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Short communication

Heat-killed yeast protects diabetic ketoacidotic-steroid treated mice from pulmonary mucormycosis

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ABSTRACT

Previous studies have shown that vaccination with heat-killed yeast, *Saccharomyces cerevisiae* (HKY), protects mice against systemic candidiasis, aspergillosis, cryptococcosis or coccidioidomycosis. Here we sought to define the potential use of HKY as a vaccine to protect mice from mucormycosis. Mice were vaccinated with different regimens of HKY prior to induction of diabetes. Diabetic ketoacidotic (DKA) mice were then treated with steroids prior to intratracheal challenge with *Rhizopus oryzae*. All regimens of HKY vaccine improved survival of DKA mice and reduced fungal burden in the primary target organ, lungs, as determined by qPCR. Furthermore, compared to mice vaccinated with diluent, vaccination with HKY substantially increased the mouse immune response as determined by detection of increased anti-Rhizopus antibody titers. Our results show that HKY protects steroid-treated DKA mice from pulmonary *R. oryzae* infection. Considering its demonstrated efficacy against other fungal infections, HKY is a promising candidate for development as a panfungal vaccine.

1. 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].

Rhizopus sp. 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 *Rhizopus 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].

2. Methods

2.1. 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

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days at 37 °C and the inoculum prepared as previously described in phosphate-buffered saline (PBS) [10].

2.2. 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 euthanized 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].

2.3. 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.

2.4. 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.

3. Results

3.1. 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 (Fig. 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 (Fig. 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 (Fig. 2) ($P = 0.048$).

3.2. 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 (Fig. 3) ($P = 0.00001$). These data clearly demonstrate the presence of shared antigens between *S. cerevisiae* and *R. oryzae*.

4. 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

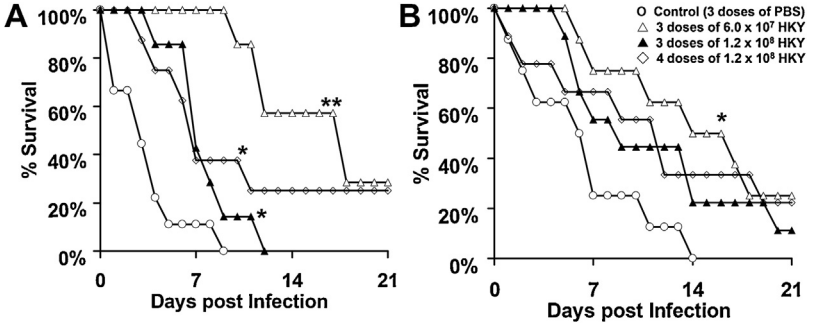

Fig. 1. *HKY vaccine significantly prolonged survival in DKA/cortisone acetate-treated mice infected with R. oryzae.* Mice were vaccinated with different doses of HKY. Control mice were given PBS alone. Mice were then treated with streptozotocin to produce DKA and dosed with cortisone acetate prior to 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).

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