Whole glucan particles as a vaccine against systemic coccidioidomycosis

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We reported previously that yeast-derived whole glucan particles (WGPs), with or without conjugation to BSA, used as a vaccine protected against systemic aspergillosis in mice. Here, we examined their utility as a potential vaccine against coccidioidomycosis. WGPs were prepared from Saccharomyces cerevisiae; conjugation with BSA (WGP–BSA) was done using 1-cyano-4-dimethylaminopyridinium tetrafluoroborate-mediated conjugation. Heat-killed S. cerevisiae (HKY) was used as a positive-control vaccine. CD-1 mice were vaccinated with WGPs or WGP–BSA, HKY or PBS once weekly, beginning 21 days prior to infection. Mice were infected intravenously with arthroconidia of Coccidioides posadasii. In the low-mortality study, 50 % of PBS-treated controls died. Only WGP–BSA at 0.6 mg per dose induced significant protection compared with PBS treatment. All surviving mice were infected in all three organs examined. Those given WGP–BSA at 0.6 mg per dose had fewer c.f.u. in liver and lungs (P<0.04), and those given WGPs at 6 mg per dose had fewer in lungs (P<0.02), compared with PBS. In the high-mortality study, 90 % of PBS mice died. Vaccination with HKY, and WGPs or WGP–BSA at 6 or 12 mg per dose significantly prolonged survival (P<0.05). No surviving mice were free of infection. HKY and WGP–BSA at 12 mg per dose reduced c.f.u. in the liver and lungs (P<0.05) and WGP–BSA at 6 mg per dose reduced c.f.u. in the lungs (P<0.05); unconjugated WGPs did not reduce infection. WGPs or WGP–BSA acted as a vaccine that protected against mortality caused by coccidioidomycosis. Thus, WGP protection against coccidioidomycosis and aspergillosis provides the basis for development of a pan-fungal vaccine.

INTRODUCTION

Coccidioidomycosis is endemic to parts of North America, Central and South America (Galgiani et al., 2005). Although effective therapy is available, the treatment duration is protracted, failures can occur while on therapy with azoles, and some patients, such as those with meningitis, require life-long therapy (Dewsnup et al., 1996). Prevention of disease through the use of a vaccine is a highly desirable option, but one that is not currently available for coccidioidomycosis. Numerous studies have been done examining various vaccine preparations, and a single preparation, formalin-killed spherules, was taken to clinical trial, but failed (Pappagianis 1993, 2001; Cole et al., 2004; Clemons & Stevens, 2011).

Abbreviations: HKY, heat-killed S. cerevisiae; WGPs, whole glucan particles; WGP–BSA, WGPs conjugated with BSA.

During the course of studies on potential vaccines, we found that heat-killed yeast of Saccharomyces cerevisiae (HKY) provided protection against experimental systemic aspergillosis (Liu et al., 2011a; Stevens et al., 2011). Furthermore, we found that HKY induced protection against infection with Candida albicans (Liu et al., 2012a), Cryptococcus grubii (Majumder et al., 2014), Mucor (Luo et al., 2014) and Coccidioides posadasii (Capilla et al., 2009). To better understand the mechanism and components responsible for protection, we performed studies on cell wall glycans, showing that mannans and glucans could induce protection against aspergillosis or coccidioidomycosis (Liu et al., 2010, 2012b). More recently, we demonstrated that highly pure particulate β-glucans alone or conjugated to BSA could induce protection against aspergillosis, whereas soluble preparations could not (Clemons et al., 2014a).

To further examine the specificity of the protective nature of these particulate β-glucans, we tested them against...
experimental systemic murine coccidioidomycosis. Our results demonstrate the potential of particulate β-glucan preparations to induce protection and serve as a basis for the development of a pan-fungal vaccine.

**METHODS**

**Animals.** Male, 6-week-old, CD-1 mice from Charles River Laboratories were acclimated for 1 week prior to use in these studies. Mice were randomized to experimental groups, housed in microisolator cages, and provided food and water ad libitum, under Animal Biosafety Level 2 standards. All animal experiments were done under an approved protocol of the Institutional Animal Care and Use Committee of the California Institute for Medical Research. All guidelines for animal care and use from the Office of Laboratory Animal Welfare, National Institutes for Health, Washington, DC, USA, were followed (National Research Council, 2011).

**Organism.** *Coccidioides posadasii* strain Silveira (ATCC 28868) was used in these studies. The organism was grown and arthroconidia for inoculation prepared as described previously (Clemons et al., 1990). All growth and handling of the organism were done under Biosafety Level 3 containment (Chosewood & Wilson, 2009).

**Vaccines.** Heat-killed yeast (HKY) of *Saccharomyces cerevisiae* strain 96–108 were prepared as described previously (Capilla et al., 2009; Liu et al., 2011a, 2011b) and served as a positive vaccine control in these studies. Whole glucan particles (WGPs) and WGPs conjugated to BSA (WGP–BSA) were prepared from *S. cerevisiae* at Biothera as described previously (Clemons et al., 2014a). Other investigators have reported that immunization with BSA with or without adjuvant provides no protection against coccidioidomycosis in mice (Li et al., 2001). Thus, we did not feel that it was necessary to include a BSA only control.

Groups of 10 mice were vaccinated with PBS, HKY, WGP–BSA or WGPs subcutaneously on days 21, 14 and 7 prior to infection. Vaccine doses were: HKY at 6 × 10² yeast per dose (2.5 mg per dose) in 0.15 ml (Capilla et al., 2009), WGPs or WGP–BSA at 0.6, 6 or 12 mg per dose in 0.3 ml volumes (Clemons et al., 2014a) or 0.3 ml of PBS. All doses were given using volumes as a split dose in two dorsal sites based on previous studies with HKY, WGP and WGP–BSA. The vaccine regimens and schedules were the same in both studies. A single mouse in the group given PBS died from unknown causes prior to infection with *Coccidioides* and was not included in any analyses. Mice vaccinated with HKY and higher dosages of WGPs or WGP–BSA often had small palpable granulomas at the site of injection. No other effects of vaccination were noted.

**Infection model.** The model of infection used in these studies was that of establishing systemic disease similar to previous investigations (Clemons et al., 1983, 1985a, b, 1990, 1995, 2000; Capilla et al., 2009). For the low-mortality study, all mice were infected intravenously with 127 arthroconidia of *C. posadasii* in a 0.25 ml volume; groups had 10 mice each, except for the PBS-treated group, which had n = 9. For the high-mortality study, all mice were infected intravenously with 275 arthroconidia of *C. posadasii* in a 0.25 ml volume; all groups had 10 mice each. The group sizes were determined using StatMate version 2 (GraphPad Software) to have approximately 80% power to detect differences in survival at the 0.05 level. These group sizes have been robust for determining differences in outcome using non-parametric statistics.

Mice were examined daily and deaths were tallied up to day 28 post-infection. Mice found severely moribund or immobilized were euthanatized using CO₂ anoxia. On day 28 of infection, all surviving mice were euthanatized using CO₂ and the number of c.f.u. remaining in the lungs, liver and spleen was determined by quantitative plating of organ homogenates as described previously; these are the primary target organs of infection in this model (Clemons et al., 1983, 1985a, b, 1990, 1995; Clemons & Stevens, 1992, 1994; Capilla et al., 2009).

**Statistical analysis.** Comparative survival was analysed by log rank test and the residual burdens of *C. posadasii* in the organs were compared using a Mann–Whitney U test using GraphPad Prism (version 3.1). A log₁₀ value of 7.5 was assigned to data points missing due to the death of an animal (Lachin, 1999; Shih, 2002). This value assured that death was assigned a worse outcome than survival with any fungal burden and is close to that found just prior to death (Clemons et al., 1983, 1985a, b, 1990, 1995; Clemons & Stevens 1992, 1994; Capilla et al., 2009).

**RESULTS**

The aim of these studies was to determine whether vaccination with WGPs or WGP–BSA could provide protection against experimental systemic coccidioidomycosis, similar to the protection they afforded mice against systemic aspergillosis (Clemons et al., 2014a).

**Low-mortality study**

In the initial study, 50% of PBS-treated controls succumbed to infection. Sixty percent or more of the mice vaccinated with HKY, WGPs or WGP–BSA survived throughout the 28 days of infection (Fig. 1). Statistically, only WGP–BSA 0.6 mg provided significant protection compared with PBS treatment (P = 0.029) and WGPs 6 mg approached significance (P = 0.052). HKY was not significantly protective in this study, but mice vaccinated with HKY did survive longer than mice given PBS. Groups of mice given any dose of WGP–BSA and the two higher doses of WGPs survived longer than the PBS-treated group or the HKY-treated group.

Vaccine effectiveness was also assessed by determination of the residual c.f.u. burdens in the lungs, liver and spleen of surviving mice (Fig. 2). All mice had detectable infection in all three organs. Mice vaccinated with WGP–BSA 0.6 mg had significantly fewer c.f.u. in the liver and lungs than did PBS-treated mice (P = 0.04), and mice vaccinated with WGPs 6 mg had significantly fewer c.f.u. in lungs than did PBS-treated mice (P = 0.02) and in the spleen than did HKY-treated (P = 0.04). No other comparisons approached significance. It should be noted for WGP doses that the median burdens of the WGPs 12 mg group were higher than those in the WGP 6 mg group (Fig. 2). Similarly, the median burdens recovered from mice given WGP–BSA 6 or 12 mg were higher than those given WGP–BSA 0.6 mg. The c.f.u. burden in the various organs corresponded with the survival data, with both supporting the lower vaccine doses (WGP–BSA 0.6 mg and WGPs 6 mg) to be protective compared with the higher doses (WGP–BSA 6 mg, WGP–BSA 12 mg and WGPs 6 mg). Thus, these data were suggestive of higher doses being less effective.
High-mortality study

As shown in the initial study, WGPs or WGP–BSA appeared to be potentially effective when used as a vaccine against coccidioidomycosis. We performed a replicate study to determine the reproducibility of this protection and to clarify the comparative protection. In addition, the replicate study was designed, through the use of a higher number of arthroconidia in the inoculum, to be a more rigorous challenge to the protective efficacy of these preparations as vaccines.

The infection proved highly lethal, with 90 % of the PBS-treated control mice succumbing to infection by day 17 (Fig. 3). Comparatively, only 40 % of HKY-treated mice died and this was significantly protective ($P<0.002$), similar to our published studies (Capilla et al., 2009). WGPs at 12, 6 or 0.6 mg per dose resulted in 50, 30 and 20 % survival, respectively. WGPs at 12 or 6 mg per dose provided significant protection compared with PBS-treated ($P=0.0005$) and were equivalent to HKY. WGP–BSA at 12, 6 or 0.6 mg per dose resulted in 60, 60 and 30 % survival, respectively, suggestive of a dose–response, as was the case with WGPs (Fig. 3). The two higher dosages of WGP–BSA prolonged survival significantly compared with PBS-treated ($P<0.05$) but were equivalent to HKY. Neither WGPs nor WGP–BSA at the 0.6 mg dosage induced significant protection.

The recovery of c.f.u. of *C. posadasii* from the organs 28 days post-infection is shown in Fig. 4. No animals in any vaccine regimen were cleared of infection in any organ. There were no significant differences in burden in the spleen of the vaccinated groups compared with PBS controls. However, HKY and WGP–BSA at 12 mg per dose both significantly reduced c.f.u. in the liver ($P=0.035$) and in the lungs ($P=0.04$ and 0.035, respectively). WGP–BSA at 6 mg per dose significantly reduced c.f.u. in the lungs compared with PBS ($P=0.035$). In contrast, no dose of the non-conjugated WGP vaccine resulted in a significant reduction in c.f.u. in any of the three organs. However, WGP–BSA and WGPs were not significantly different at all doses and were also equivalent to HKY.

Overall, the results of the replicate study corroborated those of the initial study showing that WGP–BSA or WGPs alone could provide some protection against experimental systemic coccidioidomycosis, even when the model established was a rapidly lethal and thus highly rigorous test of protection. This severity of the infection in the replicate study may also explain why the lowest dose of WGP–BSA tested was not effective, as it had been in the lower mortality study.

**DISCUSSION**

The results of our current studies demonstrate that WGPs alone or conjugated with BSA can act as a vaccine against coccidioidomycosis. Protection was found to be dose-responsive, with some doses equivalent to or somewhat better than that provided by our positive-control preparation of HKY, depending on the severity of infection. Although survival was prolonged by vaccination with WGPs or WGP–BSA in both studies, protection did not result in animals free of infection in the organs, and the severity of infection probably played a role in determining the effectiveness of a dosage. Interestingly, all the clinical evidence also indicates that successful resolution of natural coccidioidal infection results in persistence of viable organisms; to wit, reactivation of infection decades after moving from endemic areas, only when the individual is immunosuppressed (Deresinski & Stevens, 1975). In addition, the literature suggests thus far that if a vaccine preparation...
protects against one species of Coccidioides it will also protect against the other, since these species are very closely related (Clemons & Stevens, 2011). In conjunction with our previous results, it appears that WGP preparations are capable of inducing some protection against coccidioidomycosis and aspergillosis (Clemons et al., 2014b).

We have addressed the question of mechanism of induced protection in part in previous studies. Examination of the cytokine profiles in serum and BAL of WGP-vaccinated mice revealed significant differences in the levels of various cytokines, which were used to assess the immune response. These data were subsequently analyzed using the Mann–Whitney U test. In the spleen, the sole significant comparison was that WGP 6 mg had significantly lower c.f.u. than HKY (P=0.043). For the liver, WGP–BSA 0.6 mg had significantly lower c.f.u. than PBS (P=0.043). For the lungs, WGP–BSA 0.6 mg and WGP 6 mg had significantly lower c.f.u. than PBS (P=0.043 and 0.022, respectively). No other comparisons were significant. There were nine mice in the PBS-treated group and all other groups had 10 mice each. Horizontal bars represent the group median.

Fig. 2. Number of c.f.u. recovered from surviving mice. A log₁₀ value of 7.5 was assigned to data points missing due to the death of the animal. Statistical comparisons were done by Mann–Whitney U test. In the spleen, the sole significant comparison was that WGP 6 mg had significantly lower c.f.u. than HKY (P=0.043). For the liver, WGP–BSA 0.6 mg had significantly lower c.f.u. than PBS (P=0.043). For the lungs, WGP–BSA 0.6 mg and WGP 6 mg had significantly lower c.f.u. than PBS (P=0.043 and 0.022, respectively). No other comparisons were significant. There were nine mice in the PBS-treated group and all other groups had 10 mice each. Horizontal bars represent the group median.

Fig. 3. Cumulative survival of mice vaccinated with PBS, HKY, WGP 6 or WGP–BSA at the doses (mg per dose) indicated and infected intravenously with 275 arthroconidia of C. posadasii. All groups consisted of 10 mice each.
mice showed activation of an innate and adaptive immune profile (i.e., upregulation of CSFs, INF-γ, TNF-α, chemokines such as MCP-1, MIP-1α, RANTES, KC and Th17-activating cytokines such as IL-6, IL-1β, IL-17) (Clemons et al., 2014b). Interestingly, only a minimal rise in antibody to β-glucan was found, suggestive that antibodies play a minimal role in protection (Clemons et al., 2014b). The latter is in opposition to the studies using laminarin conjugated to diphtheria toxoid, which suggested antibody directed against β-glucan was a major mechanism of protection against multiple fungal infections (Torosantucci et al., 2005, 2009). As we noted previously, induction of the various pro-inflammatory cytokines and chemokines is suggestive of a priming of an active innate immune response, whereas increases in IFNγ and IL-17 suggest an induction of a cell-mediated immune response (Zelante et al., 2009; Clemons et al., 2014b; Whibley & Gaffen, 2014). Additional studies are required to fully determine the nature of the protection induced by WGPs or WGP–BSA.

It is possible that the conjugation of WGPs to proteins specific for Coccidioides could improve the effectiveness of the vaccine preparation. Similarly, the use of multivalent epitope constructs specific to Coccidioides delivered in shells of β-glucan has been shown to induce protective cell-mediated responses (Hurtgen et al., 2012; Cole et al., 2013). These data also support the concept of using glycans as carriers of fungal proteins, similar to our own results and those of Torosantucci et al. (2005), which take advantage of the immunostimulatory properties of β-glucan and reduce the need for an adjuvant such as aluminium hydroxide.

Overall, our results provide a basis for the development of a pan-fungal vaccine. That WGPs or WGP–BSA has now been shown to induce a degree of protection against two different fungal infections, in conjunction with our results showing HKY vaccination protects against five different fungi, would indicate that WGPs is a candidate as a base preparation onto which specific proteins or protein constructs could be conjugated. Additional studies are needed to further this area of study.

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REFERENCES


