Reply to "Unrealistic Nonphysiological Amounts of Reagents and a Disregard for Published Literature"

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Thank you for the opportunity to respond to Professor Ginsburg's communication regarding our *mBio* paper on combinatorial cationic and oxidative stress in *Candida albicans* (1, 2). I am writing on behalf of my coauthors, who have helped with and aligned themselves with this response.

Our main goal in this study was to examine the responses of *C. albicans* to cationic and oxidative stress (1). Most of our experiments were performed *in vitro* using stressors and doses that have been used historically in the field (3 and references therein). We then tested the relevance to phagocytic attack. Professor Ginsburg queries the nature of the oxidative and cationic stresses that we employed. We imposed cationic and oxidative stress using Na^+ and H_2O_2, respectively, to permit comparison with previous publications that have examined osmotic and oxidative stress responses in *C. albicans* by using these stressors. While K^+ is more relevant to phagocytosis, it is worth remembering that Na^+ stress is relevant to some host niches, such as the kidney. However, in our study, we showed that catalase is inhibited by both K^+ and Na^+ (Fig. 6B in reference 1).

Professor Ginsburg questions the physiological relevance of the stress doses that we examined from the perspective of the phagocyte. Previous studies indicate that *C. albicans* may be exposed to relatively high concentrations of reactive oxygen species (ROS) and cations in some host niches. In phagocytes, cation concentrations have been reported to reach the 0.2 to 0.3 M range (4). There are conflicting reports regarding ROS levels. Some studies suggest that steady-state H_2O_2 levels may be relatively low (e.g., 5), while others suggest that ROS might reach high local concentrations within the phagosome during the oxidative burst (4, 6). Frankly, because of the significant technological challenges associated with making such dynamic measurements (7, 8), there is a lack of detailed information about the maximal concentrations to which fungal cells are exposed in phagocytes as a consequence of the oxidative burst and cationic fluxes. Indeed, Winterbourne states, "We do not yet have probes that can quantify cellular production of specific oxidants" (8). In our study, we examined the impact of combinatorial stress *in vitro* by using 5 mM H_2O_2 and 1 M NaCl (1). We accept that these may lie outside normal physiological ranges. However, we performed these mechanistic studies having first shown that the synergistic effects of combinatorial oxidative and cationic stresses are observed over a wide range of concentrations (Fig. 2 in reference 1).

Professor Ginsburg highlights the importance of the antimicrobial effects of hypochlorous acid. We did not examine this; it would be interesting to do so. However, it is worth considering the following points. As Professor Ginsburg points out, H_2O_2 is converted to HOCl in the phagosome by myeloperoxidase (MPO). However, myeloperoxidase is reported to be less active at the alkaline pHs (9, 10) that are reached in the phagosome during the oxidative burst (11). Also, although the extent to which MPO deficiency increases susceptibility to microbial infection is debated in the field (a substantial proportion of individuals with MPO deficiency are asymptomatic), it is clear that MPO deficiency does not lead to the severe susceptibility to infections observed in patients with chronic granulomatous disease, a disorder of the phagocyte NADPH oxidase (12, 13).

We observed that ectopic catalase expression partially rescues synergistic killing *in vitro* as well phagocytic killing *ex vivo* (1). These findings, together with the observation that catalase-deficient *C. albicans* cells are more susceptible to neutrophil killing (14), support the idea that H_2O_2 detoxification mechanisms provide protection for *C. albicans* following phagocytosis. We also observed that the phagocytic killing of fungal cells is attenuated to similar extents by pharmacological inhibition of cationic fluxes or the oxidative burst (Fig. 8A in reference 1). This is consistent with our suggestion that the mechanisms of synergistic killing dissected *in vitro* are relevant to phagocytic killing.

We did not use azide or aminotriazole to inhibit catalase inside cells. These inhibitors are not specific for catalase. For example, azide inhibits energy metabolism, which, interestingly, affects resistance of *C. albicans* to antimicrobial peptides (15), and aminotriazole induces the amino acid starvation response and filamentation in *C. albicans* (16).

We are aware that the *C. albicans* catalase gene responds to carbon source. Indeed, one of the citations quoted by Professor Ginsburg was published by my group (17). Actually, we reported this in an earlier paper (18). Of more relevance is the observation that catalase gene expression is induced following phagocytosis by PMNs (18, 19).

We remain convinced that *C. albicans* is exquisitely sensitive to combinatorial cationic and oxidative stresses and that catalase makes a significant contribution to this phenomenon. New lab members have independently recapitulated the synergistic killing as well as the impact of catalase obtained by using independently generated mutants. Also, the major impact of such stresses on the transcriptome has been reconfirmed by RNA sequencing as part of a larger study of combinatorial stresses (in preparation). Our study was limited to combinations of cationic and oxidative stress. We did not examine other types of stress to which *C. albicans* cells may be exposed following phagocytosis, such as antimicrobial peptides (mentioned by Professor Ginsburg) or proteases and

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acidification. It would be interesting to examine other stress combinations. Indeed, we would be delighted if our study has encouraged others to examine the impact upon *C. albicans* of other types of combinatorial stress.

In closing, my coauthors and I thank Professor Ginsburg for his interest in our study and for his suggestions. However, we strongly refute the suggestion that we have intentionally used unrealistic amounts of reagents or intentionally disregarded key published data.

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