Chloroquine Enhances Survival in *Bacillus anthracis* Intoxication

Andrew W. Artenstein,1,3 Steven M. Opal,1,3 Patricia Cristofaro,1,3 John E. Palardy,1,3 Nicolas A. Parejo,1,3 Michael D. Green,4 and Jhung W. Jhung1

1Center for Biodefense and Emerging Pathogens, Division of Infectious Diseases, and 2Department of Anatomic Pathology, Memorial Hospital of Rhode Island, Pawtucket, and 3Brown Medical School, Providence, Rhode Island; 4Centers for Disease Control and Prevention, Atlanta, Georgia

The intentional release of anthrax in the United States in 2001 resulted in 11 cases of inhalational disease, with an attendant mortality rate of 45%. Current therapeutic options for anthrax are limited; antimicrobials target only replicating organisms, thus allowing bacterial toxins to cause unchecked, devastating physiological derangements in the host. Novel approaches that target the cytotoxic effects of anthrax exotoxins are needed.

Chloroquine (CQ), a commonly used antimalarial agent, endows anthrax-intoxicated murine peritoneal macrophages with a 50% and 35% marginal survival advantage at 2 and 4 h, respectively, over that of untreated control cells. The cell rescue is dose dependent and, at lower concentrations, results in delayed cell death. We subsequently studied the effect of CQ in BALB/c mice challenged with anthrax lethal toxin. CQ-treated mice demonstrated reduced tissue injury, as assessed by histopathological examination of the spleen and by peripheral blood differential cell count ratios. CQ significantly enhanced survival and may augment current treatment and prophylaxis options for this otherwise lethal infection.

Anthrax is considered to be among the most likely threat agents of bioterrorism and, as such, has been designated as a category A agent, along with the agents of smallpox, botulism, plague, tularemia, and certain viral hemorrhagic fevers, by the Centers for Disease Control and Prevention (CDC) [1]. The threat was realized in the United States in 2001 when attacks using aerosolized anthrax spores resulted in 11 cases of the inhalational form of disease; 5 of the 11 patients died [2]. Although multiple antimicrobials possess activity against *Bacillus anthracis*, they do not provide complete protection against disease, because systemic illness results from the effects of toxins produced by the organism during infection. Patients with inhalational anthrax appear to reach a critical threshold in the pathogenic process beyond which survival is unlikely [2, 3]; therefore, an early, multifaceted approach to therapy may be necessary to optimize outcomes in these cases. One such approach is to target key steps in the process of intoxication.

The pathogenesis of anthrax revolves around the actions of 2 distinct exotoxins formed from the binary combinations of 3 plasmid-encoded proteins: protective antigen (PA), lethal factor (LF), and edema factor (EF) [4]. After PA binds to a cellular receptor, it is proteolytically activated by a host serine protease, hepta-merizes, and binds either EF or LF [5]. The complex is subsequently translocated into the host cell via receptor-mediated endocytosis [5], a step that requires acidification of the endosome. Inhibitors of receptor-mediated endocytosis, such as ammonium chloride and chloroquine (CQ), have been shown previously to inhibit the action of anthrax edema toxin (PA plus EF) in mammalian cells [6]. Similarly, lysosomotropic amines that dissipate intracellular proton gradients have been shown to protect cells against the effects of anthrax lethal toxin (LeTx) [7].

LeTx, aptly named because of its lethality in experimental animals, is formed by the combination of PA and LF. It is thought to cause cytotoxicity by inducing
reactive oxygen intermediates and disrupting intracellular signaling pathways [8]. LeTx is cytolytic for murine macrophages as early as 90 min after exposure and is thought to be the primary mediator of shock and death in systemic anthrax [9]. Recent data suggest that LeTx may also disrupt other critical areas involving cell-to-cell signaling, such as the dendritic cell–T cell interaction [10]. Interventions aimed at inhibiting the effects of LeTx could significantly improve the outcome of inhalational anthrax. Toward this end, we conducted an expanded investigation into the effect of CQ on the deleterious effects of LeTx.

**MATERIALS AND METHODS**

**Animals.** Female, specific pathogen–free BALB/c mice (Charles River Laboratories) were used. Mice were maintained in accordance with Animal Welfare Assurance guidelines and university guidelines of Brown Medical School. Challenge experiments involved intravenous injections of LeTx, administered via tail vein under light CO₂ anesthesia, followed by multiple daily observations. Mice that survived for >7 days were killed and necropsied. For the assessment of hematological parameters, cytokines, and early histopathological changes in the spleen, groups of 3 mice (CQ-treated and the various controls) were killed, bled, and necropsied at various times in relation to challenge: immediately before and 6, 12, 24, 36, and 48 h after challenge with LeTx or injection of saline.

**Cells.** The methods for isolation and preparation of murine peritoneal macrophages have been described elsewhere [11]. BALB/c mice were given an intraperitoneal injection of 2 mL of sterile, aged thioglycollate broth and were allowed to rest for 3–4 days. The mice were then humanely killed, and mononuclear cells were harvested from peritoneal fluid under sterile conditions by lavaging the peritoneal cavity with 5 mL of ice-chilled, heparinized RPMI 1640 medium (Sigma) with 11.6% sucrose solution. Cell viability was confirmed by trypan blue exclusion before initiation of the experimental protocol. After centrifugation for 10 min at 800 g, the cells were resuspended in RPMI 1640 medium (supplemented with L-glutamine, penicillin, streptomycin, and 10% fetal bovine serum [FBS; Gibco-BRL]), counted, and plated on microtiter plates, in a density of 2 × 10⁵ cells/well. Cells were then washed twice in RPMI 1640 medium with 10% FBS and incubated for 16 h at 37°C in 5% CO₂, and fresh growth media with 5 ng/mL lipopolysaccharide (Escherichia coli O111:B4; Sigma) was added. Light microscopy confirmed the cell population to be >99% adherent macrophages.

**CQ.** Selected wells were incubated for an additional 2 h at 37°C in 5% CO₂ with CQ diphosphate (Sigma), in final concentrations of 0.06–51.59 μg/mL (0.12–100 μmol/L). For mouse challenge experiments, we used 0.5 mg of CQ (~15 mg/kg) in 0.5 mL of sterile water given via orogastric tube. Groups of mice were treated according to 1 of 3 dosing schedules: 1 dose/day for 2 days, followed by challenge with LeTx 4 h after the final dose; 1 dose followed by challenge with LeTx 4 h later; or
Figure 2. Chloroquine (CQ)–enhanced survival of mice after challenge with anthrax lethal toxin (LeTx). A, Survival curves of groups of BALB/c mice challenged intravenously with anthrax LeTx at varying doses: 100 μg of protective antigen (PA) plus 20 μg of lethal factor (LF) (Δ), 50 μg of PA plus 10 μg of LF (○), and 20 μg of PA plus 5 μg of LF (□). Kaplan-Meier plots comparing survival of mice challenged with LeTx (solid line) or treated with 2 daily doses of CQ followed by LeTx challenge (dashed line). There were 17 mice in the LeTx control group and 16 in the CQ-treated group; plots were compared by use of the Mann-Whitney U test.

B, 1 dose 4 h after challenge with LeTx. Control mice received 2 doses of CQ only.

Toxin. PA and LF heterodimers (initially a gift from S. Leppla, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, and subsequently obtained from List Biological Laboratories) were added to the cell preparations at doses of 50, 100, 250, 500, and 1000 ng/mL. Mouse-challenge experiments used 50 μg of PA and 10 μg of LF, given as a single injection.

Cytotoxicity assay. Cell viabilities were determined at 0, 2, and 4 h after the addition of LeTx, by use of the CellTiter 96 Aqueous One Solution Assay (Promega), according to the manufacturer’s instructions, and were calculated by use of 490-nm measurements in a microtiter plate reader (Bio-Rad Laboratories), according to the following formula: 100 × (Ax - Apc) / (An - Apc), where Ax, Apc, and An represent the signal of the sample, the positive control, and the negative control, respectively. The percentage of viability of murine peritoneal macrophages after exposure to graded doses of anthrax toxin with or without CQ was compared by use of a nonparametric, repeated-measures analysis. Percentage of survival differences in CQ-treated cells were assessed by comparison with controls exposed to LeTx alone.

CQ levels. CQ levels were measured 2 h after the second of 2 consecutive daily doses of 0.5 mg in the serum, spleen, and liver of 5 control BALB/c mice at CDC (Atlanta, GA), by use of high-performance liquid chromatography analysis, as described elsewhere [12].

Histopathological analysis. Necropsies were performed on each mouse. Spleens were removed and fixed by submersion in 10% neutral-buffered formalin, embedded in paraffin, stained with hematoxylin-eosin, and reviewed by a board-certified pathologist who was blinded to the treatment group. A splenic injury score, based on the following assignments, was derived for each splenic sample: 0, normal histological features; 1, mild-to-moderate mononuclear infiltrate mixed with neutrophils and megakaryocytes in the red pulp with reactive follicles (enlarged germinal centers with stimulated lymphocytes); and 2, moderate-to-severe necrosis of red pulp with follicular destruction and scattered nuclear debris.

Hematological assessment. Peripheral blood smears were obtained from each mouse at each time point, coded, and Giemsa stained; manual white blood cell differential counts were performed in a blinded fashion. Ratios of polymorphonuclear neutrophils to monocytes were compared by analysis of variance, with a Tukey-Kramer multiple comparisons test. P < 0.05 was considered to be significant.

Cytokine expression. Serum measurements of interleukin (IL)–1β, IL-2, IL-4, IL-5, IL-10, granulocyte-macrophage colony-stimulating factor, interferon-γ, and tumor necrosis factor (TNF)–α were performed by use of the Bio-Plex Cytokine Assay (Bio-Rad Laboratories), according to the manufacturer’s instructions, and were read via the Bio-Plex Protein Array System (Bio-Rad Laboratories).

RESULTS

We added LeTx (PA plus LF) to murine peritoneal macrophages and showed that, at doses of each constituent as low as 100 ng/mL, significant cytotoxicity was observed within 2 h. Cell viability curves appeared to be dose dependent at each time point within the LeTx dosing range of 100–500 ng/mL. At LeTx doses of 1000 ng/mL, near complete cytotoxicity was observed by 2 h. Preincubation of cells with 51.59 μg/mL CQ 2 h before the addition of 500 ng/mL LeTx resulted in a 50% and 35%
Table 1. Chloroquine (CQ)–enhanced survival in mice challenged with anthrax lethal toxin (LeTx).

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Day 3 Survivors/total, no.</th>
<th>P&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Day 7 Survivors/total, no.</th>
<th>P&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>CQ controls</td>
<td>5/5</td>
<td>...</td>
<td>5/5</td>
<td>...</td>
</tr>
<tr>
<td>LeTx controls</td>
<td>1/17</td>
<td>...</td>
<td>0/17</td>
<td>...</td>
</tr>
<tr>
<td>(CQx2)&lt;sup&gt;c&lt;/sup&gt; plus LeTx</td>
<td>8/16</td>
<td>.007</td>
<td>5/16</td>
<td>.018</td>
</tr>
<tr>
<td>(CQx1)&lt;sup&gt;c&lt;/sup&gt; plus LeTx</td>
<td>3/16</td>
<td>.335</td>
<td>2/16</td>
<td>.227</td>
</tr>
<tr>
<td>LeTx plus (CQx1)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4/16</td>
<td>.175</td>
<td>4/16</td>
<td>.044</td>
</tr>
<tr>
<td>Total CQ-treated plus LeTx-challenged</td>
<td>15/48</td>
<td>.049</td>
<td>11/48</td>
<td>.054</td>
</tr>
</tbody>
</table>

<sup>a</sup> Compared with LeTx controls at day 3 (Fisher’s exact test).
<sup>b</sup> Compared with LeTx controls at day 7 (Fisher’s exact test).
<sup>c</sup> Parentheses refer to timing of CQ relative to challenge, and the no. (1 or 2) refers to the no. of doses of CQ.

Marginal survival advantage by 2 and 4 h, respectively (figure 1A). Similar protective effects were noted at lower doses of LeTx. There was a direct, dose-response relationship between CQ concentration and cell viabilities, with delayed cytotoxicity even at lower drug concentrations (figure 1B).

For the in vivo experiments with female BALB/c mice, various doses of LeTx in a fixed 5:1 ratio of PA to LF [9] were assessed; an LeTx dose of 50 μg of PA and 10 μg of LF reliably produced mortality (figure 2A). CQ conferred a significant survival advantage, at both 3 and 7 days after challenge, compared with untreated mice (table 1 and figure 2B). Two doses appeared to enhance survival more than 1 dose, at both 3 and 7 days after LeTx challenge (table 1). In 5 control mice, mean peak serum, splenic, and hepatic levels of CQ were 38.8, 193.2, and 3092.2 ng/mL, respectively. These values are consistent with those previously observed in human pharmacokinetic studies and with the known drug sequestration in tissues [13].

Histopathological evaluation of the mouse spleens and the assignment of an “injury score” were performed by a pathologist who was blinded to treatment group. The spleens of CQ-treated, LeTx-challenged mice demonstrated overall lower scores and were closer in appearance to those of unchallenged mice (figure 3A–3C). We examined the effect of LeTx on peripheral white blood cells during the first 48 h after challenge. LeTx induced a

Figure 3. Histopathological analysis of representative anthrax lethal toxin (LeTx)–challenged BALB/c mouse spleens in the presence or absence of chloroquine (CQ) treatment, viewed by 10× and 40× objectives. Mice were either challenged with 200 μL of LeTx (50 μg of protective antigen plus 10 μg of lethal factor) or were injected with 200 μL of sterile saline (negative control) and were necropsied at the time of death or on day 7 after challenge if the mouse survived. A, CQ-treated, saline-challenged mouse survivor. Both the white and red pulp are preserved and reveal normal cellularity and architecture. B, LeTx-challenged mouse in the absence of treatment with CQ. Advanced necrosis of the red pulp associated with nuclear debris is seen. The mouse died on day 2 after challenge. C, Two daily doses of CQ followed by LeTx challenge demonstrates mild mononuclear cell infiltrate in red pulp with reactive follicular architecture. The mouse survived to day 7 after challenge.
after 12 h. all time points after challenge ( ), with at all time points challenged mice and the CQ-treated, LeTx-challenged mice was noted at with a stable ratio in the CQ-treated, unchallenged mice ( ). Plasmodium falciparum out the world, despite the high global prevalence of CQ-resistant [9], and the invariant death of LeTx [14], BALB/c mice are considered to be susceptible to LeTx [14], mouse strains are known to vary in their relative susceptibility the otherwise lethal effects of anthrax LeTx. Although inbred mouse strains are known to vary in their relative susceptibility to LeTx [14], BALB/c mice are considered to be susceptible [9], and the invariant death of LeTx-challenged control mice in the present study confirms the appropriateness of this model.

CQ remains the most widely used antimalarial agent throughout the world, despite the high global prevalence of CQ-resistant Plasmodium falciparum [15]. It is generally safe and well tolerated by humans. When used as malaria chemoprophylaxis, CQ phosphate is typically given orally at a dose of 500 mg (equivalent to 300 mg of CQ base) once weekly. Doses of up to 1000 mg are used to treat CQ-susceptible falciparum malaria. The drug is rapidly and nearly completely absorbed from the gastrointestinal tract, is widely distributed in various body tissues, and has a plasma half-life of up to 120 h. After a 500-mg oral dose, peak plasma concentrations range from 0.15 to 0.25 μg/mL; however, concentrations of CQ appear to be 200–700-fold higher in kidneys, liver, lungs, spleen, and other organs than in plasma [13, 16]. Therefore, the drug concentrations studied in our in vitro experiments correlate with therapeutically achievable tissue levels and are, in fact, likely to represent the lower end of these values. Doses used for our in vivo experiments were analogous to those used in human malaria treatment regimens.

The antimalarial action of CQ is attributed to its inhibition of hematin detoxification in the intraerythrocytic parasite forms, resulting in the accumulation of toxic metabolic products within the organism [17]. The agent is also a weak, basic lysosome- and is known to accumulate within endosomes, thereby altering the acidic environment in these organelles that is required for a variety of cellular functions [18]. That the LeTx complex requires endosomal translocation may explain, in part, the inhibitory effect of CQ on LeTx. CQ has also been widely and successfully used for the treatment of inflammatory disorders, although the mechanism of its anti-inflammatory effects is not precisely known. The recent demonstration that the drug inhibits expression of the TNF-α gene in human cell lines via inhibition of lipopolysaccharide-induced signal transduction may partially explain these effects [19]. Weber et al. have shown more recently that CQ interferes with expression of TNF-α via inhibition of mitogen-activated protein kinase (MAPK) signaling [20]. The MAPK-kinase activation system also appears to be a target of LeTx [4].

Spicic congestion and necrosis were noted to be the most common abdominal autopsy findings among victims of the anthrax attacks in 2001 [21]. Splenic histopathological analysis in the present study has confirmed the findings of Moayeri et al. of cell death and progressive red-pulp necrosis in BALB/c mice [22] after challenge with LeTx and has clearly demonstrated the beneficial effects of CQ in LeTx-challenged mice. We observed histopathological changes as early as 6 h after challenge with LeTx, and these changes were progressive in nonsurvivors (data not shown). The marked depletion of peripheral blood mononuclear cells and concomitant mild increase in neutrophils are consistent with previous data [22] and are significantly mitigated by treatment with CQ. Prospective studies to evaluate the potential utility of serial, differential peripheral blood counts as a predictor of mouse mortality and a noninvasive, surrogate marker for therapeutic success are currently in progress.

Our data support the potential use of CQ to inhibit the toxic
effects of LeTx. Additional investigations are needed to confirm and extend our observations in vivo using live mouse challenges with bacilli and, subsequently, with anthrax spores. Further experiments examining the timing of CQ dosing relative to infection will be important in delineating the precise role of this drug in the treatment and/or prophylaxis of anthrax. Given the pathogenesis of anthrax and the high mortality from systemic disease, it is likely that antitoxin approaches, such as that described here, will have to be combined with effective antimicrobials to optimize the management of this lethal infection [23]. CQ is licensed for human use, is widely available, and has a long record of safety; these attributes, in concert with our findings of its favorable effect on anthrax intoxication in mice, could facilitate the rapid transition of this drug from bench to bedside, should it be needed.

Acknowledgments

We thank Stephen Gregory for careful review of the manuscript and Margo Katz and Kathy Bollesen for administrative assistance.

References