High-dose ascorbate with low-dose amphotericin B attenuates severity of disease in a model of the reappearance of candidemia during sepsis in the mouse

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High-dose ascorbate with low-dose amphotericin B attenuates severity of disease in a model of the reappearance of candidemia during sepsis in the mouse. Am J Physiol Regul Integr Comp Physiol 309: R223–R234, 2015. First published May 20, 2015; doi:10.1152/ajpregu.00238.2014.—Amphotericin B (Ampho B) is a fungicidal drug that causes cell wall injury. Pharmacological ascorbate induces the extracellular prooxidants, which might enter the Ampho B-induced cell wall porosity and act synergistically. We tested low-dose Ampho B with a short course of pharmacological ascorbate using a mouse model of sepsis preconditioned with an injection of Candida albicans 6 h prior to cecal ligation and puncture (CLP). In this model, candidemia reappeared as early as 6 h after CLP with a predictably high mortality rate. This characteristic mimics sepsis in the phase of immunosuppression in patients. Using the model, at 12- and 18-h post-CLP, we administered isotonic (pH neutralized) pharmacological ascorbate intravenously with low-dose Ampho B or sodium deoxycholate, vehicle-controlled, administered IP. The survival rate of low-dose Ampho B plus ascorbate was 53%, compared with <11% for low-dose Ampho B or high-dose Ampho B alone. In addition, a beneficial effect was demonstrated in terms of kidney damage, liver injury, spleen histopathology, and serum markers at 24 h after CLP. Kidney injury was less severe in low-dose Ampho B plus ascorbate combination therapy due to less severe sepsis. Moreover, ascorbate enhanced the effectiveness of phagocytosis against C. albicans in human phagocytic cells. Taken together, the data indicate that the new mouse model simulates sepsis-induced immunosuppression and that the combination of pharmacological ascorbate with an antifungal drug is a potentially effective treatment that may reduce nephrotoxicity, and perhaps also increase fungicidal activity in patients with systemic candidiasis caused by Candida albicans.

Candida albicans; ascorbate; sepsis; amphotericin B

SEPSIS IS A SERIOUS, GENERALIZED immune response to systemic infection, independent of the infecting organisms (42). The

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Table 1. The minimal inhibitory concentration that demonstrated antifungal resistance to the Candida albicans used in this study

<table>
<thead>
<tr>
<th>Drug</th>
<th>MIC, ng/ml</th>
</tr>
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<tbody>
<tr>
<td>Amphotericin B</td>
<td>125</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>125</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>31.3</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>31.3</td>
</tr>
<tr>
<td>Posaconazole</td>
<td>31.3</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>31.3</td>
</tr>
<tr>
<td>Anidulafungin</td>
<td>15</td>
</tr>
<tr>
<td>Caspofungin</td>
<td>15</td>
</tr>
</tbody>
</table>

MIC, minimal inhibitory concentration.

*Candida* infection, characterized by *Candida* injection after sepsis (9), may not reflect *Candida* infections in humans, which mostly resulted from endogenous sources (50). As a result, we developed a new sepsis-induced candidemia reappearance model that is characterized by the reactivation of an exogenous *Candida* administration prior to the onset of sepsis. We used this new system to test Ampho B administered alone and in combination with pharmacological ascorbate.

**METHODS**

*C. albicans* Preparation, Characterization, and Germ Tube Test

*C. albicans* was isolated from blood samples (Mycology Unit, King Chulalongkorn Memorial Hospital), identified by morphology together with the API 20C AUX yeast identification kit (Biomuriex, Lyon, France), and stored in Sabouraud dextrose agar (SDA) (Thermo Scientific, Hampshire, UK) at −80°C. Before conducting all of the experiments, *C. albicans* was subcultured in SDA at 35°C for 24 h. The characteristics of the clinically isolated *C. albicans* were demonstrated by the minimal inhibitory concentration (MIC) (Table 1) and 50% lethal dose (LD50). MIC analysis followed a standard protocol (43), with all reagents purchased from Sigma-Aldrich (St. Louis, MO). The Clinical Laboratory Standard Institute recommended strains *Candida krusei* American Type Culture Collection (ATCC) 6258 and *Candida parapsilosis* ATCC 22019 were used for quality assurance. The LD50 of selected *C. albicans* was 4×10⁵ blastospores as counted by hemocytometer (Bright-Line, Denver, CO), which was equal to the value measured by a spectrophotometer (EL×808 absorbance reader; BioTek, Shoreline, WA) by the optical density at 630 nm at 0.45 (OD 630 nm at 0.45). The procedure for LD50 was described in detail in the animal section below. For the germ tube test, 300 blastospores of *C. albicans*, as determined by hemocytometer, were placed in 50 μl of serum from a septic mouse (24 h after CLP) or serum from a sham mouse, incubated at 37°C for 3 h, then counted by hemocytometer. The percentage of the ratio of blastospores with germ tube formation to total blastospores was calculated.

**Animal and Animal Models**

The U.S. National Institutes of Health (NIH) criteria and protocols for the use and treatment of laboratory animals were followed (NIH publication protocols no. 85-23, revised 1985). Male, 8–10-wk old BalB/C mice (National Laboratory Animal Center, Nakornpathom, Thailand) were used. The animal protocols were approved by the Institutional Animal Care and Use Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. All procedures were performed under isoflurane anesthesia.

*Candida albicans* injection model. Resuspended *C. albicans* in normal saline solution (NSS) adjusted into different doses of optical density at 630 nm in the same volume of each preparation (100 μl) was injected through the tail vein. The LD50 was extrapolated from the correlation equation of the graph of the 7-day mortality of mice, which was injected with *C. albicans* in different OD 630-nm doses (0.8, 0.6, 0.4, 0.2, and 0.1). The *Candida* dose with 50% mortality rate was calculated and identified as LD50.

Cecal ligation and puncture sepsis model. The cecal ligation and puncture (CLP) procedure was described in detail previously (34), with some modifications. Briefly, the cecum was ligated at 12 mm from the cecal tip and punctured twice with a 23-gauge needle, leaving a small amount of fecal material exposed. The abdominal incision was closed in two layers with 6-0 nylon sutures. Antibiotic (imipenem/cilastatin 7 mg/kg in 0.3 ml NSS) was administered subcutaneously at 3 h and 8 h after CLP for 24-h end-point experiments. Additional antibiotic doses were administered at days 2, 4, and 6 after operation for 7-day survival experiments (Fig. 1).

A model of reappearance of candidemia during sepsis (*candida*+CLP). One hundred microliters of *C. albicans* with the dose of OD 630 nm at 0.3 (≈2×10⁵ blastospores) or reciprocal NSS was injected through the tail vein at the −6 h time point (Fig. 1). Then at 0 h, the CLP operation or sham laparotomy was performed. The schedule of antibiotic administration was demonstrated in Fig. 1. In parallel, a reciprocal volume of NSS was injected in the sham group. Blood was collected through the right retroorbital plexus under

![Fig. 1. Timeline of a model of reappearance of candidemia in sepsis (*Candida*+candida ligation and puncture (CLP)). In 24-h experiments, *Candida albicans* was intravenously injected at 6 h before cecal ligation and puncture (CLP) (−6 h), then subcutaneously administered antibiotic (ATB sc) was administered at 3 h and 8 h. Subsequently, the intravenous (tail vein) ascorbate (Asc) or normal saline (NSS) plus intraperitoneal amphotericin B 0.1 mg·kg⁻¹·dose⁻¹ (0.1 MK) or 1 mg·kg⁻¹·dose⁻¹ (1 MK) or intraperitoneal deoxygycoclate (De-cholate) were administered at 12 h and 18 h after CLP. The mice were euthanized at 24 h after CLP. In the 7-day survival experiment, additional ATBs were injected subcutaneously at 1, 2, 4, and 6 days after CLP, and intraperitoneal amphotericin B 1 mg·kg⁻¹·dose⁻¹ (Ampho B 1 MK) or De-cholate was given at 2, 4, and 6 days after CLP.](http://ajpregu.physiology.org/doi/10.1152/ajpregu.00238.2014)
isoflurane anesthesia with a microhematocrit capillary tube (Fisher Scientific, Pittsburg, PA) and through cardiac puncture at the end-point of experiments. A blood volume of 15–50 μl was drawn at each time point, depending on the frequency of blood collection in specific models. The experiments were repeated if there were any insufficient blood sample for the analysis. At the time of euthanasia, internal organs (lungs, kidneys, spleen, and liver) and peritoneal fluids were collected in 4°C PBS for the fungal culture and fixed in 10% formalin for the organ histology.

Treatments of Ascorbate and Amphotericin B
Ascorbate (Sigma-Aldrich) was adjusted to pH 7 with sodium hydroxide (NaOH) for all experiments. Ascorbate 0.9 g/kg in sterile water or the same volume of NSS pH 7 as a control solution was administered through the tail vein at 12 h and 18 h after CLP surgery. In parallel, Ampho B (Amphotret, Maharashtra, India) at 1 mg·kg⁻¹·dose⁻¹ and 0.1 mg·kg⁻¹·dose⁻¹ or sodium deoxycholate (De-cholate) (Sigma-Aldrich) 0.8 mg·kg⁻¹·dose⁻¹, vehicle-controlled, were injected intraperitoneally at 12 h and 18 h (Fig. 1). There was no difference in the severity of sepsis between low or high doses of De-cholate, 0.08 and 0.8 mg·kg⁻¹·dose⁻¹, respectively (data not shown). For the 7-day survival experiments, additional doses of Ampho B 1 mg·kg⁻¹·dose⁻¹ or De-cholate were administered intraperitoneally (Fig. 1). The ascorbate level after the administration on day 2 could not reach the therapeutic level, as defined by serum ascorbate more than 10 mM (data not shown).

Polymorphonuclear Cell Count, Blood Chemistry, and Cytokine Concentrations
A volume of 15 μl of blood was mixed with 250 μl of 3% acetic acid, a hemolytic solution, and the total number of leukocytes was counted with a hemocytometer. Simultaneously, 10 μl of blood was smeared on a glass slide for Wright stain and counted with ×100 magnification in 100 fields to determine the percentage of polymorphonuclear cells (PMN). The total number of PMN was calculated by total leukocyte count from hemocytometer multiplied by the percentage of PMN from the Wright stain glass slide. Serum ascorbate (Asc) and alamine transaminase (ALT) were measured with colorimetric detection by ascorbic acid assay (BioAssay U.S. Biological, Salem, MS) and ALT assay (DiaSys Diagnostic Systems, Holzheim, Germany), respectively. Serum creatinine (Scr) was measured by HPLC (32–34). Serum TNF-α, IL-6, IL-10, and IL-17 were determined by the LumineX xMap-based multiplex technology MILLIPLEX MAP (multianalyte panels) 4-plex cytokine kit (Millipore, Billerica, MA), according to the manufacturer’s recommended procedure.

Microorganism Burdens in Blood, Peritoneal Fluid, and Internal Organs
Blood and peritoneal fluid in serial volume was immediately diluted in PBS, plated in SDA for 24 h at 35°C for fungal colony counting, and reciprocally placed in blood agar (Oxoid, Hampshire, UK) for 24 h at 37°C for bacterial colony counting. Internal organs were washed twice in PBS, weighed, homogenized, diluted in PBS, and incubated in SDA. The colony count in SDA after 24 h at 35°C was defined as the C. albicans burden. Moreover, to test whether there was C. albicans in feces and gastrointestinal tissues of mice without a preconditioning Candida injection, the different amounts of fecal contents were collected. Serial dilutions of gastrointestinal contents and tissue from cecum, colon, and recto-sigmoid area were collected from normal mice and septic mice at 24 h after CLP. Feces, well-mixed in PBS, and tissues were plated directly in SDA with 0.1% chloramphenicol (Bio-Rad, Hercules, CA), Sabouraud dextrose broth (SDB), and SDB with 0.05 g/l chloramphenicol (Sigma-Aldrich).

Organ Histology
Tissue was fixed in 10% formalin and embedded in paraffin; 4-μm sections were stained with periodic acid-Schiff reagent. Kidney damage was defined as tubular epithelial swelling, loss of brush border, and vacuolar degeneration. The degree of kidney damage was estimated at ×200 magnification using 10 randomly selected fields, according to the following criteria: 0, normal; 1, area of damage <25% of tubules; 2, damage involving 25–50% of tubules; 3, damage involving 50–75% of tubules; and 4, 75–100% of the area being affected (35). Spleen damage was determined by counting the number of vacuole-like areas and groups of necrotic and apoptotic cells (44) in the white pulp of the spleen at ×400 magnification.

In Vitro Experiment for the Synergistic Effect of Ascorbate and Amphotericin B
One hundred microliters C. albicans in the dose of OD 630 nm at 0.7 diluted in Rose Park Memorial Institute (RPMI) 1640 was put into a 96-well microplate. Ampho B dissolved in dimethyl sulfoxide (DMSO) to a concentration of 125 ng/ml, 10 times lower than the MIC (Table 1), with serial concentrations of ascorbate (5–40 mM) adjusted to the neutral pH (pH 7) were mixed into each well. RPMI was added, as needed, to fully and equally fill each well. OD 630 nm was read after 24 h of incubation at 35°C to determine the number of Candida. The number of Candida was determined after 24 h of the incubation at 35°C by a spectrophotometer with OD at 630 nm.

Phagocytosis and Killing Assays
Human phagocytes from healthy donors were separated by 6% dextran solution, as previously described (41). Coincubation of 3 × 10⁵ phagocytes/ml with 3 × 10⁷ blastospores/ml of C. albicans in PBS with the serial concentrations of ascorbate (10–40 mM) adjusted to pH 7 at 37°C for 1 h was performed. Then, 100 μl of well-mixed solution was isolated and stained with Wright’s stain according to the standard protocol. Phagocytic activity was determined by the percentage of phagocytes with Candida blastospores inside to the total phagocyte count at ×400 magnification from 100 fields per slide. Concurrently, to determine the phagocyte-killing function, 150 μl of the solution was centrifuged to separate phagocytes, then washed twice with PBS, recentrifuged, and filled with 100 μl of a nutritionally rich medium (Lysogeny broth; Sigma-Aldrich) to initiate complete cell disruption and the release of intracellular blastospores. The solution was then put in SDA at 35°C for 24 h and counted for fungal colonies (16). All experiments were performed in triplicate.

Statistical and Survival Analysis
SPSS version 17.0 (SPSS, Chicago, IL) was used for all statistical analyses. Data are presented as mean values and the standard error of the means (means ± SE). Differences among groups of results were examined using one-way ANOVA, followed by Bonferroni analysis. The two-tailed Student’s t-test was used to examine the difference of the optical density at 630 nm between Asc and Ampho B + Asc and also used to demonstrate the difference in serum creatinine between Candida plus CLP and sham. A P value <0.05 was considered statistically significant. Survival analyses were evaluated using the log-rank test by observation and recorded every 6–12 h for the first 72 h after Candida injection or CLP surgery, and then once daily to 7 and 14 days for CLP survival and Candida injection survival, respectively. Mice exhibiting extreme morbidity were euthanized. Some of the mice with the manifestations of the extreme morbidity, such as abnormal breathing patterns or no response to stimuli, were euthanized, and their internal organs were examined for organ histology.
administration of various doses of Candida at 24 h is demonstrated. A, inset: graph magnification is shown that indicates zero Candida blood level. B: candidemia at 7 days observation (n = 5/time point). Candida burdens in different organs at 6 h and 24 h after Candida injection at the dose of optical density (OD) 630 nm 0.3 are shown (C) (n = 4–6/group). Measured parameters (means ± SE) were significantly different among the absorbance for fixed time point using one-way ANOVA followed by Bonferroni analysis with alpha set at 0.05 and four comparisons for A and B and eight comparisons for C. (a,b,c,P < 0.05; a is OD 1.2 vs. OD 0.8, b is OD 1.2 vs. OD 0.6, and c is OD 1.2 vs. OD 0.3).

RESULTS

Characteristics of Candida Injection Model

After the injection of different doses of Candida, candidemia presented after all of the selected doses of the injection. Candidemia became undetectable after 3–6 h for low-dose injections (OD 630 nm 0.3 and 0.6) (Fig. 2A, inset) and then reappeared (Fig. 2B). Despite the disappearance of Candida from the blood after 6 h and 24 h after injection with a dose of OD 630 nm 0.3, Candida was present in all measured organs (Fig. 2C). At 6 h after injection, Candida was predominantly present in the lung and kidney 59 ± 5 and 43 ± 12 CFU·ml⁻¹·mg⁻¹, respectively. The presence of Candida was predominant in the liver and spleen, 20 ± 7 and 11 ± 3 CFU·ml⁻¹·mg⁻¹, respectively (Fig. 2C). At 24 h, the organ in which Candida was most presented was the kidney, at 18 ± 7 CFU·ml⁻¹·mg⁻¹ (Fig. 2C). These results indicated that Candida infection remained in tissues despite clearance from the blood after this inoculation dose. The candidemia clearance after the inoculation dose of OD 630 nm 0.3 (2 × 10⁵ CFU/ml) facilitated the identification of this dose level for the further experimental study of the reactivation of candidiasis.

Characteristics of a Model of Reappearance of Candidemia During Sepsis (Candida+CLP)

The goal of a superimposed Candida infection in a sepsis model is to have reappearance of Candida, so as to mimic Candida infection as it occurs after sepsis in humans. Therefore, the reappearance of candidemia was demonstrated. Candidemia spontaneously reappeared from the 4th day after Candida injection alone (Fig. 2B), but reappeared as early as 6 h after Candida+CLP (Fig. 3A), ~93 h earlier. Moreover, candidemia in Candida+CLP increased very rapidly from 34 ± 16 CFU/ml at 3 h to 2,143 ± 434 CFU/ml at 24 h after CLP (Fig. 3A). In contrast, Candida injection alone without CLP showed a delayed and more modest increase in candidemia from 15 ± 10 CFU/ml at day 4 to 1,650 ± 897 CFU/ml at day 7 after injection (Fig. 2B). Of note, CLP alone did not produce candidemia at 24 h after CLP (Fig. 3A) and at 7 days (data not shown). Moreover, C. albicans could not be detected by culture (see methods) of cecal contents and gastrointestinal tissues of normal and septic mice. Sepsis from CLP without Candida, coupled with postsurgery treatment, showed a survival rate of 73% (11 of 15 mice) at day 7 (NSS+CLP), indicating a modest severity of sepsis in our CLP model (Fig. 3B). The addition of Candida infection before sepsis (Candida+CLP) produced 100% mortality at 5 days (Fig. 3B). Moreover, mice with Candida+CLP died rapidly, with >50% mortality within 1 day and >67% mortality within 2 days (Fig. 3B). Rapid mortality in the Candida+CLP model is likely due to the severity of sepsis. Compared with controls, within the first 24 h Candida+CLP mice had higher cytokines and more pronounced neutropenia than CLP alone (Fig. 3, C–G), as well as higher fungal burden (Fig. 3A). Limited leukocytosis and/or rapid neutropenia could be factors in the severity of Candida+CLP. In contrast, germ tube production in septic serum was lower than in normal serum (Fig. 3H). High blood Candida levels in CLP mice (Fig. 3A), despite less Candida growth in vitro with septic serum, is consistent with an immunologic cell role in the control of candidemia. Candida+CLP mice at 3 days after injection showed multiple Candida pseudohyphae lesions on the heart, renal glomeruli, and renal interstitium (Fig. 4). These findings are consistent with disseminated candidiasis. The reappearance of Candida from internal sources in the Candida+CLP mice resembles human sepsis in terms of the reactivation of latent Candida, although from different sources of Candida (21, 50). Subsequently, we used the Candida+CLP model for further
studies due to the Candida reactivation property of the model.

The In Vitro Synergistic Effect of Amphotericin B with Ascorbate and In Vivo Ascorbate Concentrations

We used the Candida+CLP model to test the synergistic properties of Ampho B and ascorbate, beginning with in vitro experiments. The Ampho B concentration was selected at 10 times lower than the minimal inhibitory concentration (MIC), and incubations were performed with different pharmacological ascorbate concentrations. The Ampho B concentration was chosen such that no fungicidal effect was predicted with its use alone. Also, a very low dose of Ampho B could reduce nephrotoxicity in vivo. Ascorbate concentrations were chosen to reflect those measured in humans after intravenous administration (66, 67). As predicted, neither low-dose Ampho B (125 ng/ml) alone nor ascorbate alone had any effect on Candida growth. Ampho B combined with ascorbate concentrations ranging from 10 to 40 mM demonstrated growth inhibition of ~85% (Fig. 5). Alternatively, there was no synergistic effect of ascorbate at these concentrations with fluconazole at a dose 10 times lower than the MIC (125 ng/ml) (data not shown). It appears that the synergistic effects of high-dose ascorbate may be limited to only specific antifungal drugs.

Subsequently, ascorbate concentration at or above 10 mM was chosen as the target level of serum ascorbate for further experiments. We take advantage of renal dysfunction in the Candida+CLP model to maintain serum ascorbate (Fig. 6A). While ascorbate is rapidly cleared in mice without renal insufficiency, ascorbate concentrations are maintained at or above 10 mM for many hours in Candida+CLP mice with ascorbate injection at 12 h and 18 h after surgery (Fig. 6B). Ascorbate injections administered prior to 12 h post-CLP were cleared more rapidly (data not shown). In addition, the treatment of patients is usually performed after the onset of the diseases, which is similar to the delayed treatment in the mouse model. For these reasons, we conducted experiments with treatment
beginning 12 h after sepsis to test the synergy of pharmacologic ascorbate with low-dose amphotericin B.

**Low-Dose Amphotericin B Plus Pharmacologic Ascorbate In Vivo: Effects in a Model of Reappearance of Candidemia During Sepsis (Candida+CLP Model)**

The objective of these experiments was to test the therapeutic effects of low-dose Ampho B (0.1 mg·kg⁻¹·dose⁻¹, which is 10 times lower than the MIC) combined with pharmacological ascorbate. As controls, deoxycholate alone (NSS+De-cholate), pharmacologic ascorbate with deoxycholate (Asc+De-cholate), low-dose Ampho B alone (NSS+Ampho B 0.1 MK), and full-dose Ampho B alone (NSS+Ampho B 1 MK) showed no survival benefit at 7 days (Fig. 7, A and B). Only ascorbate combined with low-dose Ampho B (Asc+Ampho B 0.1 MK) improved the 7-day survival, from 9% (2/22) to 54% (12/22) (Fig. 7B). It is both notable and interesting that full-dose Ampho B alone improved the survival in days 1 and 2, but was reversed on day 3, leading to high mortality and a low 7-day survival rate (Fig. 7B). In bacterial sepsis alone without Candida injection (CLP), there was a tendency toward improved sepsis survival in the ascorbate administration group, but the improvement was not statistically significant (Asc+De-cholate vs. NSS+Decholate) (Fig. 7C). Although Ampho B administration in CLP mice did not noticeably worsen the severity of sepsis (NSS+De-cholate vs. NSS+Ampho B 1 MK), it seemed to neutralize the potential benefit of ascorbate administration (Asc+Ampho B) (Fig. 7C). To determine the microorganism loads, blood and peritoneal fluid were collected at 24 h for Candida and bacterial cultures. As expected, candidemia and peritoneal Candida was lower with low-dose Ampho B plus ascorbate, but not with low-dose Ampho B alone (Fig. 8, A and B), consistent with the in vitro data (Fig. 5). However, bacterial burden was not different between groups (data not shown). In addition, we evaluated sepsis severity in the Candida+CLP model at 24 h, based on the following serum markers: Scr for renal function, ALT for liver function, and cytokines for immune responsiveness. We also obtained histopathology data of the kidney, spleen, and heart (Fig. 9). Low-dose Ampho B combined with pharmacological ascorbate attenuated organ injury: kidney injury as determined by Scr (Fig. 8C) and renal histopathology (Fig. 9H), liver injury as measured by ALT (Fig. 8D), and spleen injury as evaluated by vacuole-like lesions in the white pulp of the spleen (Fig. 9I). Moreover, there was cardiac vacuolization in some mice re-
ceiving low-dose Amphot B treatment alone, but not in mice with the combined treatment (Fig. 9G). Amphot B combined with pharmacological ascorbate reduced IL-6, TNF-α, and IL-10, but not IL-17 (Fig. 8, E–H).

**Higher Ascorbate Concentration Improved Phagocytosis and Intracellular Killing of Candida by Human Phagocytic Cells**

Effective phagocytosis and phagocyte killing are mechanisms for the control of candidiasis, especially in the setting of neutropenia (32). The phagocyte killing depends on oxidative stress production, which might be associated with ascorbate (61). We tested the ability of human phagocytic cells to phagocytize and kill Candida, in the presence of different ascorbate concentrations. Phagocytic activity was enhanced with pharmacological ascorbate concentrations of 30 and 40 mM (Fig. 10A), and Candida killing activity improved at concentrations of 20 and 40 mM (Fig. 10B).

**DISCUSSION**

Prior mouse models of Candida used Candida injection after sepsis (9), but this may not be the ideal method to mimic reactivation of candidemia. In this study, we injected Candida before sepsis, which was induced by CLP surgery 6 h after Candida injection. We demonstrated that sepsis reactivated candidemia, resembling the alteration of intrinsic Candida
microbiota in patients with candidiasis (50). We found more severe injuries and a much higher mortality rate in Candida/H11001 CLP, compared with CLP alone. Using this model, we were able to investigate a new treatment modality and the combination of pharmacological ascorbate with low-dose amphotericin B (Ampho B). This combination improved survival and attenuated disease severity, compared with the use of either modality alone.

The severity of intravenous Candida albicans infection depends on the injected doses and the properties of C. albicans used in different studies (36, 56). With high-dose Candida injection, candidemia persisted at all time points. As for low-dose Candida injection, mice cleared up candidemia very rapidly within the first 24 h after injection, with no spontaneous reappearance until after 4 days. Even with rapid clearance of candidemia in the low-dose model, tissue Candida persisted in the lung, kidney, and liver, consistent with other reports (36, 66). For these reasons, low-dose injections seemed to suitably mimic the immunosuppression phase, despite the more rapid natural course in mice, compared with human patients with sepsis. It is interesting to note that Candida injection alone without CLP did not cause renal injury at 24 h after injection, as measured by serum creatinine (Scr) (Figs. 8C and 9H), despite Candida being detected in the kidney at 24 h (Fig. 2C).

Decreasing the time interval needed for Candida reappearance would improve the experimental model of sepsis immunosuppression. Candida reappeared as early as 6 h after sepsis initiation in a model of reappearance of candidemia during sepsis (Fig. 3A). In comparison, more than 4 days were needed in nonseptic mice for Candida reappearance (Fig. 2B). Several sources of Candida reactivation were demonstrated in several organs (Fig. 2C) for mice in the Candida+CLP model, which was different from the translocation across the gut mucosa in patients with superimposed candidiasis (14). However, the
reactivation of the existing internal sources of Candida may more closely resemble the human condition, compared with other current models. Sepsis immunosuppression is initially defined as a condition in which patients or experimental animals are susceptible to opportunistic infection in sepsis patients (40, 55). There are at least two hypotheses related to the sepsis-immunosuppressive phase. The serial theory states that immunosuppression takes place after the hyperinflammatory phase. The parallel theory states that immunosuppression is concomitant with hyperinflammation (3, 52). Generally, immune compromise after sepsis can be manifested by the reactivation of endogenous infections, such as cytomegalovirus, herpes virus, mycobacteria, and candidosis. We used candidemia reactivation as a marker of sepsis immunosuppression. It was necessary to inject Candida to be able to study its reactivation as part of sepsis immunosuppression. Without prior

Fig. 9. Low-dose Ampho B with high-dose Asc attenuated organ histology of a model of reappearance of candidemia in sepsis (Candida+CLP). The representative figures of renal histology at 24 h of the model with PAS staining in sham surgery models (Candida+Sham, NSS administered at 12 h and 18 h) (A), Asc or NSS administered with CLP surgery models (Candida+CLP with Asc or NSS administered at 12 h and 18 h; B and C). In parallel, spleen histology in individual group (D–F) and cardiac histology in non-Asc-treated Candida+CLP (G) is demonstrated. The original magnification in all of histology figures was ×400. The histological scoring from renal (H) and spleen histology (I) is shown (n = 5 or 6/group). Measured parameters (means ± SE) were significantly different among the Candida+Sham with NSS, Candida+CLP with Asc, and Candida+CLP with NSS groups for fixed time point using one-way ANOVA, followed by Bonferroni analysis with alpha set at 0.05 and three comparisons.

Fig. 10. Ascorbate-enhanced effective phagocytosis of human phagocytes. The positive phagocytic cells after incubation with Candida blastospore (A) and the Candida killing activity (B) with different ascorbate concentrations are shown. Data (means ± SE) were obtained from five or six independent experiments. Measured parameters were significantly different among the ascorbate concentration groups for using one-way ANOVA, followed by Bonferroni analysis with alpha set at 0.05 and five comparisons.
Candida injection, we could not identify C. albicans by culture methodology in gastrointestinal tissue and contents, the natural reservoir of Candida (14), from either normal or septic mice. Several explanations for this failure to identify C. albicans are possible, including 1) conventional culturing methods may be insufficient to detect Candida as part of normal mouse flora; 2) Candida may not be part of normal mouse flora; or 3) the presence of Candida may be dependent on mouse environment or strain. More sensitive detection methods and/or longer observation times after sepsis (60) might be necessary to demonstrate spontaneous candidemia after sepsis alone. Nevertheless, the reactivation of candidemia in Candida+CLP was very rapid, within hours after sepsis, compared with days or weeks in patients in the intensive care unit. This difference might, at least in part, be due to more focused and appropriate treatments and life support systems for patients, a notable absence in experimental studies using mice.

The Candida injection changed a modest severity of CLP model with a 30% mortality rate into more severe injury with a 100% mortality rate (Fig. 3B). A number of factors could be responsible. The germ tube findings that we report here show that the serum of sepsis mice was less effective than normal mouse serum as for fungal growth, despite an exponential increase in Candida in sepsis. This finding suggests that changes in immune response elements could be responsible for accelerated candidemia in Candida+CLP septic mice. One contributing factor may be a decreased neutrophil response in Candida+CLP compared with CLP alone, consistent with the neutrophil depletion occurring more rapidly when sepsis is combined with candidemia (31, 32). Rapid neutrophil depletion in Candida+CLP might be due to the higher sepsis severity, which leads to the higher rate of neutrophil apoptosis, as previously mentioned (18). Another factor may be associated with IL-17 responsiveness in Candida+CLP mice, as IL-17 is a cytokine responsible for the fungal infection (25, 26). While Candida+CLP mice had an increase in IL-17 compared with CLP mice, the increase may have been insufficient for host protection. IL-17 was the only cytokine, among others we tested, with a similar level at 24 h in Candida+CLP, compared with CLP alone. Moreover, IL-17 was the earliest cytokine to increase as early as 3 h after Candida+CLP, but IL-17 did not increase in CLP alone. This characteristic suggested the importance of IL-17 in controlling the severity of sepsis in the Candida+CLP model. We propose that IL-17 synthesis in Candida+CLP was more strongly stimulated by both bacterial and fungal infection (25, 26). The dual importance of IL-17 in both bacterial and fungal infection was previously published (19, 20, 25, 28). Still another contributing factor of high mortality in Candida+CLP mice could be injury to the spleen. In this study, we found groups of apoptotic/necrotic cells in the white pulp of the spleen, previously described as a “vacuole-like area”, were more prominent in Candida+CLP spleens, consistent with the description in severe sepsis (44). Apoptosis of immune cells, especially apoptosis in the spleen as reported in patients with sepsis (24) and mouse models of sepsis (34, 35, 44), was hypothesized to be one of the responsible factors for the immunosuppression and more severe injury in sepsis. The severe destruction of the spleen might be an additional contributing factor to the immunosuppression observed in the Candida+CLP model.

In animals and humans, concentrations of ascorbate (vitamin C) are normally tightly controlled when it is ingested orally, via transporter saturation (45–47). In humans, intestinal transporters saturate at doses above 200 mg, with decreased bioavailability. Tissue transporters saturate when ingested doses are above 100 mg. Likewise, the renal reabsorptive ascorbate transporter saturates at doses above 100 mg, with urinary excretion of unabsorbed ascorbate. Together, these mechanisms produce orchestrated ascorbate concentrations. When ascorbate is administered either intraperitoneally in animals or intravenously in animals or humans, tight control mechanisms are transiently saturated, until renal excretion restores homeostasis. With parenteral administration of pharmacological doses of ascorbate, plasma concentrations can reach 500 times those possible with oral administration (47). Only these pharmacological concentrations produce hydrogen peroxide in the extracellular fluid, which serves as a pro-drug for the formation of reactive oxygen species that are toxic to cancer cells, but harmless to normal tissues (4, 7, 23). It has been postulated that ROS formed from pharmacological ascorbate could also be harmful to bacteria (6). Lower doses of pharmacological ascorbate have effects that attenuate the severity of sepsis (1, 2, 58, 63–65, 67) that are not bactericidal.

Our findings showed that while pharmacological ascorbate alone was ineffective for candidemia, pharmacological ascorbate combined with Ampho B, even when the latter was reduced to 10 times lower than the MIC, was more effective than Ampho B alone. Furthermore, full-dose Ampho B showed minimal survival at 7 days in the Candida CLP model, compared with more than 50% survival at 7 days using pharmacological ascorbate with low-dose Ampho B. In vitro, for maximum synergy with low-dose Ampho B, 10 mM ascorbate was sufficient (Fig. 5). This concentration was not only easily achieved in mice but also achieved easily and safely in humans (23). Synergy between pharmacological ascorbate and Ampho B may be a consequence of each having separate actions on Candida membranes. Ampho B is a pore-forming agent that destabilizes the Candida membrane. Reactive oxygen species generated by pharmacological ascorbate from hydrogen peroxide, could also act independently to destabilize Candida membranes. In addition, hydrogen peroxide may also diffuse into Candida, with the subsequent formation of internal reactive oxygen species leading to internal membrane or protein damage. These potential actions of pharmacological ascorbate are independent of Ampho B, and synergy is reasonably predictable. Additional independent effects of ascorbate on neutrophils, phagocytosis, and killing could also contribute to the benefits of pharmacological ascorbate (15, 27, 53, 61).

Because Ampho B is nephrotoxic, lower-dose regimens are preferable. Pharmacological ascorbate was permissive for lower dosing of Ampho B in the Candida+CLP model. The CLP model induces renal insufficiency, such that only two doses of intravenous ascorbate as the delayed treatment were sufficient. In contrast, most of the successful sepsis treatments in mouse models need to be administered at the onset or the early phase of sepsis (10, 12, 13, 33–35).

A concern relating to this model is whether or not additional nephrotoxicity is induced by pharmacologic ascorbate. In the setting of renal insufficiency, intrarenal oxalate formation and crystallization are concerns with pharmacological ascorbate. Oxalic acid, an end product of ascorbate metabolism, can
crystallize as calcium oxalate in urine and renal interstitial tissues. In *Candida*+CLP mice treated with ascorbate, we did not find any crystal deposits in kidney histology, after 1 day and from autopsy (data not shown). We also found that serum creatinine was lower in mice treated with pharmacological ascorbate combined with low-dose Ampho B, compared with Ampho B alone. Moreover, we demonstrated that ascorbate injection in *Candida*+CLP did not alter the kidney function. On the other hand, full-dose Ampho B improved the mortality rate at day 2 of survival but not at day 7 of survival, a finding that suggests its toxicity in full-dose treatment (Fig. 6B). The findings from the combined treatment group demonstrate that the treatment effects of adding pharmacological ascorbate were beneficial for renal function. Another concern involved the limitations of mouse models and whether or not they are able to sufficiently represent human patient conditions and responses. The debate on this topic is still ongoing (54, 57). Nevertheless, we postulated that our two-hit models are more complex and might more accurately mimic the patient’s conditions. However, validation in patients with reciprocal sepsis condition is required.

**Perspectives and Significance**

In our new mouse model demonstrating an immunosuppressive phase with *Candida* reappearance, a synergistic effect of pharmacological ascorbate with low-dose Ampho B was demonstrated in vitro and in vivo, without apparent toxicity. Taken together, the data provide a proof of concept for pharmacological ascorbate as an adjuvant for antifungal treatment. Pharmacological ascorbate is inexpensive and can be easily available. Pharmacological ascorbate in combination with existing antifungals may indicate the use of lower doses with reduced toxicity.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


