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Heat-Killed Yeast Protects Diabetic Ketoacidotic-Steroid Treated Mice from Pulmonary Mucormycosis

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Keywords

Mucormycosis; *Rhizopus oryzae*; vaccine; heat-killed *Saccharomyces*

Introduction

Mucormycoses are rare life-threatening fungal infections caused by fungi of the order *Mucorales* [1]. These infections usually afflict patients with immunosuppressed systems due to neutropenia, hematologic malignancies, corticosteroid treatment, diabetes or trauma [1, 2]. Owing to the rising prevalence of diabetes, cancer and transplantation in aging populations in the developed world, the number of mucormycosis infections are rising [3].

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Rhizopus spp. are the most common cause of mucormycosis [1]. Despite disfiguring surgical debridement and adjunctive antifungal therapy, the overall mortality of mucormycosis remains ~50%. In the absence of surgical removal of the infected focus, antifungal therapy alone is rarely curative, resulting in ~100% mortality for patients with hematogenously disseminated disease [1]. Thus, there is a critical need to identify new prophylactic measures or therapeutic targets against this lethal disease.

Previous studies showed that vaccination with the heat-killed yeast (HKY) *Saccharomyces cerevisiae* resulted in a cross-protective effect against systemic aspergillosis [4, 5], coccidioidomycosis [6], cryptococcosis [7], and candidiasis [8]. Because HKY elicited cross-protective activities against several fungal pathogens, we studied the effect of the HKY vaccine in protecting DKA mice from pulmonary mucormycosis infection caused by *R. oryzae*. These mice were infected intratracheally to recapitulate the mode of infection in the second most common form of mucormycosis in diabetics (i.e. pulmonary mucormycosis [14–16%] vs. rhinocerebral disease [43–52%]) [1, 9].

Methods

Vaccine preparation, organisms and culture conditions

Heat-killed, endotoxin-free *S. cerevisiae* clinical strain 96-108 (HKY) vaccine preparations were made as previously described [6] and kept at 4°C at 4×10^8 cells/ml. *R. oryzae* 99-880, a clinical isolate [10], was grown on potato dextrose agar (PDA) plates for 3–5 days at 37°C and the inoculum prepared as previously described in phosphate-buffered saline (PBS) [10].

Mice vaccination and infection

The HKY vaccine was administered subcutaneously (SC) to ICR mice using two dorsal sites (0.075 ml each). The total number of HKY per dose was 6×10^7 or 1.2×10^8 cells. HKY vaccine was given either on days 28, 21, and 14; or days 35, 28, 21, and 14 prior to fungal challenge. Control mice were given PBS SC instead of HKY.

Mice were rendered diabetic using streptozotocin ten days before infection [10] Diabetic. ketoacidotic (DKA) mice were also dosed with cortisone acetate on day −2 and +3, relative to infection [10]. Sera samples (from ~100 μl blood) for ELISA testing were collected two days prior to infection by nicking the tail.

Mice were infected intratracheally with *R. oryzae* spores after sedation with ketamine/xylazine [10]. To confirm the delivered inoculum three mice were euthanatized immediately after the infection and lungs were dissected, homogenized, and quantitatively cultured on PDA plates plus 0.1% Triton.

The primary endpoint of efficacy was time to moribundity through day 21 post-infection. As a secondary endpoint, tissue fungal burdens in the lungs and brains (the primary and secondary target organs, respectively) were determined by quantitative PCR (qPCR) [10].

ELISAs

ELISA [10] was adapted for detection of antibodies against *R. oryzae* cell surface antigens extracted by high-salt treatment overnight [11]. Negative control wells received sera from PBS-vaccinated mice. Other wells received an irrelevant isotype control monoclonal antibody as an internal control. The antibody titer was taken as the reciprocal of the last serum dilution with an OD reading   mean OD of negative control samples.

Statistical analysis

The non-parametric Log-Rank and Wilcoxon Rank Sum tests were used to determine differences in survival times and in antibody titers and tissue fungal burdens, respectively. Comparisons with *P* values of <0.05 were considered significant.

Results

Vaccination with HKY protects DKA/steroid treated mice from R. oryzae infection

Mice vaccinated with HKY survived significantly longer than those vaccinated with PBS, regardless of the HKY dose and the schedule of administration of the vaccine regimen (Figure 1A) (*P*<0.02, all comparisons). Furthermore, administration of the HKY vaccine in three doses of 6.0×10^7 cells at weekly intervals significantly prolonged the survival of DKA/cortisone acetate mice when compared to administering the vaccine at the higher dose of 1.2×10^8 cells given on the same schedule (*P* = 0.001). In a repeat study, similar results were obtained with HKY vaccine in that three doses of 6.0×10^7 cells at weekly intervals significantly prolonged the survival of mice (*P* = 0.009 vs. control mice) and the other two regimens of the higher dose of 1.2×10^8 HKY trended to enhance survival vs. control PBS-vaccinated mice (Figure 1B) (*P* = 0.09). Upon combining both experiments all vaccination regimens with HKY enhanced survival of DKA/cortisone acetate mice vs. those vaccinated with PBS (*P* <0.006).

To determine the effect of HKY on the tissue fungal burden, DKA/cortisone acetate mice were vaccinated with the most protective regimen (i.e. three doses of 6.0×10^7), and then infected intratracheally. Three days post-infection, lungs and brains were harvested from euthanized mice and processed for tissue fungal burden by qPCR. Vaccination with the HKY reduced the tissue fungal burden of the lungs by ~10-fold when compared to lungs harvested from control, PBS-vaccinated mice (Figure 2) (*P* = 0.048).

Vaccination with HKY enhanced the mouse immune response against R. oryzae

Blood samples obtained from uninfected mice vaccinated with three doses of 6×10^7 HKY cells showed enhanced serum antibody titers against *R. oryzae* by more than 250-fold when compared to control mice vaccinated with PBS, as determined by ELISA plates coated with *R. oryzae* cell surface material (Figure 3) (*P* = 0.00001). These data clearly demonstrate the presence of shared antigens between *S. cerevisiae* and *R. oryzae*.

Discussion

In this study we demonstrate the activity of HKY vaccine against murine mucormycosis due to *R. oryzae* infection. Our results also show for the first time that the HKY vaccine can protect against a fungal infection in a model with a pulmonary route of infection. Moreover, results presented in this study are the first demonstration of HKY efficacy in a model employing classical immunosuppression, such as corticosteroids. In addition, our model had a second form of immunocompromise elicited by DKA. The most compromised animals previously showing protection with HKY were complement-deficient animals, with protection against aspergillosis [5]. It is important to note that these results lend support to the concept of vaccinating patients prior to therapeutic manipulations that result in severe immunosuppression (e.g., induction of neutropenia, chemotherapy, or uncontrolled diabetes etc.) where the vaccine induced protection carries over through the period the patient is at high risk of developing mucormycosis. However, our data also suggest additional experiments are required to optimize the vaccination regimens, since higher doses were not as protective as lower doses. Furthermore, the DKA/cortisone acetate mice were not completely protected, however, the challenge was severe (e.g. highly lethal, with 100% mortality in controls in 2 weeks).

The demonstration of cross reacting antibodies engendered by HKY vaccination is consistent with previous work indicating the presence of shared antigens among fungi, including key cell wall glycan components (i.e. glucan and mannan), and homologous proteins [4, 5, 8, 12]. Although HKY induced a robust immune response against *R. oryzae,* as determined by anti-*R. oryzae* antibodies, the mechanism of protection remains to be determined. In studies examining the immune response of mice to HKY, we found vaccination induced proliferation of spleen and lymph node cells, enhanced cytokine levels for interferon-γ (IFN-γ), interleukin (IL)-6, and IL-17A from stimulated lymphocytes, and TNF-α and IFN-γ in bronchoalveolar lavage fluid [13]. In addition, HKY vaccination resulted in stimulation of specific antibodies to mannan and glucan. Thus, the mechanism of HKY-induced protection against *R. oryzae* may be a result of antibody and an adaptive T cell response. In this respect, antibodies targeting the cell surface CotH invasin of *R. oryzae* were recently shown to protect against experimental pulmonary mucormycosis when administered prophylactically or therapeutically [10], thereby confirming the protective role of antibodies against mucormycosis. It is also possible that the mechanism of protection could rely largely on stimulation of innate immunity by HKY, and/or on epigenetic reprogramming ("trained immunity") [14]. More than one arm of immunity resulting from vaccination could be responsible for protection [15]. Therefore, future studies of lymphocytes of immunized animals, for cytokine profiling, before and after *Rhizopus* challenge, and of monocyte function after vaccination [14], would be of great interest.

Yeast-based vaccines are safe in man [16], and initial studies with heat-killed *Saccharomyces* have shown tolerance by cancer patients to this vaccine [17, 18]. Our results in combination with those from studies showing efficacy against aspergillosis, coccidioidomycosis, candidiasis, and cryptococcosis [4–8] warrant the further development of HKY as a pan-fungal vaccine candidate, perhaps by generating a derivative of HKY, particularly a more purified derivative that mimics or exceeds HKY in its efficacy, and

presumably with a similar mechanism of action. Such a vaccine could have a glycan(s) and/or proteins(s) that are shared among fungal species. In this respect, we have shown that purified mannan, or glucan, with their respective conjugates, can reproduce the protection shown with whole killed *Saccharomyces* against some lethal mycoses [19, 20].

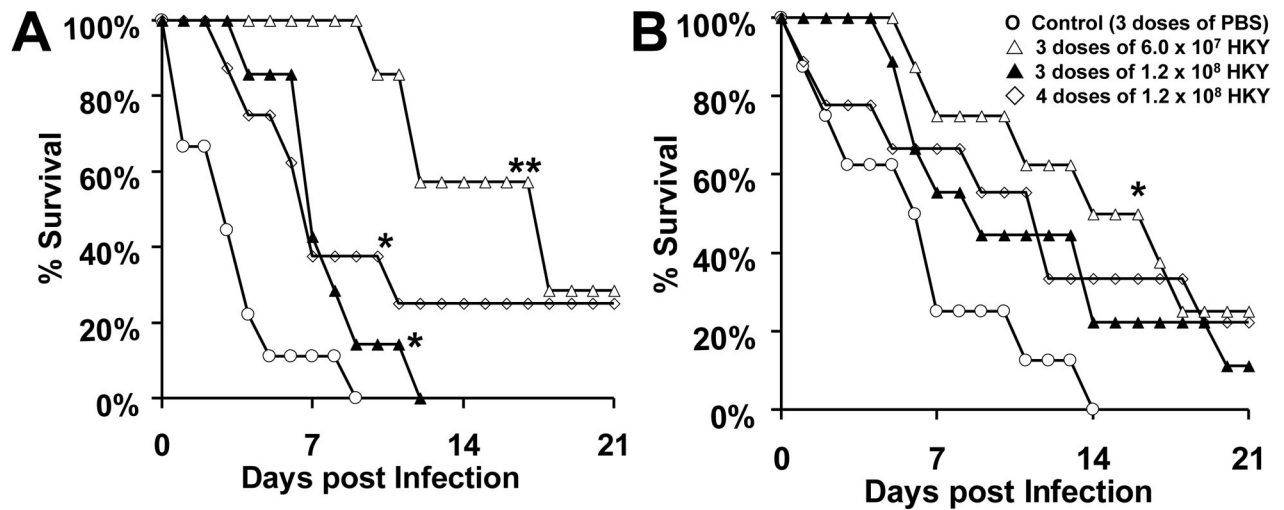## Acknowledgments

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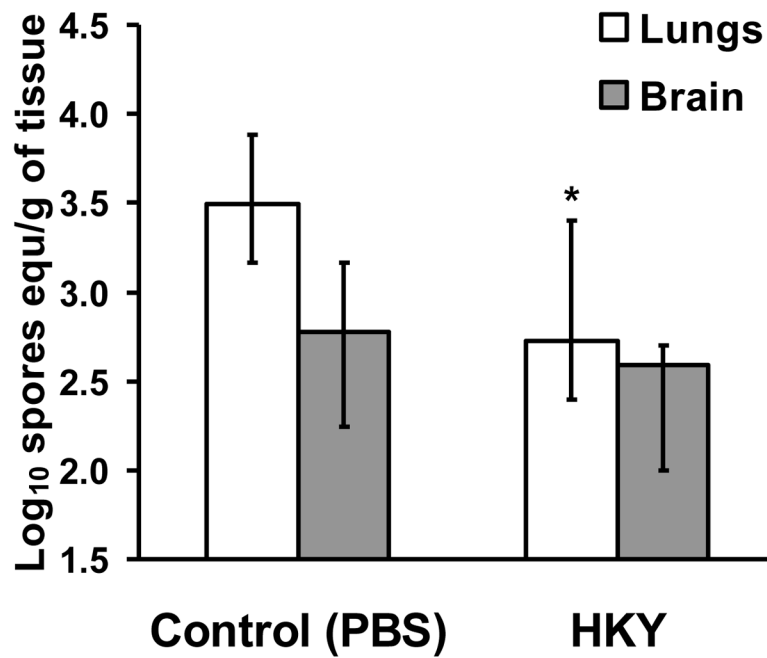
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Highlights

- HKY vaccine protects against DKA/steroid treated mice with pulmonary mucormycosis

- This is the first illustration of HKY efficacy in classically immunosuppressed mice

- HKY induces antibodies that react with *R. oryzae* cell wall antigens

- Our results broaden the HKY efficacy spectrum to a fifth mycosis

- Our data warrant the future development of HKY as a pan-fungal vaccine

Figure 1. HKY vaccine significantly prolonged survival in DKA/cortisone acetate-treated mice infected with *R. oryzae*.

Mice were vaccinated with different doses of HKY. Control mice were given PBS alone. Mice were then treated to produce DKA and dosed with cortisone acetate prior to intratracheal infection with *R. oryzae* 99-880 isolate at 2.5×10^5 spores. In the first experiment (delivered inoculum = 7.8×10^3 spore per mouse) (A), vaccinating mice (n= 7–9 per arm) with any HKY regimen prolonged survival compared to PBS-vaccinated mice (*$P < 0.02$). The lower dose of HKY, 6.0×10^7 spores, administered three times also demonstrated better efficacy than a higher dose of 1.2×10^8 HKY administered three (**$P = 0.001$) or four times. In the second experiment (delivered inoculum = 3.3×10^3 spores) (B), all HKY regimens prolonged survival compared to PBS-vaccinated mice, but only the lower dose regimen of 6.0×10^7 HKY prolonged survival significantly (* $P=0.009$) (n=8–9 mice per arm).

Figure 2. HKY vaccine significantly reduced lung fungal burden in DKA/cortisone acetate-treated mice infected with *R. oryzae*.
Mice were vaccinated with 3 doses of 6.0×10^7 of HKY. Control mice were given PBS alone. Mice were then treated to produce DKA and treated with cortisone acetate prior to intratracheal infection with *R. oryzae* 99-880 isolate at 2.5×10^5 spores (delivered inoculum $= 2.0 \times 10^3$ spores per mouse). Mice (n=10 per arm) were euthanatized at day 3 postinfection for determination of fungal burden in lungs and brains by quantitative PCR. *P <0.05 vs. Control by Wilcoxon Rank Sum test.
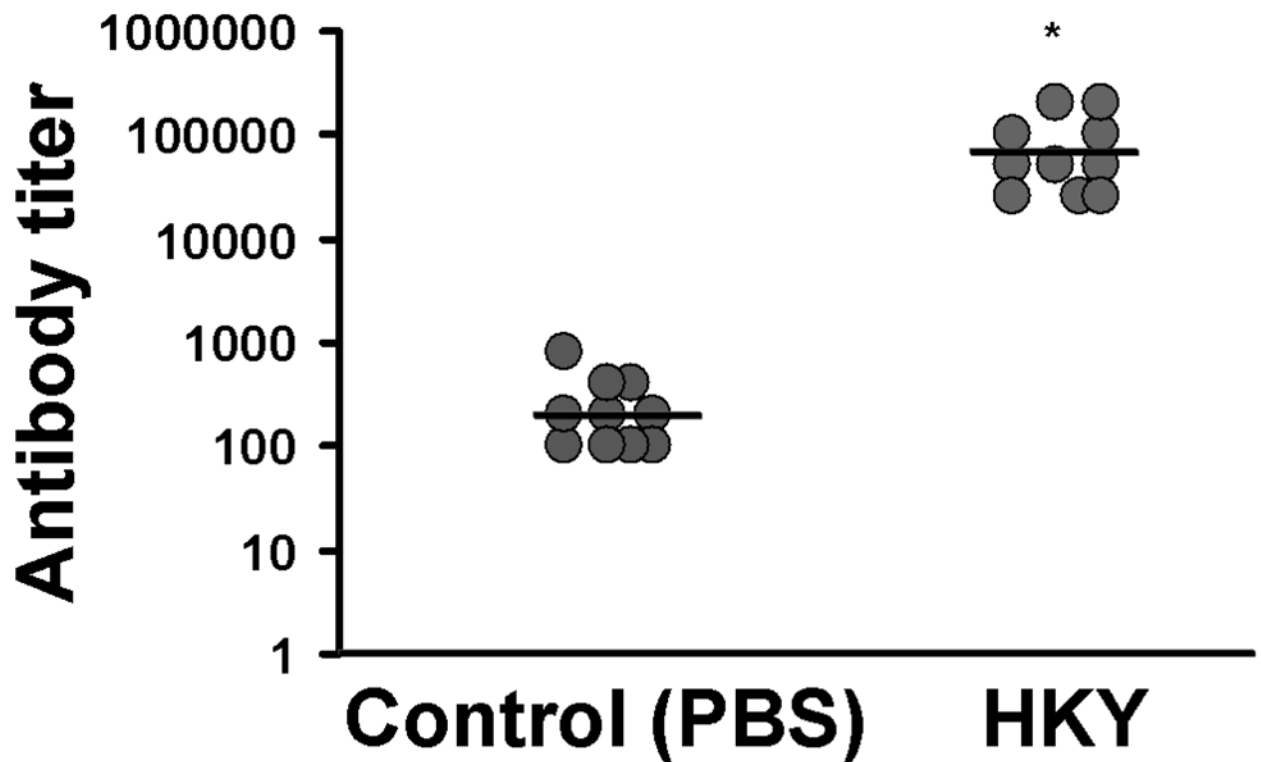
Figure 3. HKY vaccination significantly increased the mouse immune response as determined by detection of increased anti-*R. oryzae* cell surface antibody titers

Mice (n=10 per group) were vaccinated with three doses (at weekly intervals) 6×10^7 HKY cells or PBS alone as control. Serum was collected and anti-*R. oryzae* antibodies were evaluated by ELISA. * P= 0.00001 vs. PBS vaccinated control mice by Wilcoxon Rank Sum test.