated CpG dinucleotides in particular base contexts (CpG motifs), these leukocytes are rapidly activated. We now show that both B cell and monocyte-like cell line responses to DNA containing CpG motifs (CpG DNA) are sensitive to endosomal acidification inhibitors; they are completely blocked by bafilomycin A, chloroquine, and monensin. The specificity of these inhibitors is demonstrated by their failure to prevent responses to LPS, PMA, or ligation of CD40 or IgM. Acidification of endosomal CpG DNA is coupled to the rapid generation of intracellular reactive oxygen species. The CpG DNA-induced reactive oxygen species burst is linked to the degradation of IκB and the activation of NFκB, which induces leukocyte gene transcription and cytokine secretion. These studies demonstrate a novel pathway of leukocyte activation triggered by CpG motifs.

*Interdisciplinary Graduate Program in Immunology and Department of Internal Medicine, University of Iowa College of Medicine, and †University of Iowa College of Pharmacy, Iowa City, IA 52242; and ‡Department of Veteran Affairs Medical Center, Iowa City, IA 52246

Received for publication November 18, 1997. Accepted for publication January 16, 1998.

Bacterial and vertebrate DNAs differ in the frequency and methylation of CpG dinucleotides (1). CpG dinucleotides are underrepresented (CpG suppression) and selectively methylated on the 5' position of the cytosine in vertebrate DNA, but are present at the expected frequency and are unmethylated in bacterial DNA (bDNA)³. (1) This structural difference provides a possible molecular basis for the specific recognition of bDNA by leukocytes. Indeed, bDNA directly activates B cells to proliferate, become apoptosis resistant, and secrete Ig in a T cell-independent manner (2–4).³ The bDNA also induces B cells and monocytes to translocate NFκB to the nucleus and to secrete cytokines, including IL-12, TNF-α, and IFN-α/β, and activates NK cells (5–10) (A.-K. Yi and A. M. Krieg, unpublished observations). Vertebrate DNA lacks these immune effects. We have recently shown that leukocyte activation by bDNA depends on the presence of unmethylated CpG dinucleotides in particular base contexts (CpG motifs) and can be mimicked with synthetic oligodeoxynucleotides (ODN) (11–13). B cells and monocytes/macrophages treated with DNA containing CpG motifs (CpG DNA) have increased expression of c-myc, mym, egr-1, c-jun, bax, bcl-xL, TNF-α, and IL-6 mRNA and/or protein within 15 to 30 min in vivo and in vitro (4, 13, 14) (A.-K. Yi and A. M. Krieg, unpublished observations).³ These data suggest that CpG motifs act as a “danger signal” to the immune system (15).

Cellular uptake of DNA appears to occur via adsorptive endocytosis into an acidified chloroquine-sensitive intracellular compartment (16, 17). Leukocyte activation by CpG DNA is not mediated through binding to a cell surface receptor, but requires cell uptake (11). This suggested that leukocyte activation by CpG DNA may occur in association with acidified endosomes and might even be pH dependent. Here we demonstrate that endosomal acidification of DNA is required for the CpG DNA-mediated leukocyte activation and is coupled to the rapid generation of reactive oxygen species (ROS), which leads to NFκB activation and subsequent proto-oncogene and cytokine expression.

Materials and Methods

**Mice and cell culture**

Spleen cells were prepared from DBA/2 mice (5–10 wk old; The Jackson Laboratory, Bar Harbor, ME) as described previously (13). Murine B lymphoma WEHI-231 cells and murine monocyte J774 cells were purchased from American Culture Collection (Rockville, MD). All cells were cultured at 37°C in a 5% CO₂ humidified incubator and maintained in RPMI 1640 supplemented with 10% FCS, 1.5 mM l-glutamine, 50 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin.

**ODN and DNA**

*Escherichia coli* (EC) DNA (strain B) and calf thymus (CT) DNA were purchased from Sigma (St. Louis, MO). Nuclease-resistant phosphorothioate ODN (S-ODN) were obtained from Oligos Etc. (Wilsonville, OR). Phosphodiester ODN were purchased from Operon Technologies (Alamed, CA). All DNA and ODN were purified as previously described (13) using pyrogen-free solutions and had undetectable endotoxin by *Limulus* assay. S-ODN conjugated to FITC on the 5' end were prepared as previously described (18).

**Cytokine assays**

Murine spleen cells, WEHI-231 cells, or J774 cells were cultured with or without the indicated inhibitor for the indicated times and then stimulated with the CpG S-ODN 1826 (TCCATGACGGTCCGTAGCTT). *E. coli* DNA, LPS (10 μg/ml), PMA (100 ng/ml) plus ionomycin (1 nM), anti-IgM

Copyright © 1998 by The American Association of Immunologists
0022-1767/98/$02.00
(10 μg/ml), or anti-CD40 (1 μg/ml) for 4 to 24 h. The levels of cytokines in the culture supernatants were analyzed by ELISA for IL-6, IL-12, or TNF-α as previously described (13). Inhibitors used for this study were chloroquine, monensin, bafilomycin A, bis-gliotoxin, gliotoxin, N-acetyl-

\[ \text{L-cysteine (NAC), pyrrolidine dithiocarbamate (PDTC), and } \]

\[ \text{N'-tosyl-L-phenylalanine chloromethyl ketone (TPCK). Anti-mouse IgM (μ-chain specific), LPS, and all inhibitors were purchased from Sigma.} \]

Flow cytometry for inhibition of ODN acidification

Efficacy of endosomal acidification inhibitors was verified using FITC-

\[ \text{labeled S-ODN as previously described (16). Briefly, WEHI-231 cells or } \]

\[ \text{J774 cells (10^6 cells/ml) were treated with medium, chloroquine (5 μg/ml), monensin (20 μM), or bafilomycin A (250 nM) for 1 h, and then FITC-labeled S-ODN (1 μg/ml) was added. After 3 h of incubation at 37°C, cells were washed three times with PBS and then analyzed by flow cytometry.} \]

Flow cytometry for detection of ROS generation

J774 cells and WEHI-231 B cells (5 × 10^5 cells/ml) were precultured for 30 min with or without chloroquine (5 μg/ml; <10 μM) or gliotoxin (0.2 μg/ml). Cell aliquots were then cultured for 20 min in RPMI medium with or without a CpG S-ODN 1826 or non-CpG S-ODN 1911 (TCCAGGACCTTCCCTCAGGTTAC) at 1 μM or with PMA plus ionomycin (iono) in the presence of dihydorodamine-123. Cells were then analyzed for intracellular ROS production by flow cytometry as described previously (13).

Electrophoretic mobility shift assay (EMSA) and Western blot assay

J774 cells (10^5/ml) were cultured in the presence of CT, EC, or meth-

\[ \text{ylated EC DNA (methylated with CpG methylase as described (11)) at 5} \]

\[ \text{μg/ml or a CpG S-ODN 1826 or non-CpG S-ODN 1745 (TCCAGGACCTTCCCTCAGGTTAC) at 0.75 μM for 1 h. In some experiments, J774 cells were} \]

\[ \text{precultured for 2 h in the presence or the absence of chloroquine (2.5–20} \]

\[ \text{μg/ml) and then stimulated for 1 h with EC DNA, CpG ODN, non-CpG} \]

\[ \text{ODN, or LPS (1 μg/ml). Cells were harvested, and cytoplasmic extracts} \]

\[ \text{and nuclear extracts were prepared as previously described (4). EMSA was} \]

\[ \text{conducted with nuclear extracts (5 μg/lane) for NFκB activation using} \]

\[ \text{radiolabeled double-stranded phosphodiester ODN containing a consensus} \]

\[ \text{NFκB site as previously described (19, 20). The position of the p50/p65} \]

\[ \text{heterodimer was determined by supershifting with specific Abs to} \]

\[ \text{p50 and p50. Western blot for IκBα or IκBβ was performed with cytoplasmic} \]

\[ \text{extracts (50 μg/lane) as previously described (4). Abs against mouse} \]

\[ \text{p50, p52, p65, Rel B, c-Rel, IκBα, and IκBβ were purchased from Santa Cruz} \]

\[ \text{Biotechnology (Santa Cruz, CA). Anti-mouse CD40 was purchased from} \]

\[ \text{PharMingen (San Diego, CA).} \]

RNase protection assay (RPA)

J774 cells (2 × 10^6 cells/ml) were cultured for 2 h in the presence or the absence of chloroquine (2.5 μg/ml; <5 μM) or TPCK (50 μM). Cells were then stimulated with the addition of EC DNA (50 μg/ml), CT DNA (50 μg/ml), LPS (10 μg/ml), CpG S-ODN 1826 (1 μM), or control non-CpG S-ODN 1911 (1 μM) for 3 h. Cells were harvested, and total RNA was prepared using RNXol method according to the manufacturer’s protocol. Levels of mRNA of specific genes were analyzed by RPA as described previously (14). Comparable amounts of RNA (3 μg/lane) were loaded into each lane, as shown by the mRNA loading control for L32, a ribosomal protein.

Results

Inhibitors of endosomal acidification block the CpG DNA-induced cytokine secretion

To test the hypothesis that endosomal acidification of CpG DNA is necessary for its induction of leukocyte activation, B cell or monocyte-like cell lines were stimulated with CpG DNA in the presence or the absence of specific inhibitors previously shown to block endosomal acidification of DNA including bafilomycin A, chloroquine, and monensin (16). First, the efficacy of these drugs for inhibiting the intracellular acidification of CpG DNA was verified using FACS analysis. Acidification of the FITC molecule quenches its fluorescence. Therefore, increased levels of fluorescence of the FITC-conjugated ODN in the cells treated with the inhibitors indicates the effective inhibition of ODN acidification by the drugs. As shown in Figure 1, the fluorescence intensity of FITC-ODN was increased in the presence of these endosomal acidification inhibitors in the concentration range used in our experiments, indicating effective inhibition of endosomal acidification by these drugs.

These inhibitors of endosomal acidification, which have different mechanisms of action, specifically blocked the CpG DNA-mediated cytokine secretion of primary spleen cells, WEHI-231 (a murine B lymphoma cell line), and J774 (a mu-

\[ \text{rine monocyte-like cell line} \] (Tables I, II, and III and data not shown). In contrast, these endosomal acidification inhibitors, in the concentration range tested in our experiments, showed little or no effect on the cytokine production induced by other stimulants, such as anti-CD40, anti-IgM, LPS, or PMA plus iono-

\[ \text{mycin. These results indicate that leukocyte responses to CpG} \]

\[ \text{DNA may be mediated through a novel pathway involving a} \]

\[ \text{pH-dependent step.} \]

FIGURE 1. Inhibitors of endosomal acidification block acidification of ODN. Murine monocyte-like J774 cells (10^6 cells/ml) were precultured for 1 h with or without chloroquine (CQ; 5 μg/ml), monensin (MONENS; 10 μM), and bafilomycin A (BAF; 250 nM). Cells were cultured in the presence of FITC-labeled S-ODN 1276 (FL-ODN; GAGAACGCTGGACCTTCCAT; 1 μM) for 3 h and then analyzed by flow cytometry as previously described (16). Increases in fluorescence intensity indicate the inhibition of acidification of ODN. The full experiment was performed twice with similar results.
Inhibitors of endosomal acidification block the CpG DNA-induced intracellular ROS generation

Since high concentrations of chloroquine also inhibit LPS-induced TNF-α secretion without affecting LPS-mediated early signaling events (21), we evaluate whether these endosomal acidification inhibitors can specifically block the early events in the CpG DNA-mediated leukocyte activation. The earliest leukocyte activation event that we have been able to detect in response to CpG DNA is the production of ROS, which is induced within 5 min in primary spleen cells and both B and monocyte-like cell lines (Fig. 2) (13) (A.-K. Yi and A. M. Krieg, unpublished observations). As shown in Figure 2, a low concentration of chloroquine blocked the CpG DNA-induced generation of ROS, but had no effect on ROS generation mediated by PMA or ligation of CD40 or surface Ag receptor (Fig. 2). Bafilomycin A, which inhibits endosomal acidification by blocking endosomal ATPase (22), also specifically suppressed CpG DNA-mediated ROS generation without affecting ROS generation induced by other stimulants (data not shown). These results suggest that acidification of DNA may be necessary for CpG DNA-mediated early signaling events, and this process may precede the CpG DNA-induced ROS generation.

Chloroquine blocks CpG DNA-induced IκBα and IκBβ degradation and subsequent NFκB activation

CpG DNA induces rapid activation of NFκB, which is accompanied by the degradation of IκBα and IκBβ, in a sequence-specific manner (4). Therefore, we next evaluated whether endosomal acidification of CpG DNA was also required for CpG DNA-induced IκBα and IκBβ degradation and subsequent NFκB activation. As shown in Figure 3A, EC DNA and CpG ODN induced degradation of IκBα and IκBβ and activation of NFκB within 1 h of stimulation in J774 cells. In contrast, control CT DNA and non-CpG ODN failed to induce NFκB activation or IκBα and IκBβ degradation. In addition, methylation of CpG dinucleotides in EC DNA significantly suppressed EC DNA-mediated NFκB activation and IκBα and IκBβ degradation. Chloroquine selectively blocked CpG DNA (EC DNA or CpG ODN)-induced IκBα and IκBβ degradation and NFκB activation (Fig. 3B). However, chloroquine failed to inhibit any of these LPS-induced intracellular activation events (Fig. 3B). These experiments support the role of a pH-dependent signaling mechanism in specifically mediating the stimulatory effects of CpG DNA.

Inhibitors of endosomal acidification and NFκB activation block CpG DNA-induced gene expression

While NFκB is known to be an important regulator of gene expression, its role in the transcriptional response to CpG DNA was uncertain. To determine whether endosomal acidification and/or NFκB activation was required for the CpG DNA-mediated induction of gene expression, we activated cells with CpG DNA in the presence or the absence of chloroquine; PDTC, an inhibitor of IκB activation; TPCK, a protease inhibitor that blocks IκB degradation; giotoxin, an inhibitor of IκB degradation; or bisglo- toxin, an inactive congener of giotoxin (23–25). Inhibitor concentrations used in our experiments were determined based on previous reports of their specificity (16, 23–25) and on our preliminary dose titrations, which allowed us to use lower inhibitor concentrations.

Table I. Blockade of CpG DNA-induced cytokine production by inhibitors of endosomal acidification in marine spleen cellsa

<table>
<thead>
<tr>
<th>Medium</th>
<th>Bafilomycin A (500 nM)</th>
<th>Chloroquine (5 μg/ml)</th>
<th>Monensin (20 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CpG DNA</td>
<td>6 ± 5</td>
<td>≤5</td>
<td>≤5</td>
</tr>
<tr>
<td>PMA + ionomycin</td>
<td>141 ± 10</td>
<td>153 ± 3</td>
<td>151 ± 3</td>
</tr>
</tbody>
</table>

a Marine spleen cells (107 cells/ml) were cultured with or without the indicated inhibitors at the concentrations shown for 2 h and then stimulated with the CpG S-ODN 1826 (TCCATGACCTTCCTGACGTT) or non-CpG S-ODN 1911 at 0.5 μM or PMA (100 ng/ml) + ionomycin (1 nM) for 4 h. ELISA for TNF-α (pg/ml) was performed on the supernatants. Cells cultured with ODN that lacked CpG motifs did not induce cytokine secretion (not shown). Similar specific inhibition of CpG responses was seen with IL-12 assays (not shown); 2.5 μg/ml of chloroquine is equivalent to <5 μM. Differences in the cytokine levels in the samples treated with CpG DNA only and CpG DNA + inhibitors (chloroquine, bafilomycin A, or monensin) were statistically significant with p = 0.05 by Student’s t test. The experiment was repeated >10 times with similar results in spleen cells, WEHI-231 cells, and J774 cells (see Tables I and III).

Table II. Blockade of CpG DNA-induced cytokine production by an inhibitor of endosomal acidification in WEHI-231 cellsa

<table>
<thead>
<tr>
<th>Medium</th>
<th>Chloroquine (2.5 μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>IL-6</td>
</tr>
<tr>
<td>Medium</td>
<td>TNF-α</td>
</tr>
<tr>
<td>CpG DNA</td>
<td>108 ± 12</td>
</tr>
<tr>
<td>Anti-IgM</td>
<td>69 ± 3</td>
</tr>
<tr>
<td>Anti-C4D40</td>
<td>32 ± 2</td>
</tr>
<tr>
<td>Anti-IgM + CpG DNA</td>
<td>163 ± 15</td>
</tr>
<tr>
<td>Anti-IgM + anti-C4D40</td>
<td>149 ± 10</td>
</tr>
</tbody>
</table>

a WEHI-231 cells (106 cells/ml) were cultured with or without the indicated inhibitors at the concentrations shown for 2 h and then stimulated with the CpG S-ODN 1826 (TCCATGACCTTCCTGACGTT) or non-CpG S-ODN 1911 at 0.5 μM, anti-IgM (10 μg/ml), or anti-C4D40 (1 μg/ml) for 9 h (IL-6 and TNF-α). ND stands for not done. ELISA for IL-6 (pg/ml) or TNF-α (pg/ml) was performed on the supernatants. Cells cultured with ODN that lacked CpG motifs did not induce cytokine secretion (not shown). 2.5 μg/ml of chloroquine is equivalent to <5 μM. Differences in the cytokine levels in the samples treated with CpG DNA only and CpG DNA + inhibitors (chloroquine) were statistically significant with p = 0.05 by Student’s t test. The experiment was repeated >10 times with similar results in spleen cells, WEHI-231 cells, and J774 cells (see Tables I and III).

Table III. Blockade of CpG DNA-induced cytokine production by inhibitors of endosomal acidification or NFκB activation in J774 cellsa

<table>
<thead>
<tr>
<th>Medium</th>
<th>Chloroquine (2.5 μg/ml)</th>
<th>Bafilomycin (250 nM)</th>
<th>NAC (50 nM)</th>
<th>TPCK (50 μM)</th>
<th>Giotoxin (0.1 μg/ml)</th>
<th>Bisglo-toxin (0.1 μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CpG DNA</td>
<td>37 ± 13</td>
<td>27 ± 1</td>
<td>46 ± 0</td>
<td>10 ± 4</td>
<td>24 ± 5</td>
<td>17 ± 7</td>
</tr>
<tr>
<td>LPS</td>
<td>455 ± 11</td>
<td>28 ± 7</td>
<td>71 ± 8</td>
<td>54 ± 21</td>
<td>23 ± 22</td>
<td>31 ± 11</td>
</tr>
<tr>
<td>Non CpG</td>
<td>901 ± 189</td>
<td>1025 ± 43</td>
<td>1370 ± 10</td>
<td>418 ± 14</td>
<td>46 ± 6</td>
<td>≤10</td>
</tr>
</tbody>
</table>

a J774 cells (107 cells/ml) were cultured with or without the indicated inhibitors at the concentrations shown for 2 h and then stimulated with the CpG S-ODN 1826 (TCCATGACCTTCCTGACGTT) or non-CpG S-ODN 1911 at 0.5 μM, LPS (10 ng/ml) for 6 h (TNF-α). ND stands for not done. Cells cultured with ODN that lacked CpG motifs did not induce cytokine secretion (not shown). Similar specific inhibition of CpG responses was seen with IL-12 assays (not shown); 2.5 μg/ml of chloroquine is equivalent to <5 μM. Other inhibitors of NFκB activation such as calpain inhibitors I and II gave results similar to those for the inhibitors shown. Differences in the cytokine levels in the samples treated with CpG DNA only and CpG DNA + inhibitors (chloroquine, bafilomycin A, monensin, PDTC, NAC, TPCK, or giotoxin) were statistically significant with p = 0.05 by Student’s t test. The experiment was repeated >10 times with similar results in spleen cells, WEHI-231 cells, and J774 cells (see Tables I and II).
concentrations than those employed in prior studies. These inhibitors of endosomal acidification or NFκB activation completely blocked the CpG DNA-induced expression of proto-oncogene and cytokine mRNA and protein (Fig. 4; Tables I–III) (A.-K. Yi and A. M. Krieg, unpublished observations), suggesting the essential role of NFκB as a mediator of these events. As previously reported (21), chloroquine showed no effect on LPS-induced gene expression (Fig. 4). None of the inhibitors reduced cell viability under the experimental conditions used in these studies. These results suggested that both endosomal acidification and NFκB activation are necessary steps for CpG DNA-mediated immune activation.

CpG DNA-induced ROS generation precedes the CpG DNA-mediated NFκB activation

CpG DNA-induced ROS generation could be an incidental consequence of cell activation or a signal that mediates this activation. Therefore, we evaluated whether CpG DNA-induced generation of ROS precedes the NFκB activation and may be necessary for the CpG DNA-mediated NFκB activation. As shown in Figure 5, ROS generation in response to CpG DNA is not inhibited by the NFκB inhibitor gliotoxin (23), while CpG DNA-induced NFκB activation was completely blocked by gliotoxin (data not shown), confirming that the ROS generation is not secondary to NFκB activation. In addition, the ROS scavengers NAC and PDTC blocked NFκB activation, cytokine production, and B cell proliferation induced by CpG DNA or other stimulants (Tables I–III and data not shown) (13), consistent with a causal role for ROS generation in these pathways.

Discussion

CpG motifs in bacterial DNA and synthetic oligonucleotides promote B cell proliferation, Ig production, and the secretion of various cytokines, such as IL-6, IL-10, IL-12, TNF-α, and IFN-γ, from B cells, monocytes/macrophages, and NK cells (8, 9, 11–13, 4758).

**FIGURE 2.** CpG DNA-induced ROS production requires endosomal acidification. WEHI-231 B cells (5 × 10⁵ cells/ml) were precultured for 30 min with or without chloroquine (CQ; 5 μg/ml; <10 μM), Cell aliquots were then cultured as described above for 10 min in RPMI medium with or without CpG S-ODN 1826 or non-CpG S-ODN 1911 (TCCAGGACTTTCCTCAGGTT) at 1 μM (A) or 0.5 μM (B), PMA plus ionomycin (iono; A), or anti-CD40 ( @CD40; 5 μg/ml; B) in the presence of dihydrorhodamine-123. Cells were then analyzed for intracellular ROS production by flow cytometry as previously described (13). J774 cells showed similar pH-dependent CpG-induced ROS responses (not shown). The results (numbers in the histogram) are presented as the percentage of cells above a threshold level of fluorescence that was arbitrarily set such that approximately one-third of unstimulated proliferating cells were above this level (marked as M1). Each experiment was performed at least three times with similar results.
CpG DNA synergizes with Ag receptor-mediated signals to increase IL-6, TNF-α, and Ig secretion and B cell proliferation (11–13) (A.-K. Yi and A. M. Krieg, unpublished observations). CpG DNA protects WEHI-231 B cells and spleen B cells from apoptosis (4, 14). However, the molecular mechanisms by which CpG DNA mediates leukocyte activation are not clearly understood at the present time. The results in the present study indicate that endosomal acidification of DNA is required for the CpG DNA-mediated leukocyte activation and is coupled to the rapid generation of ROS that leads to IκB degradation and NFκB activation and subsequent proto-oncogene and cytokine expression.

Our previous studies indicate that B cells and monocytic cell lines do not have a specific membrane receptor for CpG DNA, and cellular uptake of the CpG DNA is required for its action (11). Cellular uptake of DNA appears to occur via adsorptive endocytosis into an acidified intracellular compartment (16, 17). This led us to evaluate whether leukocyte activation by CpG DNA occurs in association with acidified endosomes and may even be dependent on the endosomal pH. Inhibitors of endosomal acidification, such as bafilomycin A, chloroquine, and monensin, effectively block CpG DNA-mediated cytokine production in murine spleen cells, B cell lines, and monocyte cell lines (Tables I–III) (A.-K. Yi and A. M. Krieg, unpublished observations), suggesting a requirement for an intracellular pH-dependent step for CpG DNA-mediated immune cell activation.

The pH-dependent step may be the transport or processing of the CpG DNA, the ROS generation, or some later step in the activation pathway. For example, high concentrations (100–250 μM) of chloroquine inhibit LPS-induced TNF-α secretion, but not NFκB activation or gene transcription (21). This led us to evaluate whether endosomal acidification inhibitors can specifically block the early events in the CpG DNA-mediated leukocyte activation. Within 30 min after CpG DNA stimulation, B cells and monocyte-like cell lines have increased intracellular ROS generation, NFκB activation, and induction of the expression of several proto-oncogenes and cytokine genes (4, 5, 13, 14) (A.-K. Yi and A. M. Krieg, unpublished observations). At the low concentrations
signals that can lead to IκBα degradation, nuclear translocation of NFκB, and proto-oncogene expressions without affecting any of these events induced by other stimuli, such as LPS, anti-IgM, anti-CD40, or PMA plus ionomycin (Figs. 2–4). These results indicate that the chloroquine-inhibitable step takes place at a very early stage of the CpG DNA-mediated signaling pathway. Taken together, our results suggest that CpG DNA is taken up by the cell via endocytosis and is acidified in the endosomal compartment, and this process precedes and even might be necessary for the CpG DNA-mediated early signaling events such as ROS generation and NFκB activation.

ROS are widely thought to be secondary messengers in signaling pathways in diverse cell types. ROS are also known to mediate signals that can lead to IκB degradation and NFκB activation (24, 25, 27). Therefore, we tested whether ROS generated after CpG DNA stimulation precede the NFκB activation and are necessary for the CpG DNA-mediated IκBα degradation and NFκB activation. ROS generation in response to CpG DNA is selectively inhibited by chloroquine but is not blocked by giotoxin, which inhibits activation of NFκB (Fig. 5), indicating that CpG DNA-mediated ROS generation is not secondary to NFκB activation. Furthermore, the ROS scavengers NAC and PDTC suppressed CpG DNA-induced IκBα and IκBβ degradation and subsequent NFκB activation (data not shown) (4), suggesting a causal role for ROS generation in CpG DNA-mediated signaling pathway. These data are compatible with previous evidence supporting a role for ROS in the activation of NFκB (24, 25).

Our studies indicate that leukocytes respond to CpG DNA through a novel pathway involving the pH-dependent generation of intracellular ROS, which is essential for the activation of NFκB and all the downstream events observed after CpG DNA stimulation (Fig. 6).

CpG motifs have recently been shown to be required for the efficacy of DNA vaccines and may be an important signal for the activation of protective innate immune responses (15, 28, 29). CpG DNA shows promise as a novel immunotherapy for asthma, for inducing antitumor responses, and for enhancing vaccine efficacy (30–36). On the other hand, CpG DNA induces B cells to become resistant to apoptosis, a mechanism thought to be important for preventing immune responses to self antigens, such as DNA (14, 37). Indeed, exposure to bDNA can trigger anti-DNA Ab production (38, 39). Given this potential ability of CpG DNA to promote autoimmunity, it is noteworthy that lupus flares frequently are thought to be triggered by microbial infection. Chloroquine is an effective therapeutic agent for the treatment of systemic lupus erythematosus and some other autoimmune diseases, although its mechanism of action has been obscure. Interestingly, low concentrations of chloroquine also inhibit CpG DNA-mediated protection against spontaneous apoptosis of mature spleen B cells and Ag receptor-induced apoptosis of WEHI-231 B cells (A.-K. Yi, D. W. Peckham, R. F. Ashman, and A. M. Krieg, manuscript in preparation). The very low concentration at which chloroquine inhibits CpG DNA-mediated leukocyte activation (<10 μM) is noteworthy, since it is well below that required for its antimalarial activity.

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** An NFκB activation inhibitor, giotoxin, does not block CpG DNA-induced ROS generation. WEHI-231 B cells (5 × 10^6 cells/ml) were precultured for 30 min with or without giotoxin (GLIOTOX; 0.2 μg/ml). Cells were then cultured for 10 min in RPMI medium with or without a CpG S-ODN 1826 at 1 μM or PMA plus ionomycin in the presence of dihydrorhodamine-123. Cells were then analyzed for intracellular ROS production by flow cytometry. The experiment was performed at least three times with similar results.

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** Schematic diagram of proposed CpG DNA-mediated signaling pathway. The model illustrates the suggested signaling pathway for CpG DNA-mediated cytokine and proto-oncogene regulation. CpG DNA may be taken up by B cells and monocytes via adsorptive endocytosis into an acidified intracellular compartment. Acidification of CpG DNA may be a necessary step to induce sequence-specific generation of intracellular ROS, which is inhibitable by endosomal acidification inhibitors such as bafilomycin A, chloroquine, and monensin. These ROS, generated via a pH-dependent step upon CpG DNA stimulation, may be required for IκBα and IκBβ phosphorylation and degradation and subsequent NFκB activation. These steps can be inhibited by antioxidant (PDTC and NAC). CpG DNA-induced NFκB activation, which can be blocked by TPCK or giotoxin, plays a key role in CpG DNA-mediated regulation of cytokines and proto-oncogenes. Dashed lines indicate hypothetical steps.
and other reported immune effects (100–1000 μM) (40–42). Together with the likely role of CpG DNA as a mediator of the sepsis syndrome (8, 10) and inducer of pulmonary inflammation (43) and other diseases (reviewed in Ref. 15), our demonstrations of the ability of extremely low concentrations of chloroquine to specifically inhibit CpG DNA-mediated leukocyte activation suggest a possible new mechanism for its beneficial effect and possible new therapeutic applications for this class of drugs.

Note added in proof. Since the submission of this manuscript, the inhibition of CpG-induced B cell resistance to apoptosis by chloroquine and related compounds has been reported by D. E. MacFarlane and L. Manzel, 1998. J. Immunol. 160:1122.

Acknowledgments

We thank D. MacFarlane, B. Nauseef, and G. Koretzky for useful discussions, and Courtney Ward for outstanding technical assistance.

References

11. Krieg, A. K., A.-K. Yi, S. Matson, T. J. Waldschmidt, G. A. Bishop, R. Teasdale, M. L. Rottenberg, P. C. Kaper, and D. M. Klinman. 1995. CpG motifs in bacterial DNA trigger immune activation by CpG motifs in bacterial DNA: systemic induction of IL-6 and other reported immune effects (100–1000 μM) (40–42). Together with the likely role of CpG DNA as a mediator of the sepsis syndrome (8, 10) and inducer of pulmonary inflammation (43) and other diseases (reviewed in Ref. 15), our demonstrations of the ability of extremely low concentrations of chloroquine to specifically inhibit CpG DNA-mediated leukocyte activation suggest a possible new mechanism for its beneficial effect and possible new therapeutic applications for this class of drugs.

Note added in proof. Since the submission of this manuscript, the inhibition of CpG-induced B cell resistance to apoptosis by chloroquine and related compounds has been reported by D. E. MacFarlane and L. Manzel, 1998. J. Immunol. 160:1122.

Acknowledgments

We thank D. MacFarlane, B. Nauseef, and G. Koretzky for useful discussions, and Courtney Ward for outstanding technical assistance.

References