Chloroquine Modulates the Fungal Immune Response in Phagocytic Cells From Patients With Chronic Granulomatous Disease

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Invasive aspergillosis is a major threat to patients with chronic granulomatous disease (CGD). Fungal pathogenesis is the result of a diminished antifungal capacity and dysregulated inflammation. A deficient NADPH-oxidase complex results in defective phagolysosomal alkalization. To investigate the contribution of defective pH regulation in phagocytes among patients with CGD during fungal pathogenesis, we evaluated the effect of the acidotropic, antimalarial drug chloroquine (CQ) on the antifungal capacity of polymorphonuclear cells (PMNs) and on the inflammatory response of peripheral blood mononuclear cells (PBMCs). Chloroquine exerted a direct pH-dependent antifungal effect on Aspergillus fumigatus and Aspergillus nidulans; it increased the antifungal activity of PMNs from patients with CGD at a significantly lower concentration, compared with the concentration for PMNs from healthy individuals; and decreased the hyperinflammatory state of PBMCs from patients with CGD, as observed by decreased tumor necrosis factor α and interleukin 1β release. Chloroquine targets both limbs of fungal pathogenesis and might be of great value in the clearance of invasive aspergillosis in patients with CGD.

Keywords: chronic granulomatous disease; Aspergillus spp.; pathogenesis; chloroquine; polymorphonuclear cells; mononuclear cells; inflammation; antifungal activity; cytokines.

Chronic granulomatous disease (CGD) is a rare inherited immunodeficiency disorder of the NADPH-oxidase complex. Patients are at increased risk of life-threatening invasive fungal infections, particularly invasive aspergillosis [1]. Aspergillus fumigatus is the most commonly encountered species, followed by Aspergillus nidulans, with the former detected in approximately one-third of all cases of invasive aspergillosis in patients with CGD [2]. A. nidulans causes invasive infections almost exclusively in this specific host and is characterized by its aggressive behavior and high case-fatality rate [3]. Since the NADPH-oxidase complex acts both as an antimicrobial effector molecule and a regulator of inflammation, fungal pathogenesis in patients with CGD is the result of an imbalance of antifungal capacity and regulation of inflammation. The defective NADPH-oxidase complex results in a lack of superoxide production, an accumulation of protons, and impaired alkalization of the phagolysosomes. In the early 1980s, it was suggested that abnormal pH regulation within phagosomes from patients with CGD might have a role in defective killing capacities [4]. The basis of this assumption is that the initiation of superoxide production is accompanied by an influx of K+ and a rise in pH as a result of the proton-acceptor function of superoxide anions. This pH change is
proposed to be essential for the release and activation of granule-derived enzymes within the phagosome [5]. In patients with CGD, this pH dysregulation is of clinical importance, as supported by the fact that, upon normalization of the phagocytic pH in CGD phagocytes, the ability to kill Staphylococcus aureus is restored [4].

In addition to this impaired antifungal defense system, the absence of the oxidative burst in phagocytes from patients with CGD leads to a dysregulated inflammatory response [6]. Several studies indicate hyperresponsive production of tumor necrosis factor α (TNF-α) and interleukin 6 in patients with CGD, both upon encountering A. fumigatus and without any obvious stimulus [6–8]. The excessive inflammation is thought to play a role in the outcome of invasive aspergillosis in patients with CGD; limiting this exaggerated inflammatory response might decrease tissue damage and improve outcome.

The antimalarial drug chloroquine (CQ) is an acidotropic agent that passively diffuses into acidic organelles. It has shown to have a direct antifungal effect on Histoplasma capsulatum and Cryptococcus neoformans, possibly by accumulation in the fungal vacuole [9–11]. CQ, being a weak base, is able to raise the endocytic and lysosomal pH of eukaryotic cells [12, 13]. On entering the phagocytic cell, it diffuses into the acidic endosomes and can reach a concentration that is up to 10 000-fold higher than its extracellular levels [14]. Earlier studies have shown that the addition of CQ to human mononuclear cells has an additive effect on the killing of both H. capsulatum and C. neoformans [11, 15]. In addition to the antifungal effect of CQ, this drug has been effective in the treatment of diseases associated with increased release of proinflammatory cytokines, such as rheumatoid arthritis [16]. CQ is able to reduce the proinflammatory cytokine release of mononuclear phagocytes stimulated by lipopolysaccharide (LPS) and to antagonize TNF-α expression induced by fungi (such as C. neoformans and Candida albicans) by a mechanism suggested to be dependent on alkalization of endolysosomes [17–19].

To clarify the contribution of defective alkalization of phagolysosomes during fungal pathogenesis in patients with CGD, we evaluated the in vitro effect of CQ on fungicidal effector mechanisms and cytokine release by phagocytes from healthy volunteers and patients with CGD that were stimulated with A. fumigatus and A. nidulans. We hypothesized that the addition of CQ would enhance the antifungal activity of phagocytes from patients with CGD by raising the pH in phagolysosomes and downregulating the exaggerated proinflammatory response.

**METHODS**

**Human Leukocytes**

Venous blood was drawn from healthy volunteers, 3 patients with CGD and gp91phox deficiency, and 2 patients with CGD and p47phox deficiency after informed consent was obtained. Peripheral blood mononuclear cells (PBMCs) and polymorphonuclear leukocytes (PMNs) were isolated by use of Lymphoprep (Axis-Shield, Oslo, Norway). Briefly, blood was anticoagulated with lithium heparin (BD Vacutainer) and diluted with an equal volume of phosphate-buffered saline (PBS). The diluted blood was carefully added to the top of the Lymphoprep and centrifuged at 800 × g. PBMCs were harvested, washed twice in PBS, and counted by use of a hemocytometer. To remove the erythrocytes from the PMNs, the pellet was shocked at least twice with an ice-cold lysing reagent (NH4Cl, Na2EDTA, KHCO3). Subsequently, cells were washed and re-suspended in PBS. Roswell Park Memorial Institute 1640 Glutamax-I medium (Invitrogen Life Technologies) plus 10% heat-inactivated human serum was used as culture medium for all cell experiments.

**Fungal Strains**

Fully molecularly characterized A. nidulans (V44-46) and A. fumigatus (V45-07) strains, originally isolated from patients with CGD who had invasive aspergillosis, were used. The Aspergillus species were grown and prepared in a LPS-free fashion as described previously [20].

**XTT Assay**

The XTT, (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]2H-tetrazolium-5-carboxanilide sodium salt) assay of fungal viability described previously was modified as follows [20]. Each well of a 96-well plate was filled with 100 µL of CQ (chloroquine diphosphate salt; Sigma-Aldrich, St. Louis, MO) in different concentrations and with 100 µL of live, resting conidia at a concentration of 2 × 10⁶ conidia/mL. CQ was diluted in MilliQ and sterilized through a 0.2-mm filter membrane. Conidial viability was analyzed after 18 hours of incubation at 37°C in 5% CO₂ [20].

**Effect of pH on the Antifungal Activity of CQ**

To analyze the effect of pH on the antifungal activity of CQ, the pH of the medium was set at pH 6, pH 7, and pH 8. The medium containing different concentrations of CQ was buffered with 50 mM MES for pH 6, 50 mM HEPES for pH 7, and 50 mM Tris for pH 8. By titration of hydrochloric acid or sodium hydroxide, the specific pH values were obtained. Each well of a 96-well plate was filled with 100 µL of CQ solution in buffered medium with different pH values and 100 µL of live, resting conidia at a concentration of 2 × 10⁶ conidia/mL. Fungal damage was analyzed by the XTT assay. In addition, 100 µL of the supernatant was transferred to a new 96-well plate to determine effective maintenance of the pH value during the experiment. The OD of the supernatant was measured at 560 nm. Since the growth medium contains the acid-base indicator phenol red, the absorption properties of phenol
red were correlated with the pH of the growth medium, as described by Lancz et al [21].

**Antifungal Activity of PMNs in the Presence of CQ**

Each well of a 96-well plate was filled with 100 µL of PMNs from healthy volunteers (hereafter, “healthy PMNs”) or patients with CGD (hereafter, “CGD PMNs”) at a concentration of 1 × 10^5 PMNs/mL. Cells were preincubated with 50 µL of CQ for 1 hour to allow uptake of CQ, and 50 µL of live, resting conidia at a concentration 4 × 10^6 conidia/mL was added. CQ concentrations of up to 100 µM (51.6 µg/mL) were evaluated on the basis of the drug’s correlation with the immunomodulatory therapeutic dosage of 400 mg daily given to patients with rheumatoid arthritis [22]. After incubation for 18 hours, PMNs were lysed by addition of 100 µL of saponin 0.005% for 30 minutes at 37°C and underwent centrifugation at 3000 × g for 10 minutes. Cell lysis was confirmed microscopically. Conidial viability was analyzed by the XTT assay. Wells containing PMNs or CQ alone were used as negative controls, while wells containing conidia alone were used as positive controls.

**Cytokine-Stimulation Assays**

PBMCs from healthy volunteers (hereafter, “healthy PBMCs”) or from patients with CGD (hereafter, “CGD PBMCs”) were stimulated with LPS (derived from *Escherichia coli* serotype 055:B5; Sigma-Aldrich) after incubation for 1 hour with CQ at concentrations up to 100 µM. An extra LPS-purification step was performed before use [23]. Each 96-well plate was filled with 100 µL of healthy or CGD PBMCs at a concentration of 1 × 10^6 PBMCs/mL. Fifty microliters of LPS were added to obtain a final concentration of 1 ng/mL. The plates were incubated for 18 hours at 37°C and centrifuged at 3000 × g. Finally, 180 µL of culture supernatant was removed for measurement of cytokines. TNF-α and interleukin 1β (IL-1β) expression were measured by specific enzyme-linked immunosorbent assays (Sanquin Blood Supply, Amsterdam, the Netherlands, and R&D Systems, Oxon, United Kingdom), using specific monoclonal antibodies according to the manufacturer’s instruction. Wells containing only PBMCs were used as negative controls, and LPS-stimulated cytokine release without the addition of CQ was set to 100%. The release of lactate dehydrogenase was measured to assess cell viability for each condition.

**Statistical Analysis**

Each experiment with human cells was performed in quadruplicate for the XTT assay and in duplicate for the cytokine stimulation assay. Mean values ± standard errors of the mean were calculated for all of the experiments under the same conditions. Comparisons between >2 mean values were analyzed by analysis of variance, using a Bonferroni-adjusted level of significance. *P* values of <.05 were considered statistically significant.

**RESULTS**

**CQ Has a Direct Antifungal Activity Against *A. fumigatus* and *A. nidulans***

We first tested the direct antifungal activity of CQ at concentrations ranging from 0.1 mM to 100 mM. We observed a direct dose-response relationship between CQ concentration and fungal damage for both *Aspergillus* species. A CQ concentration of ≥ 0.25 mM resulted in significant fungal damage of *A. fumigatus* (*P* < .0001), while fungal damage of *A. nidulans* occurred at ≥ 0.5 mM (*P* < .05), a 2-fold higher CQ concentration (Figure 1). There was significantly more damage to *A. fumigatus* than to *A. nidulans* (*P* < .0001) in the range of 0.25–1 mM CQ (Figure 1).

**Antifungal Activity of CQ Is pH Dependent**

We next assessed the influence of the environmental pH on the antifungal activity of CQ. An alkaline environment (pH 8) increased the antifungal activity at a fixed concentration (10 mM) against *A. fumigatus*, as well as against *A. nidulans* (*P* < .0001; Figure 2). In a constant acidic environment (pH 6), no difference in fungal damage due to CQ was observed between the *Aspergillus* species (Figure 3A). However, increasing the pH of the medium to 8 resulted in more-exaggerated differences in the susceptibility of the 2 species to CQ. *A. fumigatus* was significantly more susceptible to the antifungal effect of CQ than *A. nidulans* (*P* < .0001 in a pH 8 environment (Figure 3B). Changing the pH of the culture medium in the absence of CQ did not itself result in fungal damage (data not shown). Moreover, the pH of the growth media remained stable, with only very minor deviations (pH ±0.3) over the incubation period in the different experiments performed (data not shown).
CQ Increases the Antifungal Activity of Healthy and CGD PMNs

To further investigate the antifungal activity of CQ as a lysosomotropic and acidotropic agent, we assessed its effect in the presence of healthy and CGD PMNs. The addition of 100 μM CQ increased the antifungal activity of healthy PMNs against *A. fumigatus* by 75% (*P* < .05; Figure 4A). The same trend was seen for *A. nidulans*, although these results were not significant (*P* = .23). Remarkably, a 20-fold lower concentration of CQ (5 μM) increased the antifungal activity of CGD PMNs against *A. fumigatus* by 63% (*P* < .0001; Figure 4B). CQ did not influence the antifungal activity of CGD PMNs against *A. nidulans*.

CQ Inhibits LPS-Induced TNF-α and IL-1β Release by Healthy and CGD PBMCs

To investigate the immunomodulating effect of CQ, we assessed its effect on cytokine release by PBMCs. We observed a higher LPS-induced release of TNF-α and IL-1β by CGD PBMCs as compared to healthy PBMCs (data not shown). We showed a dose-dependent decrease of IL-1β release by healthy and CGD PBMCs at a CQ concentration of ≥5 μM (*P* < .001). A decrease in TNF-α release by healthy PBMCs was observed in the presence of ≥10 μM CQ (*P* < .01), while higher concentrations of CQ (≥50 μM) were needed to inhibit TNF-α release by CGD PBMCs (*P* < .0001; Figure 5). No loss of cell viability in the presence of the different concentrations of CQ used was observed, as indicated by lactate dehydrogenase concentrations in the media. These results are consistent with previous cell-viability studies [19, 24].

CQ Inhibits *A. fumigatus*– and *A. nidulans*–Induced TNF-α and IL-1β Release by Healthy and CGD PBMCs

Finally, we evaluated the fungal immunomodulatory potential of CQ by incubating *A. fumigatus* and *A. nidulans* conidia with healthy and CGD PBMCs. *A. fumigatus* and *A. nidulans* stimulation in the presence of CQ resulted in a dose-dependent decrease of TNF-α and IL-1β release by both healthy and CGD PBMCs (Figure 6). A significant decrease in *A. fumigatus*–induced TNF-α and IL-1β release by healthy PBMCs was observed in the presence of ≥5 μM (*P* < .01) and ≥10 μM (*P* < .0001) CQ, respectively, while higher concentrations of CQ (≥50 μM) were needed to significantly inhibit TNF-α and IL-1β release by CGD PBMCs (*P* < .01 for both). In contrast, significant inhibition of TNF-α and IL-1β was already observed with 5 μM CQ upon *A. nidulans* infection of both healthy PBMCs (*P* < .01 and *P* < .0001, respectively) and CGD PBMCs (*P* < .05 and *P* < .0001, respectively).

**DISCUSSION**

In this study, we demonstrate that CQ has antifungal properties and downregulates the exaggerated proinflammatory cytokine
A direct antifungal effect of CQ on both *A. fumigatus* and *A. nidulans* was found. This effect is enhanced in an alkaline environment. *A. fumigatus* is more susceptible than *A. nidulans* to CQ, and this difference is even more pronounced in an alkaline environment. Furthermore, CQ increases the antifungal activity of healthy and CGD PMNs. Interestingly, this enhanced antifungal activity occurs at a significant lower CQ concentration in association with CGD PMNs as compared to healthy PMNs. Finally, CQ decreases both LPS-induced and *A. fumigatus*- and *A. nidulans*-induced release of TNF-α and IL-1β by healthy and CGD PBMCs.

Previous studies showed accumulation of CQ into the acidic food vacuole of the parasite *Plasmodium falciparum* [25]. Accumulation of quinacrine, an antiprotozoal drug like CQ, has been linked to its increased antifungal effect on *C. neoformans* [9, 10]. *Aspergillus* species also contain acidic vacuoles [26], which are therefore a reasonable location for the accumulation of CQ. The accumulation of CQ or quinacrine in parasites and yeasts has been shown to be maximally enhanced in an acidic environment and diminished in an alkaline environment, as expected for a weak base [9, 25].

We observed a direct pH-dependent antifungal effect of CQ on both *A. fumigatus* and *A. nidulans*. A higher extracellular pH resulted in increased fungal damage at a constant concentration of CQ, suggesting an increased diffusion rate of CQ into the fungal organisms. Direct antifungal activity of CQ against *A. fumigatus* conidia has only been investigated in a study evaluating the contribution of the pksP gene of *A. fumigatus* in intracellular processing of conidia. At a 6-hour time point, no effect on conidial viability was observed at CQ concentrations of ≤1 mM in a pH-neutral or acidic environment [27]. These data do not conflict with ours. Higher concentrations of CQ and an extended incubation period are needed to observe the antifungal effect, and an alkaline environment would lead to an increased antifungal activity of CQ at a given concentration. In an acidic environment, less CQ would diffuse into the Aspergillus species and, consequently, less killing would be observed.

Interestingly, a higher concentration of CQ was necessary to cause fungal damage of *A. nidulans*. We hypothesize that the difference might be explained by 2 mechanisms: variation in diffusion rates of CQ into the fungus or/and different intracellular resistance against the activity of CQ. Since diffusion of CQ is pH dependent, a lower intracellular pH of *A. fumigatus* as compared to that for *A. nidulans* might result in different diffusion rates of CQ and, consequently, antifungal activity. No study has compared the intracellular pH of *A. fumigatus* and *A. nidulans*, but findings from study a study could strengthen this hypothesis. Once accumulated, CQ has an antifungal effect through yet unknown mechanisms. Differences in alkalinization of the fungus or inhibition of the activity of proteins and enzymes may result in the different susceptibility between *A. fumigatus* and *A. nidulans*. Future research is required to investigate the mechanisms underlying the antifungal activity of CQ and the increased susceptibility of *A. fumigatus*, compared with *A. nidulans*.

The next step was to investigate the additional antifungal activity of PMNs on *Aspergillus* species in the presence of CQ. We found an enhanced antifungal activity of both healthy and CGD PMNs. Remarkably, for healthy PMNs this increase occurred at 100 µM CQ, while for CGD PMNs this increase was already seen at 5 µM CQ, a 20-fold lower concentration. The absence of phagolysosomal alkalinization in CGD PMNs might be responsible for an increased diffusion of CQ into the relative acidic environment of the CGD cell. Consequently, the same...
extracellular concentration of CQ would result in a higher intracellular concentration in the CGD PMNs as compared to the healthy PMNs, resulting in increased antifungal activity of CGD PMNs at lower CQ concentrations.

Lack of phagosomal alkalization in the CGD phagocytic cell is suggested to play a key role in the direct antimicrobial activities of the phagocyte. Reeves et al demonstrated that killing activity of PMNs is not directly related to reactive oxygen species but is mediated through activation of proteases by a K+ flux that crosses the membrane in a pH-dependent manner, followed by release and activation of granule-derived antimicrobial enzymes [5]. Considering that one property of CQ involves alkalizing the neutrophil lysosome, it is suggested that CQ improves the antimicrobial function of neutrophils via pH change, mainly by non-oxidative mechanisms. The enhanced antifungal activity and the differences between healthy and CGD PMNs suggest a clear relationship with the alkalizing properties of CQ.

An additional proposed mechanism of enhanced fungal killing through CQ is by limiting iron availability [28–30]. Iron acquisition is critical to the survival of *A. fumigatus* and *A. nidulans* [32, 33]. PMNs are able to provide iron uptake by the reduction of Fe3+, resulting in the release of iron from transferrin. Iron restriction has been proposed to cause increased antifungal activity of monocyte-derived macrophages against *C. neoformans* [11]. Considering the iron-dependent survival of *Aspergillus* species, CQ might reduce its growth via restricting the iron release from transferrin of PMN.

Absence of the respiratory burst is associated with dysregulated cytokine production, resulting in more proinflammatory cytokine release, which further contributes to the pathogenesis of invasive aspergillosis in patients with CGD [8]. Reactive oxygen species likely dampen inflammasome activation, and NADPH-oxidase complex–defective PBMCs are a source of elevated IL-1β release [34, 35]. Therefore, immunomodulating agents, besides antifungal treatment, that target the dampening of inflammation during invasive aspergillosis in patients with CGD may be worthwhile to explore. CQ is known for its immunomodulatory properties and is used in patients with rheumatoid arthritis and lupus [16, 36]. Furthermore, CQ has been shown to antagonize the proinflammatory cytokine response to

Figure 5. The effect of chloroquine on cytokine release by peripheral blood mononuclear cells (PBMCs) from healthy volunteers (hereafter, “healthy PBMCs”) and PBMCs from patients with chronic granulomatous disease (hereafter, “CGD PBMCs”). Bars indicating 0 on the x-axis are lipopolysaccharide (LPS)–stimulated PBMCs without chloroquine. The LPS-stimulated cytokine release without chloroquine was set to 100%. Chloroquine reduces LPS-induced tumor necrosis factor α (TNF-α; A) and interleukin 1β (IL-1β; B) release by healthy PBMCs (n = 6). Chloroquine reduces IL-1β release by CGD PBMCs (n = 4–5; D), whereas higher concentrations are necessary to reduce the release of TNF-α (C). Error bars indicate standard error of the mean. *P < .001, **P < .0001.
opportunistic fungi such as C. neoformans and C. albicans by alkalizing the fungal phagolysosome. The therapeutic efficiency of hydroxychloroquine was reported in treatment of granulomatous complications in CGD [37]. Our results show that IL-1β and TNF-α release, upon stimulation with LPS and Aspergillus species, by both healthy and CGD PBMCs can be inhibited by CQ. Interestingly, during A. nidulans infection a significant decrease of both TNF-α and IL-1β release by healthy and CGD PBMCs was already seen at lower concentrations, compared with observations during A. fumigatus infection. 

Jang et al have studied the mechanisms by which CQ leads to a diminished TNF-α and IL-1β production in human monocytes and showed that different mechanisms were involved for specific cytokines [17]. In LPS-stimulated monocytes, CQ blocks the conversion of cell-associated TNF-α precursor to mature soluble protein, whereas it reduced the levels of IL-1β messenger RNA, at least in part, by decreasing their stability and by a pH-dependent mechanism. The alkalizing effect of CQ as a weak base was a more important factor responsible for the decrease in IL-1β release, compared with TNF-α release. The more pronounced effect on IL-1β release might be especially beneficial in patients with CGD, since infection of human CGD leucocytes with A. fumigatus or A. nidulans leads to exaggerated IL-1β secretion. The increased immunomodulatory effect of CQ on A. nidulans-induced IL-1β release is remarkable, especially because we observed a significantly higher level of IL-1β release after infection with A. nidulans, compared with infection with A. fumigatus (unpublished data). Studies to unravel IL-1β processing in patients with CGD infected by A. nidulans and A. fumigatus and the influence of CQ on this process are ongoing.

In summary, CQ has antifungal properties and dampens the inflammatory response of CGD cells in vitro. The antifungal activity of CQ might be beneficial in the clearance of fungal infections in patients with CGD. More importantly, the immunomodulating effect of CQ should be considered for dampening the hyperinflammatory state in patients with CGD and thereby reducing their inflammatory complications and further improving life expectancy and quality of life. Future research is necessary to translate these promising findings to clinical practice.

**Note**

*Potential conflicts of interest.* All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.
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