Saccharomyces as a vaccine against systemic aspergillosis: ‘the friend of man’ a friend again?

Min Liu,1,2,3 Javier Capilla,1,2,3† Maria E. Johansen,1,2,3‡ Danielle Alvarado,1,2,3 Marife Martinez,1 Vicky Chen,1 Karl V. Clemons1,2,3 and David A. Stevens1,2,3

1California Institute for Medical Research, San Jose, CA, USA
2Department of Medicine, Santa Clara Valley Medical Center, San Jose, CA, USA
3Stanford University, Stanford, CA, USA

The mortality of clinical Aspergillus infections necessitates consideration of the utility of a vaccine. We have found that Saccharomyces species can act as a protective vaccine against a lethal systemic Aspergillus infection, and describe experiments optimizing a subcutaneous regimen with killed yeast. Three injections of 2.5 mg given a week apart, 2 weeks prior to challenge, consistently, significantly, provided survival protection and reduction of infection in organs in survivors. The protection was independent of the strain of Saccharomyces, and possibly even the species, and could be demonstrated in several inbred (including C- deficient) and outbred mouse strains. The protective moiety(ies) appeared to reside in the cell wall and was resistant to 100 °C, but not to protease or formalin. Alum potentiated the protection. The protection was comparable or superior to that of several Aspergillus-specific preparations described in the literature. Other studies have indicated that heat-killed Saccharomyces can protect against infection with at least three other fungal genera, raising the possibility of development of a panfungal vaccine, and such a vehicle has been studied in clinical trials, without dose-limiting toxicity.

INTRODUCTION

Aspergillosis has become the leading fungal killer of the immunocompromised host (Lin et al., 2001), and currently available therapy has a high failure rate (Walsh et al., 2008). Vaccinating a susceptible population that is largely immunodeficient would seem a formidable challenge, but there are some groups of susceptible individuals who might be the initial targets of a vaccine effort (Stevens et al., 2011) as further development proceeds: chronic granulomatous disease patients, transplant candidates prior to transplant, leukaemias after successful induction therapy, solid tumour patients at diagnosis, patients with rheumatic or inflammatory bowel disease before immunosuppression, and intensive care unit patients in the high-risk groups for aspergillosis but who lack the classical risk factors such as steroids and cytotoxic chemotherapy. Another approach would be to immunize the donors of haematopoietic stem cell transplants (Stevens et al., 2011). Encouraging evidence has been provided that CD4-deficient hosts can be successfully vaccinated, and in them CD8 cells can substitute to develop vaccine-induced immunity (Fierer et al., 2006; Wuthrich et al., 2003; Zheng et al., 2005). Moreover, prevention of disease may not be required for a vaccine to be successful; a vaccine could be useful if it augmented an impaired immune response, ‘held the fort’ until immunity recovered (immunosuppression reduced, or neutrophils return) or acted synergistically with antifungal therapy (Stevens et al., 2011).

Our path began with vaccine studies to utilize Saccharomyces cerevisiae as a recombinant delivery system for cloned Aspergillus protein antigens. This strategy is based on observations that such yeasts stimulate innate CD8 reactions (Heintel et al., 2003), and uptake of yeasts by dendritic cells leads to increased expression of CD40, many cytokines, co-stimulatory molecules and increased MHC I- and II-restricted T-cell (especially CD8) responses (Bernstein et al., 2008; Stubbs et al., 2001). Although we found that Saccharomyces expressing a candidate Aspergillus immunodominant antigen induced protection, to our surprise we found Saccharomyces without the plasmid vector containing the Aspergillus gene, or with an empty plasmid vector gave just as much protection against Aspergillus challenge in mice (Capilla et al., 2006). The present studies investigate an approach to develop a vaccine...
against aspergillosis – even a panfungal vaccine – stemming from that observation.

**METHODS**

* Saccharomyces vaccines

**HKY vaccine.** All experiments included *S. cerevisiae* strain 96-108 (Capilla *et al.*, 2009). Other principal strains studied are listed in Table 1. All *S. cerevisiae* strains were grown in yeast peptone glucose broth (1 % yeast extract, 1 % peptone, 1 % glucose) for 24 h at 37 °C in an orbital shaker, then heat-killed at 70 °C as previously described (heat-killed yeast, HKY) (Capilla *et al.*, 2009). In one set of experiments described, strain 96-108 was heat-killed at 100 °C in a boiling water bath for 3 h. All methods used for killing included sterility and viability testing by culturing on a Sabouraud dextrose agar (SDA) plate, to assure that no growth was found after 5 days incubation at 37 °C. HKY appear intact cells, and were adjusted to 4 x 10^8 cells ml^-1 by haemocytometer count for subsequent manipulations.

**Endotoxin concentration.** Endotoxin in the HKY vaccine preparations was assayed by a rapid gel-clot method using a Pyrosate kit (Associates of Cape Cod). The endotoxin content in the HKY preparation was < 0.25 EU ml^-1.

**Protease treatment.** To test the contribution of any protein components in the HKY vaccine, intact HKY was pre-treated with a protease. Protease digestion of HKY was done by incubating 5 ml HKY with 5 mM DTT (Sigma-Aldrich) for 1 h at 37 °C followed by 1 h of incubation with 500 μg proteinase K (Sigma-Aldrich) ml^-1. Protease-digested HKY suspension was washed by centrifugation and the cells were suspended in PBS (pH 7).

**Fractions of HKY.** *S. cerevisiae* 96-108 was grown and killed at 70 °C as described above. HKY cells were counted and adjusted to 4 x 10^8 cells ml^-1 in 10 ml PBS. Cells were centrifuged at 500 g for 10 min. The supernatant of this step was collected, lyophilized and redissolved in 10 ml PBS. The cell pellet was washed three times by centrifugation with PBS and suspended in 10 ml fresh PBS. Each mouse was given 150 μl of the supernatant fraction or pellet fraction for vaccination.

**Subcellular fractions.** HKY suspension (150 μl) was mixed with acid-washed 0.5 mm silica beads (BioSpec Products), and beaten at a speed of 2500 oscillations min^-1 for 30 s using a Mini-Beadbeater-8 (BioSpec Products). Cells were kept on ice for 1 min followed by repeating the bead-beating cycle five times. The broken yeast suspension was decanted and the beads were washed three times with PBS. The wash supernatants were pooled with the initial broken yeast suspension and centrifuged at 1000 g at 4 °C for 10 min. The supernatant was collected, lyophilized and dissolved in 150 μl PBS (soluble fraction). The pellet was suspended in 150 μl PBS (crude cell wall fraction). Each 150 μl of either final preparation served as a single vaccine dose for a mouse.

**Formalin-killed cells.** Formalin (Thermo Fisher Scientific) was added to kill the yeast to a final concentration of 0.5 % (v/v). The suspension was held for a minimum of 8 days at 4 °C. Prior to use as a vaccine, the yeast cells were pelleted and washed with PBS three times. The cells were suspended in PBS in a volume equal to the original.

**Adjuvants.** Oligodeoxynucleotides CpG (Operon Biotechnologies), monophosphoryl lipid A (MPL) (Sigma-Aldrich) or Injekt alum (Thermo Scientific) were studied as adjuvants (10 μg per dose, 12.5 μg per dose or 50/50 volume, respectively) administered with HKY, following the same regimen used for HKY vaccination.

* Aspergillus vaccines. Several Aspergillus vaccine preparations were prepared, following the description in prior publications, with minor modifications: heat-killed conidia (HKC) (Cenci *et al.*, 2000; Richard *et al.*, 1982), heat-killed germlings (Richard *et al.*, 1982), culture filtrate (Cenci *et al.*, 2000; Kurup *et al.*, 1988) and a crude hyphal extract (Ito *et al.*, 2006). In brief, the clinical strain of *Aspergillus fumigatus* (strain 10AF) from our laboratory (Hanson *et al.*, 1995) was grown on SDA plates at 35 °C until sporulation occurred (5–7 days). Conidia on the plates were harvested with 0.9 % saline and 0.05 % (v/v) Tween 80 for the germling preparation or Tween 80 (0.025 %, v/v) for the conidial preparation. The conidial suspension was filtered through sterile gauze and centrifuged (500 g for 10 min). The conidia were washed three times by centrifugation in PBS and suspended in PBS. Germlings were obtained by incubating the conidial suspension in RPMI 1640 at 37 °C for 4 h and then at room temperature for 24 h. Germlings were collected by centrifugation, washed and suspended in PBS, and killed by heating at 100 °C for 2 h. HKC were prepared by heating at 100 °C for 2 h (Richard *et al.*, 1982) or autoclaving as described previously (Cenci *et al.*, 2000). Sterility and lack of viability of these preparations was assessed as described in the first section of Methods. HKC or germlings were adjusted by haemocytometer count to the desired number of cells and stored at 4 °C in sterile sealed vials.

**Table 1. Principal *Saccharomyces cerevisiae* strains studied**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Original designation</th>
<th>DNA typing*</th>
<th>Virulence†</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>YJM237</td>
<td>S288c‡</td>
<td>A</td>
<td>Low</td>
<td>Laboratory</td>
</tr>
<tr>
<td>YJM332</td>
<td>UCD 91-1</td>
<td>A</td>
<td>Intermediate</td>
<td>Wine yeast</td>
</tr>
<tr>
<td>YJM264</td>
<td>Red Star</td>
<td>B</td>
<td>Low</td>
<td>Bread yeast</td>
</tr>
<tr>
<td>YJM263</td>
<td>Fleischmann’s</td>
<td>B</td>
<td>Intermediate</td>
<td>Bread yeast</td>
</tr>
<tr>
<td>96-108 (HKY)</td>
<td>96-108</td>
<td>B</td>
<td>ND</td>
<td>Clinical strain§</td>
</tr>
<tr>
<td>YJM436</td>
<td>B70302(b)</td>
<td>A</td>
<td>High</td>
<td>Clinical strain§</td>
</tr>
</tbody>
</table>

ND, Not done.

* DNA typing methodology and interpretation are detailed in Clemons *et al.* (1997). A and B are RFLP groupings.

† Ranking of virulence in vivo is detailed in Clemons *et al.* (1994), and refers to results in a murine model using intravenous injection.

‡ Further detail regarding this strain can be found in Mortimer & Johnston (1986).

§ Clinical strains were obtained from a patient culture. Strain 96-108 was a vaginal isolate, and YJM436 was an oral isolate from an AIDS patient.
For the hyphal-based vaccine (Ito et al., 2006), in brief, a conidial suspension prepared from *A. fumigatus* 10AF was cultured on potato dextrose agar at 37 °C until sporulation occurred. Conidia were harvested as described above and inoculated into Czapek–Dox medium supplemented with 1% Tryptone (BD/Difco) and cultured for 72 h at 37 °C on a gyratory shaker at 140 r.p.m. The hyphal mass was collected by filtration, suspended in PBS and homogenized mechanically in two steps: first using a high-speed mechanical homogenizer (Tissumizer; Tekmar), followed by breaking with the Beadbeater at 2500 oscillations min⁻¹ for 30 s, held on ice for 1 min and the cycle repeated five times. The broken hyphal mass was lyophilized. The lyophilized material was suspended in saline and stored at 4 °C in sterile sealed vials until use. Culture filtrate antigens were obtained from stationary cultures of *A. fumigatus* strain 10AF (Cenci et al., 2000; Kurup et al., 1988). Briefly, 10AF cultures were incubated at 37 °C for 4 weeks in a synthetic medium consisting of AOAC broth (Difco) and Czapek’s broth (Sigma-Aldrich) at a ratio of 1:1 and supplemented with 1% (w/v) glucose. The broth was separated from the mycelium by filtration, freeze-dried after extensive dialysis against distilled water at 4 °C, and the freeze-dried sample was dissolved in 0.9% saline for injection, as detailed by Cenci et al. (2000) and Kurup et al. (1988).

**Animals.** The mouse strains studied included noncompromised male CD-1, DBA/2, BALB/c and C57BL/6 mice (Charles River Laboratories). The standard diet used at the California Institute for Medical Research for mice was irradiated Harlan 7956 (Harlan Laboratories). For one experiment, diet 5L79 (Charles River Laboratories) was purchased and autoclaved prior to use. Animals were housed under standard conditions with five animals per cage. All animal studies were done with the approval of the Institutional Animal Care and Use Committee of the California Institute for Medical Research. Each study group consisted of 10 animals.

**Vaccinations.** Vaccinations were given subcutaneously (SC) using two dorsal injection sites (0.075 ml each) or orally (PO) by gavage. Unless otherwise specified in Results, the standard design of the experiments was: 4-week-old male CD-1 mice were allowed to acclimatize in our facility for 1 week, followed by three vaccinations each 1 week apart, and 2 weeks later by intravenous fungal challenge. Thus the mice were 9 weeks old at the time of fungal challenge. Each variable (e.g. dose, regimen, adjuvant, etc.) was tested on a group of 10 mice and compared to controls. All experiments included a control group receiving PBS (control) and a second group receiving HKY SC at 2.5 mg HKY per mouse (i.e. 6 x 10⁶ cells ml⁻¹) given 28, 21 and 14 days prior to infection. Other regimens used in specific experiments are described separately in Results.

**Infection.** Mice were infected with a conidial suspension of *A. fumigatus* strain 10AF as described previously (Capilla Luque et al., 2003; Clemons & Stevens, 2004; Clemons et al., 2000, 2002; Denning & Stevens, 1991; Hanson et al., 1995). Inoculum viability was assayed by placing samples of 10-fold dilutions on SDA plates. Animals were infected intravenously via the lateral tail vein with 0.25 ml inoculum (5.2 x 10⁶–9.4 x 10⁶ viable conidia per mouse in various experiments).

**Fungal burdens.** Sixteen days after infection, surviving animals were euthanized by CO₂ anesthesia. The kidneys and brain were removed aseptically, homogenized in 0.9% saline and samples of 10-fold dilutions were placed on SDA plates for c.f.u. determination as described previously (Capilla Luque et al., 2003; Clemons & Stevens, 2004, 2009; Clemons et al., 2000, 2002; Denning & Stevens, 1991; Hanson et al., 1995).

**Passive transfer.** Mice were vaccinated with the HKY regimen described. Two weeks after the last vaccine dose, mice were anaesthetized and exsanguinated. Blood was collected from vaccinated and similarly handled nonvaccinated animals, and allowed to clot at room temperature. Serum was collected after centrifugation at 1500 g for 30 min and stored at −80 °C until use. Three animal groups were used (10 mice per group) per experiment and received intraperitoneally polyclonal anti-HKY serum (0.5 ml per animal), serum from unvaccinated animals, or PBS, 1 day before challenge with *A. fumigatus* 10AF.

**Statistics.** Comparisons of survival were performed by log rank test. Fungal tissue burden was analysed by a non-parametric Mann–Whitney U test using GraphPad 3.0 for Windows. For statistical comparisons of c.f.u., an arbitrary value of log_{10} 6 c.f.u. was assigned to data points missing due to the death of an animal from infection (Lachin, 1999; Shih, 2002). This value is higher than that recovered from any surviving mouse, assures death is assigned a worse outcome than survival with any burden, and approximates the c.f.u. present in the organ just prior to death.

**RESULTS**

**Regimen and reproducibility**

Because of our results defining a regimen of HKY that was protective against coccidoidal challenge (Capilla et al., 2009), the regimen most studied against *Aspergillus* challenge is 2.5 mg HKY (strain 96-108) given SC days 28, 21 and 14 prior to infection (‘standard regimen’) (Fig. 1a).

If the challenge in any experiment is insufficiently lethal, statistically significant survival protection from the standard regimen may not be able to be demonstrated, even though there is a favourable trend. We have assessed the standard 2.5 mg regimen effect on survival independently in CD-1 mice 25 times, with protection P<0.05 20 times (and P<0.01 16 times). Conversely, for similar reasons, if the challenge is too lethal to controls, there are few survivors, and it may not be possible to demonstrate a statistically significant reduction in organ burden of *Aspergillus* infection in survivors, even though a favourable trend is evident. The kidney is the principal target organ in this model (Capilla Luque et al., 2003; Clemons & Stevens, 2004; Clemons et al., 2000, 2002; Denning & Stevens, 1991; Hanson et al., 1995), and protection (c.f.u. reduction) was P≤0.05 in 19 of 22 independent assessments (P≤0.01 13 times) (Fig. 1b). Because of the lesser infection (lower c.f.u. numbers) in the brain, and its spontaneous resolution in this organ in this model (Capilla Luque et al., 2003; Clemons & Stevens, 2004; Clemons et al., 2000, 2002; Denning & Stevens, 1991; Hanson et al., 1995), significant c.f.u. reduction is more difficult (in 21 assessments, P<0.05 13 times and P≤0.01 11 times) to show.

Fig. 1 illustrates (a) that the vaccine is not curative (at the time of sacrifice in these experiments) and (b) the complexities of conveying infectious burden reduction in the setting of a lethal challenge. Protection against lethality and reduction of residual infection both give some information, but cannot be regarded as two fully independent assessments, since the statistical method (Lachin, 1999; Shih, 2002) for handling censored data (assignment of fatalities an outcome worse than any survivor, points at
1 x 10^6 c.f.u. per organ) in reduction of residual infection is so influenced by the many deaths in the control group.

We also assessed whether our standard control, of three PBS injections in the 28-21-14 format, might affect host resistance (e.g. stress of manipulations). Such mice proved to have no resistance differences to those not given any injections and then challenged (data not shown).

Alternative regimens

Other HKY SC regimens were examined. In one study, the '28-21-14' regimen was compared to (a) 2.5 mg given days 35, 28 and 21 or (b) 42, 35, 28 and 21 prior to infection, as was (c) 25 mg given on the 28-21-14 schedule. In two other studies, (d) 2.5 mg given days 49, 42, 35, 28, 21 and 14, or (e) days 42, 35 and 28, or (f) 41.7 mg given on the 28-21-14 schedule were compared. None of the latter six regimens were superior to the 2.5 mg 28-21-14 regimen in prolonging survival (all seven regimens P<0.02 vs PBS). Regimens (a), (b), (c), (d) and (f) were modestly superior to the 2.5 mg 28-21-14 regimen in reducing c.f.u. in the kidney (the latter P≤0.05, all the five alternative regimens mentioned P<0.02, in the various experiments) and brain (all five alternative regimens P<0.02). Regimen (e) was slightly inferior to the standard regimen in reducing c.f.u., but also not significantly different.

With the idea that possibly weekly injections could be producing some tolerance, we also tested (g) only two doses given at days 35 and 14 before challenge, and one-tenth the dose at day 35 coupled with either (h) twice or (i) 2.5 times the usual dose at day 14. Regimen (i), one of these two-dose regimens, was modestly superior (P>0.05) to the standard regimen in survival, but inferior in clearing infection from the kidney, and the other two regimens offered no advantage over the standard regimen in any parameter.

We concluded that doubling (or reducing) the number of 2.5 mg injections, increasing 10- or 17-fold the dose at days 28, 21 and 14, or increasing the interval between the last vaccine dose to challenge, from 14 to 21 or 28 days, did not improve (or worsen) the degree of protection in any important way, and all alternative regimens lacked the convenience of the standard.

Alternative methods of presenting Saccharomyces

SC injection of 2.5 mg live strain 96-108 in the 28-21-14 regimen was also protective (survival P=0.009 vs PBS), and not significantly different from the standard HKY regimen in direct comparison (Fig. 2), though slightly better in reducing Aspergillus c.f.u. (P=0.035 vs PBS, brain and kidney) than the standard HKY regimen.

However, 25 mg PO of live strain 96-108 in the 28-21-14 regimen did not demonstrate a protective effect by any measure, in contrast to our experience with PO Saccharomyces vaccination against Coccidioides (Capilla et al., 2009), nor did 2.5 mg HKY PO (which modestly decreased survival compared to PBS).

Thus a live vaccine may offer a modest advantage, but the route appears critical.

Protection is strain-, even species-, independent

Five other strains of S. cerevisiae were compared as HKY, in the standard regimen, to 96-108 (Table 1). These were selected to include differences in genotype (Clemons et al., 1997), virulence in previous in vivo Saccharomyces challenges (Clemons et al., 1994) and origin (commercial, clinical, laboratory strains). All six strains studied provided survival protection (P<0.02), with no significant differences among...
them. Vaccination with all six reduced kidney ($P < 0.01$) and brain ($P < 0.03$) c.f.u. YJM 263 was also studied in a separate experiment, with similar results, as we have reported with another strain, W303 (a descendent of a standard laboratory strain, S288c; Mortimer & Johnston, 1986), with an empty vector (control) (Capilla et al., 2006). Another (eighth), commercial, strain, 96-116 (Lievito verde), was also studied as a vaccine in the standard regimen, and it prolonged survival ($P < 0.03$) and reduced c.f.u. in kidney and brain.

Protection was studied with another species of Saccharomyces, Saccharomyces servazzi. S. servazzi (ATCC 58439) was selected as it is not even in the S. cerevisiae sensu stricto complex of species (Wightman & Meacock, 2003). Vaccination with S. servazzi prolonged survival ($P = 0.008$) (Fig. 3) and reduced kidney ($P = 0.04$) and brain ($P = 0.03$) c.f.u. This was even modestly superior to results with 96-108 in that experiment.

**Genetic composition of the host**

CD-1 mice were selected because they are an ‘outbred’ strain, and it was thought this was a situation resembling more closely vaccine use in the human population, as opposed to an inbred strain, and CD-1 is a robust strain. BALB/c and DBA/2 inbred mice were compared to CD-1 mice (standard vaccination regimen) (Fig. 4a, b). The infection proved more severe in DBA/2 mice (controls), $P < 0.0001$ versus CD-1 controls, as anticipated from prior reports on aspergillosis (Hector et al., 1990). Protection was achieved in both inbred strains (survival $P = 0.046$ and $P < 0.001$ in BALB/c and DBA/2). It particularly is noteworthy that vaccination protected against the much more severe infection in DBA/2, as DBA/2 mice are immuno-compromised [complement (C5) deficient]. Another inbred strain, C57BL/6, was studied in a separate experiment (Fig. 4c). Improvement in survival from vaccination was significant ($P = 0.003$).

**Effect of diet on the host**

A standard mouse diet, Harlan 7956, used in our institution contains dried S. cerevisiae. We questioned whether this could affect responsiveness to a Saccharomyces vaccine. A yeast-free diet, 5L79, containing the same other ingredients, was purchased. Groups were randomized to receive either Harlan 7956 diet (irradiated) or 5L79 (autoclaved). After challenge, both the Harlan- and 5L79-fed mice were protected (survival $P = 0.007$ and $P = 0.015$, vs their respective diet PBS controls). Although the survival of the Harlan-fed PBS control mice was better than that of the 5L79 PBS mice, and the survival of the vaccinated Harlan-fed mice was better than that of the 5L79-vaccinated, in neither case did this reach statistical significance. Fungal burden reduction was seen in both diet group vaccinees (kidneys $P = 0.005$ and $P = 0.015$, and brains $P = 0.007$ and $P = 0.015$, respectively), but with no significant differences between diets.

We concluded that a diet containing S. cerevisiae did not significantly affect vaccine efficacy, though there was a trend for such a diet to increase resistance and to enhance vaccine efficacy. These results should be taken together with the conclusion of the study of PO HKY efficacy described in the second Results section.

**Treatments of the vaccine**

In a study of the possible contribution of surface proteins to vaccine efficacy, HKY efficacy was compared to treatment of HKY with protease and DTT. This treatment resulted in loss of efficacy, as measured by survival ($P = 0.004$ and $P > 0.05$, respectively) or reduction of brain
or kidney c.f.u. ($P<0.02$ and $P>0.05$, respectively, both organs).

In a separate experiment, the mixture resulting from the standard heat-killing method was separated into pellet and supernatant by centrifugation, and each component was separately studied. As alternatives to heat-killing, we also studied breaking the yeasts by bead-beating, heating to the higher temperature of 100 °C or treatment with formalin. The mixture resulting from bead-beating was also further separated into pellet and supernatant by centrifugation.

The pellet of the HKY, the pellet of the bead-beaten cells, and the 100 °C-treated yeasts all were protective, as was HKY, as assessed by survival (Fig. 5), reduction of kidney c.f.u. or reduction of brain c.f.u. ($P \leq 0.001$ in all three parameters of efficacy for all these four reagents), but the supernatant of the HKY material, the supernatant of the bead-broken yeasts, and the formalin-treated cells lost protective activity ($P>0.05$ for all three outcome parameters for all these three reagents).

We concluded from this series of experiments that proteins in HKY are a component of the protection, though these proteins are resistant to 100 °C (in situ in the whole yeast particle), that the protective elements reside in the insoluble cell wall after standard heat treatment or mechanical breakage, but not in the cytosol after these methods of killing, and that the protective component(s) are susceptible to formalin treatment.

**Effect of adjuvants**

To assess whether the HKY effect could be boosted by adjuvants, CpG was studied in two experiments. In one, survival was actually inferior when CpG was added, whereas reduction of c.f.u. in kidney and brain were both $P \leq 0.05$ with HKY alone, in neither organ was there a significant protective effect with HKY + CpG. In the second experiment, survival was unaffected by CpG + HKY compared to HKY alone, and reduction of c.f.u. in brain and kidney was better than with HKY alone, but not significantly different.
In an experiment with MPL + HKY, the combination gave equivalent survival to HKY alone (both \( P < 0.02 \)) (Fig. 6a), and reduction of c.f.u. in both organs was better than with HKY alone, but also not significantly different.

In a third experiment, alum + HKY was tested. Alum + HKY, and HKY alone, significantly prolonged survival (both \( P < 0.05 \)). Alum + HKY improved survival and reduction of brain c.f.u. compared to HKY alone, but the differences were not significantly different. However, whereas both HKY and HKY + alum reduced kidney c.f.u. (\( P = 0.002 \) and \( P < 0.0001 \), respectively), the advantage for alum + HKY was highly significant (\( P < 0.0001 \) vs HKY alone), and eliminated infection in 60% of the animals’ target organ of infection (vs 0% for HKY alone) (Fig. 6b). Thus, in the doses studied, only alum, of the adjuvants studied, appeared to hold promise of future potential.

**Passive transfer**

Two experiments were performed to assess whether antibody and other serum factors from vaccinated animals could reconstitute the immunity conferred by vaccination. In one experiment, the recipients were 5-week-old naïve mice, and in a second experiment, the recipients were 9-week-old (the age at which a cohort vaccinated with the standard regimen would have been challenged) naïve mice. In both experiments, no protection was noted comparing the recipients of serum from vaccinated mice, from unvaccinated mice, and the groups given no serum, whereas mice given only the vaccine, concurrently studied, showed the protective vaccine effect described (data not shown).

**Comparison to other vaccines**

The relative potency of HKY to previously defined vaccines was of interest. However, it was not logistically possible to test each previously described vaccine with the same Aspergillus strain and in the model, dosing regimen, and mouse strain, age and sex in which it was previously described; such specific circumstances possibly may have been optimal for the demonstration of efficacy of that vaccine. Nor was it possible to test our standard regimen, reproducing every previously described setting. We therefore compared the alternative preparations using our regimen for HKY.

These included heat-killed *A. fumigatus* conidia (Cenci *et al*., 2000; Richard *et al*., 1982) (HKC), heat-killed *Aspergillus* germlings (Richard *et al*., 1982), crude hyphal extract (Ito *et al*., 2006), live conidia and *Aspergillus* culture filtrate (Cenci *et al*., 2000). Batches of HKC were also killed by two different methods and studied at two different concentrations previously described in the literature, and the HKC and heat-killed germlings were studied with and without CpG adjuvant.

The crude *Aspergillus* culture filtrate, given SC, had no efficacious effect.

Heat-killed germlings, given SC, did not improve survival or reduce renal or brain c.f.u. compared to PBS, and adding CpG only improved brain clearance (\( P = 0.06 \)), still inferior to HKY.

HKC killed by heating at 100 °C for 2 h (Richard *et al*., 1982) and given SC in the same number of particles as HKY, with or without CpG, did not improve survival or reduce kidney burden compared to PBS. Brain c.f.u. were reduced by HKC (\( P = 0.015 \)), but this effect was lost when these HKC were given with CpG.

Given PO, these HKC, \( 6 \times 10^7 \) or \( 6 \times 10^9 \), or live conidia \( 6 \times 10^9 \) PO, did not result in any measure of efficacy.

Conidia killed by autoclaving and given at \( 2 \times 10^7 \) SC (Cenci *et al*., 2000) improved survival (\( P = 0.01 \)) and reduced kidney c.f.u. (\( P = 0.02 \)) compared to PBS, both \( \ldots \)
superior to HKY, but reduction of brain c.f.u. was inferior to that with HKY.

The crude hyphal extract, given SC, did give indistinguishable protective results to HKY (P>0.05) in two experiments.

Thus some Aspergillus-specific vaccine preparations, compared directly to HKY in the same SC regimen, did show efficacy, but, even with adjuvants in some cases, were not significantly better than HKY.

DISCUSSION

We show that heat-killed or live SC Saccharomyces injection was protective against systemic aspergillosis. The protection was independent of the strain studied, and apparently independent of the Saccharomyces species. The protection was independent of the mouse strain studied, even a C' deficient host (in this most severe infection, the protection was most pronounced). The protective component(s) reside in the cell wall, and surface proteins seem to be one component. The protection appears comparable to that with particulate or crude Aspergillus derivatives previously reported. This last comparison is reminiscent of conclusions drawn on the anti-coccidoidal efficacy of HKY compared to previously studied vaccines specific to Coccidioides (Capilla et al., 2009).

Taken together with our prior publication on protection against systemic coccidioidomycosis (Capilla et al., 2009), Saccharomyces appears to present a possible pathway toward development of a panfungal vaccine. Preliminary data indicate that this same vaccine is also protective against systemic candidiasis (Liu et al., 2010c; Stevens et al., 2011) and cryptococcosis (Majumder et al., 2011). Candidates in Saccharomyces for a protective effect against such diverse fungi (opportunist and primary pathogens; dimorphic fungi, filamentous fungi and yeasts; fungi with markedly diverse methods of replication) include proteins, glycans and lipids.

In addition to the protease studies, in further preliminary studies we have identified homologous cross-reacting proteins in Saccharomyces, Aspergillus and Coccidioides (Stevens et al., 2011), and others have described other homologous proteins in these three fungi (Bozza et al., 2009; Hearn et al., 1998; Ito et al., 2006; Kuranda & Robbins, 1991; Pishko et al., 1995; Xue et al., 2009), with suggestion that some Saccharomyces proteins may be cross-protective against aspergillosis (Bozza et al., 2009).

The glycan, glucan, conjugated to protein has been shown to protect against Candida and Aspergillus, and neither the glycan nor the protein was derived from those fungi (Torosantucci et al., 2005). The enhancement of an immune response to carbohydrates by conjugation to a protein is long-known (Finn, 2004; Lees et al., 1996; Lucas et al., 2005; Salehen & Stover, 2008; Stevens et al., 2011), and this has been well-exploited in development of antibacterial vaccines (Finn, 2004; Goldblatt, 2000). Our ongoing studies (Liu et al., 2009, 2010a, b; Stevens et al., 2011) support the possible contribution of glycans to the protective effect of Saccharomyces. We have found that the cell wall glycans alone, mannann or glucan, as a vaccine each provide significant protection against Coccioidoides or Aspergillus challenge, which is greatly enhanced by conjugation to a non-specific protein (BSA) (Liu et al., 2009, 2010a, b; Stevens et al., 2011). We have most extensively thus far studied mannann as a vaccine against aspergillosis, and the protective mannann was derived from Candida (Liu et al., 2009, 2010b; Stevens et al., 2011). Preliminary studies, stripping the Saccharomyces cell wall of mannann with successive alkaline digestions, have also suggested that glucan is an important component of protection (Stevens et al., 2011), as also indicated by others’ direct study of glucan (Bozza et al., 2009; Torosantucci et al., 2005). Other observations have also suggested that cell wall lipids may be a component of the protection afforded by Saccharomyces against aspergillosis (Stevens et al., 2011). The composition of the cell walls of Saccharomyces and Aspergillus has been studied in some detail, noting similarities and differences, as detailed elsewhere (Bernard & Latgé, 2001; Klis et al., 2002).

As described above, further study is directed to defining the protective components of Saccharomyces, as well as optimizing the delivery system. In regard to the latter, we note whereas alum potentiates the protective effect against aspergillosis, it did not against coccidioidomycosis (Capilla et al., 2009). Of interest were the results with the formalin-killed yeast, in that this preparation showed no significant prolongation of survival when administered at the same dose as HKY. This result was somewhat surprising, in that the most effective vaccine preparation for Coccioidoides is the formalin-killed spherule preparation (Levine et al., 1970), where the formalin does not apparently affect the induction of protective immunity to the extent that we observed with the S. cerevisiae preparation. Although the reason for the lack of protective activity is unknown, it is possible that the formalin treatment and resultant cross-linking of the amino groups of cell-associated (cell-wall-associated and cytoplasmic) proteins altered these components such that cross-protective activity was lost. In addition, it is possible that the formalin treatment of the yeast affects the cell wall polysaccharide integrity directly, altering the glycosidic linkages or through damage to cell-wall-associated structural proteins such that cross-protection is lost. A question raised is what antigen(s) is/are formalin destroying that are not affected by 100°C treatment.

Another central future issue is the nature of the protective host response. Our studies to date indicate that HKY vaccination stimulates antibodies to glucan and mannann, that spleen cells and lymph node cells of vaccinees proliferate in response to HKY (CD3 and CD8 lymphocytes) and produce proinflammatory cytokines, and that

M. Liu and others
proinflammatory cytokines are found in the bronchoalveolar lavage of vaccinated mice (Liu et al., 2011). Although the potentiation of the protective response by alum is suggestive of a role for humoral immunity (HogenEsch, 2002; Hunter, 2002), and passive transfer of serum from glucan-immunized animals offered protection in the prior studies cited (Torosantucci et al., 2005), passive transfer of serum from HKY-vaccinated mice failed to protect recipients. Our results with the C′-deficient strain may suggest the importance of regulatory T cells and Th17 cells in the protective response (Weaver et al., 2010). Much more work needs to be done, including transfer of lymphocytes and lymphocyte subclasses, and possibly testing vaccination in animals where various limbs of immunity have been compromised by suppression, ablation or congenital immunodeficiency. This will illuminate the critical arms of vaccine-induced immunity, but it is also essential that pursuit of any vaccine against aspergillosis be studied for efficacy in animals whose immunity is immunocompromised, as well as in animals challenged by other routes of infection, particularly the respiratory route (Clemons & Stevens, 2005).

We have initially seen HKY as nature’s experimental reagent, a natural conjugate, showing the way to a purified vaccine once the protective elements have been discovered and defined. We should note, however, that heat-killed Saccharomyces has been studied in man as a vehicle for delivering tumour antigens, and subcutaneous administration did not exhibit dose-limiting toxicity in phase I human clinical trials (Franzusoff et al., 2005; Lu et al., 2004; Munson et al., 2007), raising the possibility of efficacy of HKY itself as a panfungal vaccine in man.

REFERENCES


