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Vaccination with an alkaline extract of *Histoplasma capsulatum* packaged in glucan particles confers protective immunity in mice

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ABSTRACT

Infection with the dimorphic fungus, *Histoplasma capsulatum*, occurs world-wide, but North and South America are regions of high endemicity. Interventions to mitigate exposure and consequent disease are limited to remediating a habitat harboring the fungus. The development of a vaccine to prevent infection or lessen its severity is an important advance in disease prevention. Accordingly, we prepared an alkaline extract from the yeast phase of *Histoplasma* and encased it in glucan particles that act as an adjuvant and delivery vehicle. Immunization of C57BL/6 mice with this encapsulated extract decreased the number of CFUs in lungs and spleens at days 7 and 14 following intranasal infection. Moreover, this vaccine conferred protection against a lethal challenge with the fungus. Cytokine assessment in lungs at a time when the CFUs were similar between controls and vaccinated groups revealed increased quantities of interferon-γ and interleukin-17 in vaccine recipients. This finding was supported by increased generation of both Th1 and Th17 cells in lungs and draining lymph nodes of vaccinated mice compared to controls. Neutralization of interferon-γ or interleukin-17 blunted the effectiveness of vaccination. To identify the proteins comprising this extract, liquid chromatography tandem mass spectrometry was performed. Thus, an *H. capsulatum* alkaline extract packaged in glucan particles confers protection in an interferon-γ and interleukin-17-dependent manner. Discovery of a single protein or a few proteins in this admixture that mediate protective immunity would represent significant progress in efforts to prevent histoplasmosis.

1. Introduction

The mammalian pathogen, *Histoplasma capsulatum*, is a dimorphic fungus with a global distribution. It resides in the soil of numerous regions of the world but is highly endemic to the Midwestern and Southeastern US and Central and South America. Humans are coincidentally infected via the lungs upon disturbance of the soil. The spectrum of illness ranges from asymptomatic to a life-threatening progressive infection. It is estimated that 250,000 to 500,000 new infections occur annually in the US alone. Among those infected the majority will require medical intervention. However, approximately 10% of individuals develop serious infection that necessitates antifungal therapy [1].

The implementation of a targeted vaccination strategy could reduce the severity of disease if not completely prevent it. The at-risk populations include those with HIV infection, recipients of immunosuppressive agents such as glucocorticoids, individuals with inflammatory diseases that are treated with biologicals especially the tumor necrosis factor antagonists, and workers with frequent exposure to soil that is laden with fungal spores [2–6]. To date, there is no licensed vaccine for this fungal infection or for any of the medically important human fungal pathogens. We previously reported that *H. capsulatum* heat shock protein 60 is immunogenic for mouse and human cells and protected against challenges with yeast cells. Protection required $CD4^+$ and $CD8^+$ T cells [7–10]. However, a concern about the use of this immunogen as a vaccine candidate is its identity with the corresponding mammalian gene. Theoretically, this could induce an autoimmune reaction [7]. Moving forward other vaccine candidates are needed. While genetically manipulated yeast cells that exhibit impaired

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growth might confer excellent protection, the likelihood that such an agent could be deployed for human use is remote.

Given those considerations, we utilized an alkaline extract from *H. capsulatum* to generate a protective immunogen. This material was packaged in glucan particles (GPs) which combine antigen presenting cell targeting with potent adjuvant activity. Our data demonstrate that GPs containing the alkaline extract protected mice against sublethal and lethal challenges with *H. capsulatum*. The protective efficacy was associated with enhanced interferon (IFN)-γ and interleukin (IL)-17 generation by T cells, in particular $CD4^+$ T cells. These results provide a fresh approach to creating a vaccine for *H. capsulatum*.

2. Materials and Methods

2.1. Mice

Male C57BL/6 were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were housed in isolator cages and maintained by the Department of Laboratory Animal Medicine, University of Cincinnati or the University of Massachusetts Medical School. Both are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. All animal experiments were performed in accordance with the Animal Welfare Act guidelines of the National Institutes of Health, and all protocols were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati and the University of Massachusetts Medical School. Infected animals were housed in an ABSL2.

2.2. Preparation of H. capsulatum and infection of mice

H. capsulatum strain G217B yeast cells were grown for 72 h at 37° C as described (15). Mice were inoculated intranasally with 2×10^6 yeasts or 1.5×10^7 yeasts in ~30 μ l of Hank's Balanced Salt Solution (HyClone, Logan, UT).

2.3. Organ culture for H. capsulatum

Organs were homogenized in sterile HBSS and serially diluted and plated onto Mycosel™-agar plates containing 5% sheep blood and 5% glucose. Plates were incubated at 30° C for 7 days. The limit of detection was 10^2 colony forming units (CFU).

2.4. Preparation of alkaline extract

The extract was prepared similar to that for *C. neoformans* [11]. Yeast cells were grown to mid to late log phase in Ham's F12 medium, collected, washed three times in phosphate-buffered saline (PBS). Pellets were stored at −20 °C. For alkaline extraction, the cell pellets were thawed at 22 °C, suspended in 1.5 volumes of 0.1 M potassium hydroxide (KOH) and rotated for 10 min at 22 °C. Particulate material was removed by centrifugation, as described above. For each ml of supernatant, 25 μl 1 M sodium acetate buffer, pH 4.5, was added to neutralize the KOH, followed by mixing with 1.5 volumes of isopropanol and storage at − 20 °C. The precipitate was collected following centrifugation at 6000 ×*g* for 10 min at 4 °C, suspended in 15 ml 0.1 M ammonium acetate, then re-precipitated with 2 volumes of ethanol, and stored at −20 °C. The precipitate was lyophilized. Subsequently, it was then suspended in 7.5 ml 0.01 M ammonium acetate and centrifuged at 6000 ×*g* for 10 min at 4 °C. The supernatant containing soluble alkaline extracted antigens was stored at −80 °C. Protein concentration was assayed by the bicinchoninic acid assay.

2.5. Glucan particle vaccine

Saccharomyces cerevisiae cells were converted into GPs following a series of hot alkali, organic, and aqueous extraction steps, as described previously [12]. The final product consisted of a highly purified 3- to 4-μm-diameter yeast cell wall preparation devoid of cytoplasmic contents and outer mannoproteins and bounded by a porous, insoluble shell of β-glucans. The final GP vaccine consisted of *Histoplasma* alkaline extract, mouse serum albumin (Equitech-Bio, Kerrville, TX), and yeast RNA (yRNA; Sigma, St. Louis, MO) complexed within the glucan shells. Samples to be loaded into GPs were concentrated in 6 M urea, 20 mM Tris-HCl, pH 7.9 at 10 mg/ml or 50 mg/ml by protein content. Antigens were loaded into GPs and complexed with MSA and yRNA as described elsewhere [12]. Vaccines were diluted in sterile 0.9% saline for injection. A control vaccine consisted of GPs loaded with MSA and yRNA but without *Histoplasma* alkaline extract. Vaccines were stored in 0.6-ml aliquots at −80 °C, thawed, and vortexed prior to use. Vaccines were also extracted and analyzed by SDS-PAGE to quantify antigen/MSA loading.

To vaccinate mice, they were given 10^8 particles i.n. (in two 50 μl aliquots) followed by subcutaneous injections of 10^8 particles 2 wk and then 4 wk later. In initial experiments mice were injected subcutaneously 3 times with 100 μl (10^8 particles) of GPs. This number of particles contained 50 μg of extract or GPs containing mouse serum albumin. Each injection was separated by 2 wks. Mice were challenged with *H. capsulatum* 2–4 weeks following the last injection. In another set of experiments, we injected 10^8 particles once i.n. followed by infection with 2 x 10^6 yeasts i.n. 2 weeks later.

2.6. Analysis of alkaline extracts by electrospray ionization Liquid Chromatography-Mass Spectroscopy/Mass Spectroscopy (LC-MS/MS)

Alkaline extracts were processed for LC-MS/MS analysis as described [11]. Analysis and database searching was with Mascot (Matrix Science, London, UK; version 2.1.1.21) against the *H. capsulatum* database (GenBank, *Histoplasma capsulatum*, taxid 5037). Scaffold software (Proteome Software, Inc., Portland, OR; version 4.8.4) was used to validate the identity of peptides (trypsin digest products) and proteins. Probabilities were assigned by using the Protein Prophet algorithm at 70% for peptides and 90% for proteins that included at least 2 identified peptides. The relative amount (normalized iBAQ value) of each protein was determined so that protein abundance could be ranked. Protein sequences were analyzed using SignalP 4.1 for signal peptide cleavage (http://www.cbs.dtu.dk/services/SignalP/).

2.7. Flow cytometry analysis

Fluorescence-activated cell sorting (FACS) flow cytometry was performed as previously described with slight modifications [13]. Briefly, single-cell suspensions were prepared from mouse lungs by using a lung dissociation kit (Miltenyi Biotec) according to the manufacturer's protocol. The cell pellets were resuspended in 40% Percoll in PBS, layered on top of 67% Percoll in RPMI, and centrifuged at 600 ×*g* for 20 min. The lung leukocytes at the interface were collected and washed twice with PBS. For lymphoid tissue, spleens and lymph nodes were collected minced through a stainless steel mesh and red cells lysed. Lung leukocytes ($2–4 \times 10^5$) were incubated in 200 μl of RPMI + 10% fetal bovine serum in each well of a 96 well plate; lymphoid cells (2 x 10^6) were incubated in 500 μl of medium in each well of a 48 well plate. Cells were incubated with GPs at a ratio of 3 particles for every 10 leukocytes. For heat-killed yeast cells, they were incubated at a ratio of 3 yeast cells for every 10 leukocytes. Cells exposed to the indicated stimuli

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