Application of immunoproteomics and bioinformatics to coccidioidomycosis vaccinology

Eric James Tarcha  
*Medical University of Ohio*

Follow this and additional works at: [http://utdr.utoledo.edu/theses-dissertations](http://utdr.utoledo.edu/theses-dissertations)

Recommended Citation

[http://utdr.utoledo.edu/theses-dissertations/1388](http://utdr.utoledo.edu/theses-dissertations/1388)

This Dissertation is brought to you for free and open access by The University of Toledo Digital Repository. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of The University of Toledo Digital Repository. For more information, please see the repository's About page.
Application of Immunoproteomics and Bioinformatics to coccidioidomycosis
Vaccinology

Submitted by
Eric J. Tarcha

In partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Medical Sciences

Date of Defense:
May 3, 2006

Major Advisor
Garry Cole, Ph.D.

Academic Advisory Committee
Eric Lafontaine, Ph.D.
R. Mark Wooten, Ph.D.
Venkatesha Basrur, Ph.D.
Robert M. Blumenthal, Ph.D.

Dean, College of Graduate Studies
Keith K. Schlender, Ph.D.
Application of Immunoproteomics and Bioinformatics to Coccidioidomycosis Vaccinology

Eric James Tarcha
Medical University of Ohio at Toledo
2006
DEDICATION

I would like to dedicate this work to my beautiful wife Jeannette. Without your constant source of unconditional love and support, none of this would be possible. You have brought me light and hope throughout our relationship and for that, I thank you.

"Hope is a good thing, maybe the best of things, and no good thing ever dies."

-Andy Dufresne "Shaw Shank Redemption"

I would also like to thank my family and friends who have supported me through my journey through graduate school.

To my parents, thank you for all your sacrifices that have allowed me to succeed in life. Your constant source of love, support and advice has provided Jeannette and I the strength to achieve what we have. No words can be said to thank you for everything you have done for me.

To my brother and sisters, Julia, Matthew and Alexis, I would like to thank you for your support, love and inspiration to try to be the best big brother I can be. I love you with all my heart.

Lastly, I would like to thank all my friends who have helped me keep things “real.”
ACKNOWLEDGEMENTS

I would like to acknowledge and thank my major advisor, Garry T. Cole, Ph.D., and my academic advisory committee for all of their assistance and encouragement (Bob Blumenthal, Ph.D., R. Mark Wooten, Ph.D., Eric Lafontaine, Ph.D., Chiung-Yu Hung, Ph.D., and Venkatesa Basrur, Ph.D.).

I would also like to thank the following collaborators for their contributions:

- Dr. Malcom Gardner at The Institute for Genomics Research for the *C. posadasii* genome and protein databases;
- Dr. Thomas Forsthuber for his gift of the human MHC class II transgenic mice;
- Dr. Venky Basrur for all his assistance and guidance in all things proteomics; and
- The past and present members of the Cole lab. for their help and support throughout the past 5 years.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dedication</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>iii</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>iv</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Literature Review</td>
<td>7</td>
</tr>
<tr>
<td>Manuscript 1</td>
<td>42</td>
</tr>
</tbody>
</table>

A recombinant aspartyl protease of *Coccidoides posadasii* induces protection against pulmonary coccidioidomycosis in mice.

| Manuscript 2                  | 102  |
| An immunoproteomics and bioinformatics approach to identification of *Coccidoides posadasii* cell-wall associated and T cell reactive antigens yields a protective multivalent recombinant vaccine. |

| Summary and Discussion        | 159  |
| References                    | 169  |
| Abstract                      | 196  |
INTRODUCTION

*Coccidioides* (*C. posadasii* and *C. immitis*) is a primary fungal pathogen and the etiologic agent of a human respiratory infection known as coccidioidomycosis or San Joaquin Valley fever (Fisher et al., 2002; Smith, 1940). *Coccidioides* is endemic to the alkaline desert soil of Southern California, Arizona, Nevada, New Mexico and West Texas, as well as parts of Mexico, Central America and South America (Negroni, 1996; Pappagianis, 1988). The saprobic phase of the fungus grows as septate hyphae that when dry, disarticulate and break apart to form the infectious propagules of the pathogen. Humans acquire coccidioidal infection by inhalation of these propagules or arthroconidia, which once in vivo, grow isotropically and differentiate into large, endosporulating spherules inside the host tissue.

Clinically, coccidioidomycosis can present as many manifestations, from a flu-like pneumatic infection that resolves spontaneously, to a hematogenously and lymphogenously disseminated mycosis. The disseminated form of the disease in which the pathogen can migrate from the lungs to multiple other organs causing severe debilitation, and even death, is considered to be the most difficult for the patient to overcome and for clinicians to treat (Dooley et al., 1994; Einstein and Catanzaro, 1996). Approximately 50% of immunocompetent individuals exposed to *Coccidioides* develop symptomatic infection, manifesting 1-4 wks after inhalation of the fungal spores. Most infected individuals recover during the subsequent few weeks to several months, while approximately 5 - 10 % will show evidence of active disease for a much longer period of time. There are approximately 150,000 new cases that occur each year in areas of
endemicity within the United States (Galgiani, 1993; Rosenstein et al., 2001). The massive numbers of immunonaive persons who have moved to or visited the endemic areas over the past 20 yr has resulted in a large population of individuals who have never been exposed to the fungus and are at risk of infection (Cole et al., 2004). As a result, the number of reported coccidioidal infections each year has continued to rise dramatically.

Antifungal drug treatment of persons with diagnosed coccidioidomycosis is commonly maintained for 3 to 6 mo (Galgiani, 1999). Conversely, coccidioidomycosis contracted by patients infected with HIV or other immunosuppressing conditions often requires life-long antifungal medication to suppress reactivation of the fungal disease (Sugar, 1990). The total yearly cost to infected patients, based on a retrospective study conducted between 1991 and 1993 at the Kern Medical Center in Bakersfield, California, was estimated to be over $8,000 (Caldwell et al., 1996). Non-disseminated cases averaged $5,400/year, while patients who contracted the disseminated form of the disease faced an average cost of $48,000. Currently, costs incurred by patients with severe coccidioidal infection can climb to $300,000 over the course of the disease. A conservative estimate of the annual health care expenses for treatment of patients with coccidioidomycosis in the U.S. is $60 million (www.valleyfever.com). Vaccination against Coccidioides infection has been argued to be a cost-effective intervention to the financial drain of coccidioidomycosis on the medical system (Barnato et al., 2001) and, on this basis alone, is worthy of serious consideration.

Immunization of mice with formaldehyde killed parasitic spherules, avirulent strains of Coccidioides, or subcellular protein extracts has been shown to confer protective immunity to the animals against infection following a lethal, pulmonary
challenge with *Coccidioides* arthroconidia (Pappagianis et al., 1979, 1961). These data, along with the observation that resolution of natural human infection with *Coccidioides* often results in life-long immunity (Smith, 1940) suggests that development of a vaccine against coccidioidomycosis is feasible. Studies imparting the importance of T-cell mediated immunity for effective defense against *Coccidioides* has been provided by clinical as well as experimental data, while the role of B cells and antibody in protective immunity to this pathogen has been disputed (Cox and Magee, 1998, Kirkland et al., 2006; Magee et al., 2005). As a result, the exploration for novel vaccine candidates against coccidioidomycosis has focused on individual T cell reactive proteins that confer protection in a murine pulmonary model of infection.

The majority of protective antigens that have been characterized to date are products of the parasitic phase of the fungus (Kirkland and Cole, 2001). The most promising of these have been cell wall-associated or secreted antigens of parasitic spherules (Delgado et al., 2003; Shubitz et al., 2002). It has been hypothesized that the extracellular location of these antigens promotes the accessibility of the proteins to the host immune system and thereby the subsequent activation of the proper subsets of protective immune cells. Although many of these individual antigens have shown degrees of protection in the murine model of disease, no single protein has been able to confer the levels of immunodefense observed by immunization with whole cell or complex protein extracts. Even more, there is a dearth of data exploring the protective efficacy of polyvalent vaccines composed of multiple protective antigens against coccidioidomycosis, which may be the key to the development of an efficacious vaccine.
The post-genomic era has ushered in many new and exciting fields that revolve around the massive amount of genomics data provided by genome sequencing projects. These fields fall under the categorization of functional genomics and center on the study of gene expression at a functional level. Functional genomic studies rely on high-throughput techniques for measuring the mRNA (the transcriptome), protein (the proteome), and metabolite (the metabolome) components of cells, tissues, organs, and whole organisms. This has given scientists the ability to address specific questions not on an individual basis, but en masse through strategies that examine not a single biological object but upwards of thousands, or even hundreds of thousands.

The proteome is the protein complement of a cell corresponding to the genome and transcriptome, and proteomics is the science developed to study the proteome. Recent applications of proteomics to study the immunogenic proteins and/or antigenic peptides within microbial pathogens (the immunome), has opened a proverbial Pandora’s box of antigens that could be further characterized as vaccine candidates (Doytchinova et al., 2003). Thus, the application of proteomics and bioinformatics to fungal vaccinology studies seems to offer the greatest opportunity for the efficient discovery of safe, efficacious, and novel subunit vaccines. The utilization of proteomics and bioinformatics to study the immunome of *Coccidioides* has not been fully explored. Therefore, it can be argued that these types of studies provide the best opportunity to identify new antigenic proteins that can be used in the development of an effective vaccine against coccidioidomycosis.

The literature suggests that an ideal coccidioidal vaccine must be cost effective to produce, physically stable and contain an adequate number of essential protective
epitopes to induce long-lasting immunity against infection (Cole et al., 2004; Cox and Magee, 2004). Based on these suggestions, a human vaccine against coccidioidomycosis could be composed of recombinant proteins that contain genetically unrestricted ("promiscuous") protective T cell epitopes, and be polyvalent in nature. In this study it was hypothesized that there are immunogenic cell wall and secreted parasitic phase expressed antigens in the immunome of *Coccidioides posadasii* that contain epitopes eliciting a protective immune response and that can be used to develop a protective multivalent vaccine against coccidioidomycosis. It was proposed to test this hypothesis using a combined immunoproteomics and bioinformatics approach to map and identify immunogenic proteins in these coccidioidal fractions as well as to predict which antigens have promiscuous MHC II restricted T-cell epitopes.

The specific aims of this study were to:

1. Map and identify the major IgG-specific (T cell-dependent) seroreactive proteins in the parasitic phase cell wall and culture filtrate of *Coccidioides posadasii*, and assess these antigens for the presence of predicted promiscuous MHC II restricted T-cell epitopes,

2. Evaluate T-cell reactivity of selected seroreactive full-length recombinant proteins and synthetic peptides containing predicted promiscuous T-cell epitopes using human MHCII transgenic mouse and C57BL/6 mouse based immunoassays,
3. Determine the protective efficacy of seroreactive and T cell reactive recombinant antigens containing promiscuous T-cell epitopes in a murine (C57BL/6) model of coccidioidomycosis, and

4. Develop a multivalent subunit vaccine to be evaluated for protective efficacy in a murine (C57BL/6) model of coccidioidomycosis.
LITERATURE REVIEW

Historical Perspectives of Coccidioidomycosis

Wernicke and Posadas separately published on the first reported case of coccidioidomycosis in 1892, when they observed a distinct, previously uncharacterized organism in what was thought to be cancerous skin lesions of a South American soldier (Posadas, 1892; Wernicke, 1892). The infection was originally characterized as protozoan in nature due to the spherule like morphology of the pathogen observed in biopsies of the infected tissue. Four years later, Rixford and Gilchrist (1896) published on the first reported cases of coccidioidomycosis in the United States, once again observing the protozoan-like organism in infected tissue, and named it Coccidioides. Interestingly, the two patients in their report had contrasting presentations of disease. The first patient presented with a slowly developing chronic disease that led to his death 9 yr after he first reported symptoms, while the second patient died from the disease within 4 mo. The dramatic differences in clinical presentation led Rixford and Gilchrist to believe that these unique cases were caused by different species of Coccidioides. They proposed that the species name of immitis (meaning "not mild") be used for the causative organism of the first case and pyogenes (producing pus) be used for the causative organism for the second case.

At the turn of the century, Ophuls and Moffitt (1900) discovered the fungal nature of the causative agent by observing that cultures of naturally infected tissue always yielded mycelial growth. They concluded that the mold was the etiological agent of the disease when spores of the fungus were injected into the ear vein of a rabbit, and the
rabbit developed nodules in its lungs, spleen, and kidneys similar to those observed in infected humans. Further microscopic examination of the nodules from the infected rabbit revealed that these abscesses contained the round protozoan-like structures observed in clinical samples and no mycelium, strengthening their argument and suggesting that the pathogen relied on a dimorphic life cycle for in vitro and in vivo growth.

Severe disseminated disease was thought to be the only form of infection until 1929 when a laboratory accident provided insight into milder forms of disease (Fiese, 1957). A graduate student studying *Coccidioides* at University of California, in the laboratory of Ernest Dickson, was accidentally infected by breathing in aerosolized arthroconidia, and developed a pneumatic like illness with erythematous nodules on his shins. Endosporulating spherules were observed in his sputum samples taken during a doctor’s examination suggesting coccidioidomycosis. The student was eventually diagnosed with coccidioidal infection, but never developed severe disseminated disease, and soon after completely recovered. This led Dickson to further explore the nature of similar infections in people living in the surrounding area of Bakersfield California (Dickson, 1937). From his studies he concluded that “the fungus *Coccidioides* is sometimes the cause of a symptomatic complex of acute illnesses identical with what has been known locally in the San Joaquin Valley as ‘Valley Fever’ characterized by acute pulmonary symptoms and erythema nodosum.” He later proposed that the term "coccidioidomycosis" should include all forms of infection caused by *Coccidioides* not only the severe disseminated forms.
Biology of Coccidioides

Ecology

*Coccidioides* is a haploid ascomyceteous fungus phylogenetically classified in the family Onygenaceae, order Onygenales, along with the other dimorphic human fungal pathogens (Currah, 1985). This respiratory pathogen has a dimorphic life cycle containing both saprobic and parasitic phases (Figure 1). The saprobic phase grows in the alkaline desert soil and is represented by septate hyphae that produce enterothallic arthroconidia. These conidia are the infectious propagules of the fungus. The multinucleated arthroconidia, are formed when the septate mycelia dry and disarticulate. The arthroconidia of *Coccidioides* are usually barrel-shaped spores, measuring 2.5 to 4 µm in width and 3 to 6 µm in length and are small enough to reach the alveoli of the lungs when inhaled. The parasitic phase of *Coccidioides* is characterized by mature spherules that are derived from single arthroconidia, containing hundreds of endospores (Cole, 2003). The parasitic phase of coccidioidal growth relies on the conversion of the arthroconidia into spherules. This transition is thought to be induced by increased temperature (between 34 and 41°C) and CO₂ concentration (10 to 20%), although the overall process is poorly understood. The start of spherulation begins with isotropic growth of the single cells and is characterized by a rounding up and swelling of the cells followed by synchronous nuclear divisions and segmentation (Kirkland and Cole, 2001). At the center of the immature spherules is a vacuole that is considered to be important in the process of endospore release. As the spherules mature there is progressive
FIGURE 1. Summary of *Coccidioides* life cycle.
invagination of the cell wall and isolation of sections of cytoplasm that surround the vacuole, and give rise to uninucleate compartments. These compartmentalized sections eventually differentiate into endospores. The mature spherule can measure upwards of 60-120 µm and can contain 200 to 300 endospores. Once mature, the full spherule ruptures, releasing the endospores into the surrounding environment. The endospores typically measure 2 to 4 µm in diameter, and each can give rise to another mature spherule, repeating this parasitic life cycle. This process gives *Coccidioides* a high reproductive capacity and prolific fecundity in the host. The development of a defined liquid medium by Converse (1955, 1956, 1957) has enabled scientists to study the parasitic phase in vitro. In vitro studies have allowed for the delineation of the minimal requirements to induce the parasitic growth phase (Breslau and Kubota, 1964; Converse, 1956, 1957). Along with increased temperature and CO₂ concentration, parasitic growth is stimulated by the addition of a surfactant such as Tamol N. Typically, under ideal conditions, 100% of the viable arthroconidia will undergo the synchronous transformation to parasitic spherules in the growth medium and will reach the endosporulation stage between 96 h and 120 h. Although the differentiation of arthroconidia into endosporulating spherules in vitro appears to be identical to that observed in vivo (Drutz and Huppert, 1983), further studies need to be performed before this conclusion can be assured.

**Phylogenetic Speciation of Coccidioides**

Recent phylogenetic analyses of *Coccidioides* strains using single-nucleotide polymorphisms, genes, and microsatellites have shown the evolution of two genetically
different *Coccidioides* clades, a California and a non-California clade (Fisher et al., 2002). It was previously thought that *C. immitis* was the sole etiologic agent of coccidioidomycosis. The new phylogenetic data led the investigators to propose the designation of *C. immitis* for the California clade and *C. posadasii* for the non-California clade, in memory of Alejandro Posadas. While there are clear differences in genotype between *C. immitis* and *C. posadasii*, the lack of substantive phenotypic differences, as well as the observation of no demonstrable variation in virulence between the proposed species, has promoted arguments against separation of the two species (Cox and Magee, 2004). Minor differences in amino acid sequence of proteins of *C. posadasii* and *C. immitis* have been reported (Koufopanou et al., 1997; Peng et al., 1999), but there are currently no publications reporting differences in antigenicity, virulence, or morphology of the two species.

**Coccidioides as a Select Agent**

The virulence of *Coccidioides*, ease of infection by inhalation of dry, air-dispersed spores, and biogeographic range in heavily populated regions of North America (including military bases) has raised fears of the biohazardous nature and bioweaponizing potential of this human pathogen (Dixon, 2001). It has been argued, however, that *Coccidioides* is an unlikely choice as a biological weapon because (a) *Coccidioides* is relatively difficult to isolate from the soil, (b) most primary infections are fairly innocuous, (c) there is no vaccine available to protect people who would be working to weaponize *Coccidioides*, and (d) *Coccidioides* is not contagious (Fierer and Kirkland, 2002). Nevertheless, the Centers for Disease Control now mandates that research
involving live cultures of the infectious phase of *Coccidioides* be conducted under the containment conditions of a biological safety level 3 (BSL3) laboratory. *Coccidioides* spp. is the only fungal pathogen on the “select agent” list, which includes viruses, bacteria and toxins considered to be potential biowarfare agents (www.nih.gov/od/ors/ds/pubs/appendxa.html), and research involving these agents is currently regulated under the Public Health Security and Bioterrorism Preparedness and Response Act of 2002. Though the use of *Coccidioides* as a bioweapon remains remote, it indeed underscores the need for a human vaccine against coccidioidomycosis.

**Epidemiology of Coccidioidomycosis**

The distribution and frequency of coccidioidal infections and the occurrence of the fungus in nature has been established on the basis of human skin testing, observation of clinical cases, and ecologic investigations during epidemics. These studies have concluded that coccidioidomycosis is endemic to the Western Hemisphere, with the areas of highest endemicity being in southwestern United States and northern Mexico and with regions of lower endemicity in Central and South America (Figure 2) (Pappagianis, 1988). Currently approximately 8-9 million people live in the most highly endemic region of the Southwestern United States, and millions more in the neighboring regions of coastal Southern California; and in 2000, nearly 28 million travelers visited Arizona (Cox and Magee, 2004). Even greater numbers of travelers visit California and other areas of endemicity. A dramatic increase in the number of reported cases of
coccidioidomycosis has occurred in Arizona over the past 20 yr (Ampel et al., 1998). During 1997, laboratory reporting of coccidioidal infections became mandatory in Arizona, after which a significant increase in the number of cases was noted. In 2001, a total of 2,203 cases were reported (rate of 43 per 100,000 population), compared to 1,551 cases in 1998 (rate of 33)(Cox and Magee, 2004). Persons aged $\geq 65$ yr and individuals with HIV infection had the highest incidence, although symptomatic infections in all age groups increased (Ampel et al., 1998).

The regions of highest endemicity for coccidioidomycosis correspond to the Lower Sonoran Life Zone, with the fungus growing in alkaline desert soil (Maddy, 1965).
Coccidioides grows in the soil as mycelia (saprobic phase) and with the onset of soil dryness the hyphae form arthroconidia. The arthroconidia become aerosolized as a result of the disturbance of the soil by natural or mechanical means, allowing human inhalation of the infectious propagules. The highest incidence of coccidioidomycosis occurs in late summer and early fall, when the soil is the driest (Polesky et al., 1999). It is not known how many of the infectious spores must be inhaled by humans to establish symptomatic disease (Nicas and Hubbard, 2002). However, in a murine model of infection, intranasal (i.n.) inoculation with approximately 10 arthroconidia is sufficient to kill BALB/c mice within 21 days post-challenge (Shubitz et al., 2002). Coccidioides is not considered to be contagious and person-to-person transmission is not known to occur except in rare cases of maternal-fetal transfer (Linsangan and Ross, 1999).

Clinical Manifestations of Coccidioidomycosis

The early literature estimates that 40% of persons infected with Coccidioides develop symptomatic disease. New estimates of the percentage of people in endemic regions who contract symptomatic coccidioidomycosis are based on data derived from studies of recent outbreaks of coccidioidal infection and have been shown to be greater than 50%. Most of these individuals (85%) present with a mild influenza-like illness, while 8% may develop severe pulmonary disease requiring hospitalization, and 7% develop the disseminated extrapulmonary form of the mycosis (Morbidity and Mortality Weekly, 2000). Cox classifies five categories of coccidioidomycosis: 1) primary
pulmonary infection, 2) cutaneous infection, 3) Valley fever complex, 4) pulmonary coccidioidomycosis, and 5) disseminated disease (Cox and Magee, 2004).

Primary pulmonary coccidioidal infection usually results in symptomatic disease in only 40-50% of primary infections, and the others are evident only by a positive delayed-type hypersensitivity reaction to *Coccidioides* antigens in a skin test (Smith et al., 1961). In the symptomatic cases, patients typically exhibit only mild flu-like symptoms whose onset may be anywhere from 7-21 d post exposure. The most common symptoms of primary pulmonary coccidioidal infection are cough, fever, chest pain, headache, fatigue, chills, malaise, diffuse erythematous rash and anorexia. Chest radiographs in these patients also may show pulmonary infiltrates hilar or basal in location.

Cutaneous infections with *Coccidioides* most often occur through percutaneous inoculation in laboratory accidents (Wilson et al., 1953). Primary cutaneous coccidioidomycosis is characterized by a painful suppurative lesion at the site of infection, often with regional lymphadenopathy, and most remain localized.

Valley fever or valley fever complex surfaces in approximately 5% of individuals with primary coccidioidal infections (Drutz and Catanzaro, 1978; Smith, 1940). This complex tends to develop coincidentally with the development of delayed-type hypersensitivity reactions to coccidioidal antigens. The principal manifestations of the valley fever complex are skin conditions characterized by small tender reddened nodules under the skin (as over the shin bones) often accompanied by fever and transitory arthritic pains or popular/vesicular lesions termed erythema nodosum and erythema multiforme.
These conditions are often accompanied by joint pain (desert rheumatism) and conjunctivitis. The clinical manifestations are thought to be attributable to a hypersensitivity to the fungus, a concept that is supported by the hyperreactivity of patient to skin testing with coccidioidin (a preparation of soluble *Coccidioides* antigens). *Coccidioides* cells have not been observed in the lesions of erythema nodosum or multiforme, and they have not been demonstrated in coccidioidal arthritis or conjunctivitis. The immunological basis of the valley fever complex is currently attributed to circulating immune complexes composed of anti-*Coccidioides* IgG and/or IgM and coccidioidal antigen(s), but this hypothesis has yet to be tested and the basis of the valley fever complex has not been fully determined (Yoshinoya et al., 1980).

Another manifestation of *Coccidioides* infection is persistent pulmonary coccidioidomycosis. This form of chronic infection occurs in 5% of individuals with primary coccidioidomycosis and is characterized by chronic progressive pneumonia, miliary lesions in the lungs, pulmonary nodules, or pulmonary cavitation (Winn, 1968). Although approximately half of these cases will resolve without surgery or therapy, many patients develop chronic progressive pulmonary involvement, with symptoms of cough, weight loss, fever, hemoptysis, dyspnea, and chest pain that may persist for years.

Epidemiological studies have established that 1-6% of patients with primary coccidioidomycosis develop disseminated disease (Johnson et al., 1996; Schneider et al., 1997; Smith et al., 1961). Dissemination of *Coccidioides* usually occurs early in the infectious process and may be acute, subacute, or chronic (Drutz and Catanzaro, 1978). Extrapulmonary disease spreads through the blood and lymphatics of the host, allowing the fungus to spread to virtually any part of the body, with the general exception of the
gastrointestinal tract (Forbus and Bestebreurtje, 1946). If dissemination is multifocal, the overall mortality rate is greater than 50%, while patients with single lesions have shown to have a better prognosis. Meningitis occurs in 30 to 50% of cases of disseminated disease, and in some patients this is the only site of extrapulmonary disease. Without treatment, most cases of disseminated coccidioidomycosis are eventually fatal.

Risk factors for severe pulmonary and disseminated disease include African-American, Filipino or Asian heritage, pregnancy, immunocompromising conditions (e.g., organ transplantation, AIDS), diabetes, smoking and older age (65 yr +) (Ampel, 1999; Blair et al., 2003; Jones et al., 1995; Rosenstein et al., 2001; Tripathy et al., 2002; Virgili et al., 2002). In a study observing *Coccidioides* specific antigens and skin testing reactivity in patients with active coccidioidomycosis, the authors noted that although African Americans were more likely to have disseminated disease than were Caucasians, their skin test reactivity was not significantly different than other ethnicities (Gifford and Catanzaro, 1981). In agreement with this study, Williams et al. (1984) reported that African Americans and Filipinos developed similar T cell reactivity as Caucasians after vaccination with a killed spherule vaccine. Therefore, though it is assumed that the increased susceptibility of African Americans, Asians, and Hispanics has an genetic or immunogenetic basis, there has not been any studies published that have been able to identify the underlying reasons, and it does not appear to reside in an inability of these persons to mount a cellular immune response to *Coccidioides*. 
**Immunity to Coccidioidal Infection**

Histological preparations of infected murine lung tissue reveal strikingly different host cell interactions with spherules of *Coccidioides* prior to and after endospore release. As the contents of the endosporulating spherule are released, the host mounts an intense suppurative response dominated by polymorphonuclear neutrophils (PMNs). The PMNs are the dominant innate cells found at sites of endospore release from ruptured spherules (Chandler et al., 1980) and are the first cell type to interact with arthroconidia (Savage and Madin, 1968). Ingestion of arthroconidia or endospores by neutrophils triggers an oxidative burst by the host cells, but less than 20% of the fungal cells are killed (Brummer, 1989; Frey and Drutz, 1986). The intermediate stages of *Coccidioides* spherules are impervious to phagocytosis and killing by PMNs due to their relative size (60-80 µM compared to 12-15 µM) and the neutrophil cell set may only play a role in activation of the adaptive immune response at these points of *Coccidioides* development.

Neutrophils have been reported to be a major source of interleukin (IL)-12 and IL-10, both of which play important roles in the development of adaptive immunity (Romani et al., 1997).

While neutrophils seem to play a limited role in coccidioidal killing, immune monocytes and macrophages have been shown to have increased killing capacity. Beaman et al. performed a number of studies evaluating the role of monocytes and macrophages in coccidioidal immunity (Beaman et al., 1979, 1981, 1983; Beaman and Holmberg, 1980a, b). Beaman observed that non-immune macrophages from mice and humans were extremely inefficient in killing arthroconidia and endospores in vitro (Beaman et al., 1979, 1981, 1983; Beaman and Holmberg, 1980a, b). In contrast,
activation of macrophages by coincubation with immune T lymphocytes or interferon gamma (IFN-γ) significantly enhanced the anti-coccidioidal killing, demonstrating the importance of activation of these effector cells in controlling Coccidioides infection (Beaman, 1987, 1991).

Natural killer (NK) cells are another major subset of immune cells important in innate immunity. Upon activation and translocation to sites of infection, NK cells secrete cytokines and chemokines that induce inflammatory responses and control the differentiation of monocytes and granulocytes such as PMNs (Moretta et al., 2002a, b). Before adaptive immunity has fully developed, NK cells are thought to contribute the main source of IFN-γ at the site of infection in response to macrophage-derived IL-12 and IL-18. An article demonstrating NK cell cytotoxic properties against Coccidioides cells has been published, but the nature of the antifungal activity needs to be confirmed (Petkus and Baum, 1987).

Another group of cells important in innate as well as adaptive immunity are dendritic cells (DCs). The DCs are a complex, heterogeneous group of multifunctional antigen-presenting cells (APCs) that are central to the induction of both primary innate and adaptive immune responses (Banchereau and Steinman, 1998). It has been shown that DCs become activated with different external stimuli and achieve maturation characterized by their expression of high levels of MHC class I and II, accessory molecules CD40, CD80, CD86 and early activation markers such as CD83 on their cell surface (Janeway, 2005). The strong capacity of DCs to initiate primary immune responses is due to their ability to deliver specific costimulatory signals that are essential for T cell activation from the resting or naive state into distinct classes of effector cells.
These immunogen-specific immune responses are critical for fighting microbial infections. In addition, DCs play an important role in adaptive immunity by secreting cytokines that polarize the T helper (Th) response during interactions with B and T cells in the secondary lymphoid organs, which in turn can direct the immune systems overall response to infection. DCs also can alter the function of regulatory T cells that control activated T cells through their suppressive signals.

Dendritic cells play an important role in the activation of T helper 1 (Th1) response during *Coccidioides* infection. A recent study investigated the efficacy of a DC-based vaccine against *C. posadasii* by immunizing C57BL6 mice with bone marrow-derived DCs transfected with a cDNA encoding the protective *Coccidioides*-Ag2/proline-rich Ag (Awasthi et al., 2005). The immunized mice were shown to have reduced fungal burden in the lungs and spleens compared to controls and the lung tissue homogenates of immunized animals showed higher levels of IFN-γ, a hallmark of dominant Th1 response. The biodistribution and trafficking of injected DCs was observed by nuclear imaging techniques. A significant amount of injected DCs were observed in the lungs suggesting that the antigen specific DCs migrated to the sites of infection. These data show the potential of DCs in anti-coccidioidal immunotherapy and their important role in directing the immune response during *Coccidioides* infection.

Both clinical and experimental evidence have demonstrated that T cell immunity is key for an effective immune response against coccidioidomycosis (Cox and Magee, 1998). Two of the functionally distinct subsets of CD4+ T cells, Th1 and Th2 T cells, are distinguished by different patterns of secreted cytokines after activation (Mosmann and Coffman, 1989). Th1 lymphocytes are characterized by production of IL-2, IL-12, tumor
necrosis factor (TNF-α), and gamma interferon (IFN-γ), while Th2 cells produce IL-4, IL-5, IL-6, and IL-10. The Th1/Th2 dichotomy of the adaptive cellular immune response plays a pivotal role in the immunopathogenesis of *Coccidioides*. These two cytokine/chemokine profiles correlate with resistance and susceptibility to *Coccidioides* infection, respectively (Cox and Magee, 1995, 1998; Magee and Cox, 1995, 1996). T helper 1 cells secrete cytokines that initiate and participate in cell-mediated immune responses, including the activation of macrophages. The Th2 subset of T lymphocytes secrete cytokines that stimulate B-cells to produce antibodies, activate mast cells and eosinophils, and may down-regulate cellular immune responses (Munder et al., 1999). Protective cell mediated immunity against coccidioidomycosis is thought to be derived from CD4+ T cell activation of macrophages through IFN-γ to enable these cells to efficiently engulf endospores and kill them through phagolysosomal fusion and oxidative burst. Arthrospores are phagocytized by macrophage, but in vitro data suggest they may be resistant to killing, and may not be cleared before they convert to small spherules (Beaman, 1981).

Most clinical data suggest that Th2 immunodominance compromises host protection against coccidioidal infection and exacerbates the course of disease (Kirkland and Cole, 2001). Patients with asymptomatic, or benign disease have been documented to possess strong skin test reactivity to *Coccidioides* antigens and low or nondemonstrable levels of anti-*Coccidioides* complement fixation (CF) antibody (Catanzaro et al., 1975; Cox et al., 1977). Contrarily, patients who have developed severe, chronic, or progressive pulmonary or disseminated disease tend to be hyporesponsive or anergic to skin testing antigens but have high levels of anti-
*Coccidioides* IgG antibody to the CF antigen (Catanzaro et al., 1975; Cox et al., 1977). Recovery from active disease, either spontaneous or in response to antifungal therapy, is in many patients associated with a reacquisition of T-cell reactivity to *Coccidioides* antigens and decreased CF antibody titers, supporting the current dogma.

Cytokine data from clinical samples also have pointed to a correlation between the immunodominant T cell response and disease. A study of 20 healthy subjects who were skin test positive to *Coccidioides* antigens and 15 healthy, skin-test negative persons demonstrated that peripheral blood mononuclear cells (PBMCs) from skin test-positive but not skin test-negative donors secreted both IL-2 and IFN-γ in response to in vitro stimulation with *Coccidioides* spherule antigens (Ampel and Christian, 2000). In another study, the production of the Th1 cytokines IFN-γ and IL-12 and the Th2 cytokines IL-4 and IL-10 by PBMCs from healthy, skin test-positive and skin test-negative subjects and patients with active pulmonary or disseminated coccidioidomycosis were compared (Corry et al., 1996). The data showed that IFN-γ production was significantly lower in cells from the patients with disseminated disease than in those from healthy, skin test-positive persons. In contrast, cells from patients with primary pulmonary disease secreted IFN-γ levels that were similar to those of healthy, skin test-positive donors. None of the groups differed in their production of IL-12, IL-4 or IL-10. While not complete, clinical data suggest a strong relationship between Th1/Th2 response of host T cells and the type of clinical manifestations after infection with *Coccidioides*.

Recently, the idea of host-induced damage during infection due to inflammation has come to light in regards to *Coccidioides*. Intense inflammation at sites of infection resulting in host tissue damage has been hypothesized to further exacerbate the disease
process. This has led to interest in the function of regulatory T cells (T\(_R\)) in coccidioidal disease (Powrie and Maloy, 2003). This subset of CD4\(^+\) T lymphocytes is present in healthy individuals (approximately 5 to 10%) and is distinguished by expression of the activation marker CD25. These naturally occurring thymus-derived T\(_R\) cells (CD4\(^-\)CD25\(^+\)), have the ability to inhibit the proliferation of T effector lymphocytes (CD4\(^+\)CD25\(^-\)) by their secretion of cytokines (e.g., IL-10), and remain functionally stable for long periods in the absence of antigen (Nagler-Anderson et al., 2004). Typically, the roles of CD4\(^+\)CD25\(^+\) T cells are to support the mobilization of T cell-mediated immune response against an invasive microbe, while simultaneously minimizing host tissue damage due to immune pathology. Recent evidence has suggested that persistently elevated production of IL-6 observed during coccidioidomycosis may contribute to the inability of regulatory T cells to control the activation of T effector cells in response to the presence of the pathogen leading to out of control inflammation and exacerbation of disease (Pasare and Medzhitov, 2003).

Human clinical data have been corroborated using the mouse model of coccidioidomycosis in the research laboratory. This model also has been the workhorse for scientists performing *Coccidioides* vaccine studies. Inbred mouse strains have shown variable susceptibility to infection. BALB/c mice have been shown to have the highest susceptibility to *Coccidioides* infection, requiring only 10 arthroconidia to establish disseminated disease (Cox et al., 1988; Kirkland and Fierer, 1983). Conversely, DBA/2 mice have the highest documented resistance to infection, while C57BL strains fall in between the two (Kirkland and Fierer, 1983). Table I shows the lethality of *Coccidioides* in tested inbred mouse strains.
**TABLE I. Lethality of *Coccidioides* for Inbred Mouse Strains Challenged by an i.p. Route**

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Log$<em>{10}$ LD$</em>{50}$ (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/cAnN</td>
<td>1.67 (0.60)</td>
</tr>
<tr>
<td>C57BL/10N</td>
<td>2.77 (0.33)</td>
</tr>
<tr>
<td>C57BL/6N</td>
<td>2.83 (0.23)</td>
</tr>
<tr>
<td>C57L/J</td>
<td>1.65 (0.56)</td>
</tr>
<tr>
<td>DBA/2N</td>
<td>5.25 (0.36)</td>
</tr>
</tbody>
</table>

Adapted from Kirkland and Fierer, 1983

Genetic mapping of susceptibility has yielded conflicting results, with one study suggesting that a single gene was responsible for resistance, while other studies suggested polygenic associations (Fierer et al., 1999; Kirkland and Fierer, 1985). Both susceptible BALB/c and resistant DBA/2 mouse strains have the $H-2^d$ haplotype, indicating susceptibility is not controlled primarily by the $H-2$ locus. There is currently no convincing data showing which distinct genetic loci are responsible for the susceptibility or resistance to *Coccidioides* infection.

Studies using murine models of coccidioidomycosis have provided undeniable evidence that cellular immunity is crucial to the infected host’s defense against *Coccidioides*. The first unambiguous study demonstrated that adoptively transferred immune splenic T cells but not B cells or serum from mice vaccinated with a protective formalin killed spherule (FKS) vaccine provided the recipient mice substantial protection against subsequent infection (Beaman et al., 1977). Depletion of T cells from the immune spleen cells ablated the protective transfer. A more recent report showed that...
adoptive transfer of protective immunity was dependent primarily on CD4^+ T cells, but optimal transfer was achieved with the transfer of both CD4^+ and CD8^+ T cells (Cox and Magee, 1998).

Similarities between human coccidioidomycosis and the murine model of disease have been demonstrated in the correlation between resistance and the production of Th1 cytokines and, between susceptibility and the production of Th2 cytokines. It has been observed that mice which are highly susceptible to *Coccidioides* infection (e.g. BALB/c) produce more IL-10 and IL-4 during early stages of disease than more resistant strains (e.g., DBA/2) (Fierer et al., 1998). *Coccidioides*-infected, IL-10 deficient mice are as resistant to coccidioidomycosis as DBA/2 mice, suggesting that up-regulation and persistence of elevated levels of IL-10 expression may contribute to increased susceptibility of mice to disseminated coccidioidomycosis. The ability of IL-10 to suppress accessory cell function required for optimal T cell activation makes it an important inhibitor of cell-mediated immunity (Wille et al., 2002).

An early Th1-dominated immune response has been suggested to be important in controlling a *Coccidioides* infection. Interluekin-12, a Th1-promoting cytokine produced by DBA/2 mice, has been shown to influence early host defenses against coccidioidal infection by promoting the production of IFN-γ and a protective cellular immune response (Magee and Cox, 1996). Both IFN-γ and TNF-α have been shown to contribute to the ability of host phagocytes to kill arthroconidia and endospores in vitro (Beaman, 1987). Magee and Cox have shown that administration of recombinant IL-12 to BALB/c mice before and after intraperitoneal (i.p.) challenge with *Coccidioides* significantly improved the host response to infection, as measured by reduction of the fungal burden in
lungs, liver and spleen (Magee and Cox, 1996). The protective effect of IL-12 in these infected animals was accompanied by a shift from a Th2 to a Th1 response. Common to most infections is that IL-12 regulates the magnitude of the IFN-γ response at the initiation of infection, thus potentiating natural resistance, favoring Th1-cell development, and inhibiting Th2 responses (Romani et al., 1997).

Persistently high levels of IL-4, IL-5 and IL-6 during later stages of *Coccidioides* infection in mice appear to depress cell mediated immunity. Magee and Cox have shown that neutralization of endogenous IL-4 in *Coccidioides*-infected BALB/c mice, by administration of neutralizing anti-IL-4 antibody, led to a significant reduction of fungal burden in their tissues and further demonstrated the role of a Th2 dominant immune response to coccidioidal immunopathogenesis (Magee and Cox, 1995).

The function of cytotoxic T cells in anti-*Coccidioides* immunity is essentially unknown. Adoptive transfer of protective immunity has been determined to be dependent on CD4+ T cells, but as stated above, protection was shown to be most complete with the transfer of both CD4+ and CD8+ immune T cells (Cox and Magee, 1998). Recent work by Kirkland et al. suggested that protection mediated by a vaccine composed of rAg2/Pra and CpG-ODN required IL-12, IFN-γ and MHC II-restricted T-cells, while cytotoxic CD8 T-cells were not needed (Kirkland et al., 2006). Contrastingly, a new report by Fierer et al. supports the findings of Cox and Magee, in that immune CD8+ T cells can provide protection (Fierer et al., 2006). They found that adoptively transferred CD4+ and CD8+ T cells from mice vaccinated with a temperature sensitive mutant of *C. immitis* can protect mice lacking cellular immunity against coccidioidomycosis and that TNF-α is a necessary component of the acquired immune response (Fierer et al., 2006). These data
suggest that cytotoxic T cells may play an important, yet undefined role in protection against *Coccidioides* infection, which needs to be explored further.

The role of antibody in the host defense against *Coccidioides* has been disputed. Studies dismissing the effect of antibody in protective immunity have shown that adoptive transfer of sera and B cells from mice vaccinated with FKS did not confer protective immunity to the recipient mice (Beaman et al., 1977), and that protective immunity provided by a single protective recombinant antigen (Ag2/PRA) required IL-12, IFN-γ and MHC II-restricted T cells, while CD8+ T cells and B cells were not required (Kirkland et al., 2006). In contrast, a recent study by Magee et al (2005) investigated immunity against coccidioidomycosis using gene microarrays. They found that a high percentage of B-cell-related genes were associated with protective immunity, and confirmed the importance of B cells against coccidioidomycosis by showing that vaccination was not fully protective in B-cell-deficient MuMT mice when compared to controls. Therefore, while there is conflicting evidence for antibody-mediated protection in coccidioidomycosis, the possibility of the existence of protective antibodies is intriguing. The fact that such antibodies could be identified by using monoclonal antibody technology is exciting because of the implications of these protective antibodies in therapeutic intervention or even vaccine development.

*Coccidioides Vaccinology*

*Coccidioides* vaccine research (vaccinology) has been a subject of much interest to members of the *Coccidioides* research community since the early 1900s. As previously stated, the arguments for development of a vaccine against
coccidioidomycosis include the increasing incidence of this disease, a fast growing population in the areas of endemicity, the lack of a highly effective drug treatment and the exceedingly high expenses incurred by the medical system in treatment of this disease. The feasibility of developing an effective vaccine is spurred by the ideas that first, the fungus is geographically restricted thereby limiting the areas where potential infections can be obtained, and second, the target population is well defined including individuals who are genetically predisposed to the disease or have a high probability of exposure to *Coccidioides* (Cox and Magee, 2004). The most compelling argument for the feasibility of a vaccine against coccidioidomycosis is that natural human infection with *Coccidioides* results in immunoresistance to exogenous reinfection indicating the fungus itself has immunizing capacity (Fiese, 1957; Smith, 1940; Smith et al., 1961; Stevens, 1980).

1. **Whole Cell and Subcellular Fraction Vaccines**

   The earliest studies of experimental coccidioidomycosis in animal models were centered on immunization of the hosts with either live cells or attenuated strains of the pathogen. These experiments showed that these live cell vaccines protected against a lethal, intranasal infection of the host (Converse, 1965; Converse and Besemer, 1959; Pappagianis et al., 1960, 1961). Unfortunately there were many adverse side effects observed at the sites of vaccination, such as extensive inflammation and percutaneous persistence of the fungus (Converse, 1965; Converse and Besemer, 1959; Pappagianis et
These unfavorable consequences led researchers to explore the possibility of non-viable cellular vaccines.

Levine and coworkers compared the protective efficacy of formaldehyde killed arthroconidia, mycelia, and parasitic stage spherules (FKS) in mice (Levine et al., 1960). They found that mice vaccinated with FKS survived a challenge with a high dose of arthroconidia (1000 spores). Mice vaccinated with killed mycelia or arthrospores had a mortality rate of over 50% with same challenge dosage. Interestingly, Levine et al. also discovered that the immunogenicity of mature parasite-phase cells was far greater than that of endospores and were resultantly more protective (Levine et al., 1960). The possibility of quantitative and qualitative differences in the protective components in the two preparations needs to be further explored.

The efficacy of the FKS vaccine was further studied, with investigators examining the impact of vaccine dose, schedule and route of immunization in the effectiveness of the vaccine, as well as its use in different animal models of disease (Levine et al., 1962, 1965). The vaccine was found to be most effective in mice given as an intramuscular or subcutaneous vaccine at three separate dosages of 0.7 mg spaced out over 3 wk. The FKS preparation also was investigated as a vaccine in cynomolgous monkeys, and was found to provide a measure of protection to these non-human primates after lethal coccidioidal challenged (Levine et al., 1962). Although the vaccinated monkeys showed significantly improved survival and pathology compared to the mock-vaccinated monkeys, the post-mortem investigations revealed that most of the vaccinated group still had detectable amounts of residual fungus in their organs, suggesting that FKS was still
not providing complete protection at the infective dosage of *Coccidioides* used in this study.

The demonstrated protective efficacy of FKS in both the murine and primate model of infection prompted a clinical trial to assess the toxicity and effectiveness in humans. Toxicity trials showed that persons receiving a total of 2.7 mg of FKS intramuscularly in one to three doses presented with localized tenderness or induration at the site(s) of injection but otherwise tolerated the vaccine well. When vaccine doses of 10 mg or greater were provided, the recipients had unacceptable levels of toxicity as observed by the severe localized or systemic inflammation (Williams et al., 1984). The results of these trials showed that approximately 7% of recipients had some sort of adverse reaction to FKS when volunteers were vaccinated with at a 1.75 mg dosage. In addition, only 50% of the FKS recipients converted to skin test positive, and there was no bias observed in the immunologic responses of Caucasians or the more susceptible African Americans and Filipinos.

Between 1980 and 1985, a double-blinded human trial was conducted using the FKS vaccine versus a placebo (Pappagianis, 1993). The study involved almost 3000 volunteers who were skin test negative to both coccidioidin and spherulin, but only a minority of the vaccinated individuals became skin test-positive to *Coccidioides* after vaccination. There was no difference in the number of cases of coccidioidomycosis or the severity of the disease in the FKS-vaccinated group compared to the placebo group after a 5-yr follow-up period. The incidence of *Coccidioides* infections was relatively low during the period of the trial and given the marked increase in infections that occurred during the early 1990s, it is of interest to clinicians to determine if subjects
enrolled in the FKS vaccine study acquired coccidioidomycosis during the epidemic compared to those who received the placebo vaccine.

One possible explanation for the ineffectiveness of this vaccine is that relatively small numbers of killed, parasitic cells could be injected into humans without unacceptable local side effects of pain and swelling. Based on comparative body weight, the amount of FKS vaccine used for the human trials was less than 0.1% of the dose needed to immunize mice. Human peripheral blood mononuclear cells (PBMCs) obtained from skin test-positive and -negative volunteers produced elevated levels of inflammatory cytokines upon exposure to FKS, which may account for the toxicity of the vaccine in humans (Dooley et al., 1994).

The sub-optimal outcome of the FKS clinical trials, and the resulting toxicity observed in humans after administration of higher doses of the killed spherules prompted scientists to explore the possibility of protective subcellular vaccines. A study examining the protective fractions of the killed spherules vaccine reported that the protective components of the cells were found mainly in their cell walls (Kong et al., 1963). As a result most investigators focused on the subfractionation and identification of cell wall associated immunoprotective proteins. Multicomponent subcellular fractions such as a PBS extract of spherule cell walls and a 27K fraction derived from disrupted FKS showed significant protection in vaccinated mice (Pappagianis et al., 1979; Zimmermann et al., 1998). The 27K vaccine was derived from the soluble proteins of mechanically disrupted FKS after a 27,000 x g centrifugation. Interestingly, a major protein component of the 27K vaccine fraction was found to be a cell wall associated aspartyl
protease protein (Pep1) that was never analyzed further as a vaccine candidate (Johnson et al. 2001).

In 1983, Lecara et al. demonstrated that an alkaline soluble, water-soluble (C-ASWS) extract from mycelia cell walls induced protective immunity in DBA/2 mice (Lecara et al., 1983). Subsequent analyses showed that the spherule-derived C-ASWS was more protective than the mycelium-derived extract, suggesting that there was a protective component(s) unique to the parasitic phase of the fungus. Antigenic characterization of the proteins in the C-ASWS by two-dimensional immunoelectrophoresis against goat antisera to C-ASWS and coccidioidin established that C-ASWS from spherule cell walls contained distinct antigenic components. One was shown to be antigenically identical to a polymeric antigen in coccidioidin, designated antigen 2 (Cox and Britt, 1985). This research led to the subsequent identification and cloning of the first singly protective vaccine candidate against coccidioidomycosis, Ag2/Pra, discussed below.

Recombinant Protein and DNA Vaccines

Complex subcellular vaccines have not been considered viable options for a human coccidioidal vaccine due to their heterogeneous antigenic nature as well as the difficulty in reproducing the exact antigenic components in batch-to-batch preparations of the protective extracts. These issues have prompted investigators to explore the identity of component antigens in these vaccines that can confer immunoprotection to the host. Currently, the focus in coccidioidomycosis vaccine development has been the identification of singly protective antigens that can be recombinantly expressed, purified
and administered as single or defined multi-component subunit vaccines. The majority of protective antigens that have been characterized to date are products of the parasitic phase of the fungus, and are cell wall-associated (Kirkland and Cole, 2001). To date, the most promising of these are cell wall-associated antigens (Delgado et al., 2003; Shubitz et al., 2002).

The Cox laboratory cloned a protein that was present in the protective C-ASWS preparation of the spherule wall, and referred to it as antigen 2 (Ag2) (Zhu et al., 1996a, b). Simultaneous investigations of vaccine candidates in the Galgiani laboratory identified a protective 33-kDa proline-rich antigen (Pra) in SDS-PAGE gel separations of a hydrogen fluoride-deglycosylated lysate of *Coccidioides* (Dugger et al., 1996). The *PRA* gene was cloned and shown to be identical to the *AG2* gene sequence. The recombinant antigen (rAg2/Pra) was expressed by *Escherichia coli* and shown to elicit both patient T cell response and antibody reactivity (Galgiani et al., 1996; Wieden et al., 1996; Zhu et al., 1997), and immunolocalization experiments found that the antigen was deposited in the parasitic cell wall (Galgiani et al., 1992). Immunization of BALB/c mice with rAg2/Pra plus complete Freund’s adjuvant protected the mice against i.p. challenge of *C. posadasii*, based on significant reduction of the fungal burden in the lungs and spleen at 14 d post-infection (Kirkland et al., 1998). This same antigen also was expressed in bacteria as a glutathione-S-transferase (GST) fusion protein, which demonstrated protection against i.p. challenge in BALB/c mice as assessed by decreased fungal burden (Jiang et al., 1999b). However, in a survival experiment, BALB/c mice immunized with this recombinant antigen failed to show protection compared to control mice during a 30-d period post-challenge (Jiang et al., 1999b). Recent survival
experiments with this antigen have shown to that immunization with rAg2/Pra results in significant protection in C57BL/6 mice based on survival 30 - 60 d post-challenge compared to survival of control animals, however persistent CFUs were found in the lungs of mice surviving to 90 d post-challenge (R. Herr, Personal Communication).

A second promising protective recombinant protein is a cell wall-associated, GPI-anchored β-1, 3-glucanosyltransferase (rGel1) (Delgado et al., 2003). This vaccine candidate was discovered by utilizing bioinformatic searches of the newly developed C. posadasii genome database for genes that encode GPI-anchored cell wall-associated proteins. The predicted structure of this protein contained a 447 amino acids, with an 18 amino acid signal peptide and a 34 amino acid GPI anchor with two N-glycosylation sites. The protein was cloned, expressed in E. coli and purified. Antiserum raised against the purified protein revealed that the antigen is expressed on the surface of endospores, and the recombinant protein was recognized in immunoblots by sera from patients with confirmed Coccidioides infection, but not with human control sera. Immunization of BALB/c or C57BL/6 mice with rGel1 plus bacterial derived synthetic CpG-oligodeoxynucleotides (ODN) (discussed in the next section) and incomplete Freund’s adjuvant (IFA) elicited a protective response against coccidioidal infection via the i.p. or i.n. routes in both strains. Immunized mice challenged intraperitoneally with the pathogen showed approximately 3-4 log_{10} units reduction in fungal burden in their lungs and spleen, and when challenged by the i.n. route, 70% of the immune mice survived compared to none of the non-immunized animals.

Another recombinant protein tested as a vaccine candidate is the Coccidioides-specific antigen (Csa). The Csa protein was originally observed as a secreted antigen in
the media of saprobic and parasitic phase cultures, and the gene was cloned several years ago (Pan et al., 1995). Only recently has the Csa protein been tested for its ability to protect mice against coccidioidal infection. The recombinant Csa protein was expressed by *E. coli* and used as a vaccine in combination with rAg2/Pra expressed by *Saccharomyces cerevisiae*. C57BL/6 mice were immunized intradermally with the combined vaccine plus CpG/MPL adjuvant. Survival experiments showed that rCsa + rAg2/Pra-immunized mice challenged intranasally with a lethal inoculum of *Coccidioides* showed 90% survival at 60 d post-infection compared to none of the infected animals immunized with adjuvant alone. Mice immunized with rCsa or rAg2/Pra alone plus adjuvant showed 30% and 60% survival, respectively (Yu et al., 2003). This is the first evidence that a multivalent recombinant vaccine may enhance protective immunity against coccidioidomycosis.

Most of the other recombinant antigens of *Coccidioides* which have been tested to date have failed to meet the benchmarks of protection established in murine vaccine trials using rAg2/Pra and rGel1, and are no longer considered candidates for a human vaccine. These include the first purified recombinant T-cell-reactive protein of *Coccidioides* tested in a murine model (Wyckoff et al., 1995), a heat shock protein (rHsp60) (Thomas et al., 1997), and a spherule outer wall glycoprotein (rSOWgp) (Hung et al., 2002).

The use of DNA vaccines against coccidioidomycosis has been explored providing variable results (Abuodeh et al., 1999; Jiang et al., 1999a, b; Jiang et al., 2002). One example of genetic vaccination against coccidioidomycosis was demonstrated by Li et al. (2001) in which they examined the protective efficacy of a DNA vaccine construct of the *C. posadasii* urease gene (*URE* DNA vaccine). In this study the protective efficacy
of the *URE* DNA vaccine was compared with that of the recombinant urease (rUre) protein. The maximum survival observed in BALB/c mice immunized with the recombinant protein + CpG/IFA was 40%, while 80% of the animals survived after immunization with the *URE* plasmid construct, indicating that the DNA vaccine provided better protection. DNA vaccination with the *PRA* gene revealed slightly less promising results. There was not a significant difference in the fungal load in the lungs of BALB/c mice vaccinated with *PRA* DNA vaccine compared to mice vaccinated with the vector alone. In contrast, immunization of C57BL/6 mice with the *PRA* DNA vaccine construct significantly reduced the fungal load in the lungs compared to that in mice given the vector alone (Abuodeh et al., 1999). The Cox laboratory employed DNA vaccination in efforts to discover new coccidioidomycosis vaccine candidates. Ivey et al. (2003) used a recently developed technology called Expression Library Immunization (ELI) in efforts to explore the *Coccidioides* parasitic phase expressed genes for candidate vaccines. A cDNA library was cloned into mammalian expression vectors and pools were used to vaccinate mice. The pools that showed significant protection were subdivided into smaller gene pools of until they were able to identify a single protective clone. Their efforts were rewarded with the discovery of ELI antigen 1 (ELI-Ag1). This gene encoded a putative GPI anchored cell wall-associated protein with predicted chitin binding capabilities. Vaccination with the ELI-Ag1 cDNA vaccine construct conferred significant protection to i.p. challenged BALB/c mice compared to mice receiving the vector alone. Although this work represents the first report of ELI leading to the identification of a single protective gene for vaccination against a fungal disease, DNA vaccines are not considered a viable option for humans for multiple reasons. While DNA
vaccines are relatively easy to produce and less expensive than recombinant protein vaccines, evidence from clinical trials suggest DNA vaccines will not induce the same level of robust immune responses in primates, including humans, that have been reported in mice (Huygen, 2003; Seder and Mascola, 2003). Another drawback of DNA vaccines is that there has been no such vaccine approved for general human application in the U.S. to date, thus eliminating DNA vaccines as current option for coccidioidomycosis.

Consideration of Adjuvant

Since the focus of Coccidioides vaccine research has shifted to the use of recombinant protein subunits, a necessity for the use of highly immunogenic adjuvants with these vaccines has developed. Whole killed spherules or subcellular extract vaccines did not require potent adjuvants due to the reactogenicity of not only the proteins, but also the other cellular components in the vaccines such as cell wall carbohydrates. These “other” molecules could provide the secondary signals through the host pattern recognition receptors, such as Toll like receptors (TLRs), required to activate and direct the host immune response. Soluble proteins will not, in general, induce cellular immune responses by themselves (Freund, 1956). Therefore, with the requirement of cell-mediated immunity for protection against Coccidioides and the recent use of soluble recombinant protein subunit vaccines in Coccidioides vaccine research, it is imperative that an adjuvant that can stimulate the cellular compartment of the vaccinated host’s immune system is developed and tested along with the protein vaccine candidates. Unfortunately, the only adjuvants licensed for human use by the U.S. Food and Drug Administration are aluminum-salts such as aluminum hydroxide and aluminum
phosphate (alum) which skew the immune response towards a Th2 bias as characterized by the secretion of IL-4 and IL-5 and the generation of IgG1 and IgE (Yip et al., 1999). A protective immune response against *Coccidioides* requires a predominant Th1 response and active cell-mediated immunity, which would seem to eliminate the use of alum with a vaccine against coccidioidomycosis. Recently developed Th1 biased adjuvants, such as CpG-ODN, have not been licensed for use in humans but have been evaluated in preclinical trials with humans and in experimental-animal models (Carpentier et al., 2003; Weeratna et al., 2000, 2005). Synthetic oligodeoxynucleotides expressing CpG motifs mimic the immunostimulatory activity of bacterial DNA and are detected by Toll-like receptor 9 (TLR9)-bearing B cells and dendritic cells triggering an immune cascade characterized by polyclonal-B-cell activation, improved antigen uptake/presentation by antigen-presenting cells, and the secretion of chemokines and proinflammatory cytokines that foster a strong Th1 response. The CpG ODN has been shown to be superior to alum and similar to complete Freund’s adjuvant, the traditionally used Th1-type adjuvant in the induction of cell-mediated immune responses including the production of reactive oxygen intermediates and secretion of inflammatory cytokines, including IL-1, TNF-α, IL-12, and IFN-γ (Brazolot Millan et al., 1998; Sun et al., 1998; Weeratna et al., 2000). The fact that CpG-ODN has potential as a possible FDA- approved Th-1 type adjuvant, and provides a robust reactogenic Th1 dominated immune response with whatever antigens it is administered, provided an adequate argument for it to be tested with *Coccidioides* vaccine candidates. The rUre and the URE gene vaccines, as well as with rGel1, were tested with CpG-ODN as adjuvant. These vaccines induced protective immunity in the
vaccinated mice, affording further evidence of CpG-ODN’s prospective use with candidate vaccine antigens (Delgado et al., 2003; Li et al., 2001).

**Future of Coccidioidomycosis Vaccinology**

The *C. posadasii* C735 genome sequencing project was initiated approximately 5 yr ago at The Institute for Genomic Research (TIGR) in Rockville, MD. The genome was sequenced by a whole genome shotgun strategy, supported by the generation of expressed sequence tags from the saprobic and mycelial phases of the life cycle ([www.tigr.org](http://www.tigr.org)). The preliminary annotation of the genome was released March 3, 2006, but genomic and EST sequences have been available to collaborating groups during the past 2-3 yr of the project. The *C. immitis* strain RS genome is being sequenced by the Broad Institute of Harvard and MIT and is also near completion ([www.broad.mit.edu/annotation/fungi/coccidioides_immitis](http://www.broad.mit.edu/annotation/fungi/coccidioides_immitis)). With the availability of *C. immitis* and *C. posadasii* genome data, new technologies employing bioinformatic, genomic and proteomic screenings can be used for the identification of *Coccidioides* vaccine candidates (Doytchinova et al., 2003, BenMohamed et al., 2003, De Groot et al., 2005a). These approaches should by design, be more robust and streamlined compared to previous strategies used by researches for identification of vaccine antigens.

Proteomic and bioinformatic strategies are the logical principal technologies that will be applied to the vast amount of data provided by the sequencing of the *C. immitis* and *C. posadasii* genomes for future vaccine development. The ability to identify the components of a protective subcellular vaccine using protein separation techniques such as 2-dimensional polyacrylamide gel electrophoresis or 2-dimensional liquid
chromatography, followed by mass-spectrometry will provide researchers with a large number of putative vaccine candidates to explore (Johnson et al., 2005; Pavkova et al., 2005; Pitarch et al., 2006). Bioinformatic approaches can be employed to analyze genomic or proteomic data in order to streamline the antigen discovery process. One such approach is dubbed “epitope-driven vaccine design” in which epitope prediction algorithms are used to screen putative protein sequences for T-cell-reactive epitopes (BenMohamed et al., 2003; De Groot et al., 2005a, b). By utilizing bioinformatics to find epitopes that can be screened directly for reactivity with immune T cells and T-cell subsets, or to identify proteins that can be further evaluated in animal models of protection, researchers will be able to quickly and efficiently find new vaccine candidates.
A Recombinant Aspartyl Protease of *Coccidioides posadasii* Induces Protection Against Pulmonary Coccidioidomycosis in Mice

Eric J. Tarcha¹, Venkatesha Basrur¹, Chiung-Yu Hung¹, Malcolm J. Gardner², and Garry T. Cole¹*

Department of Medical Microbiology and Immunology
Medical University of Ohio, Toledo, OH 43614 ¹

and The Institute for Genomic Research, Rockville, MD 20850²

INFECTION AND IMMUNITY
Jan. 2006, p. 516–527 Vol. 74, No. 1
ABSTRACT

Coccidioidomycosis is a respiratory disease of humans caused by the desert soil-borne fungal pathogen, *Coccidioides* spp. Recurrent epidemics of this mycosis in Southwestern United States have contributed significantly to escalated health care costs. Clinical and experimental studies indicate that prior symptomatic coccidioidomycosis induces immunity against subsequent infection, and activation of T cells is essential for containment of the pathogen and its clearance from host tissue. Development of a human vaccine against coccidioidomycosis has focused on recombinant T-cell-reactive antigens which elicit a durable protective immune response against pulmonary infection in mice. In this study we fractionated a protective multicomponent parasitic cell wall extract in an attempt to identify T-cell antigens. Immunoblots of electrophoretic separations of this extract identified patient seroreactive proteins which were subsequently excised from 2D-PAGE gels, trypsin-digested and sequenced by tandem mass spectrometry. The full-length gene which encodes a dominant protein in the immunoblot was identified using established methods of bioinformatics. The gene was cloned and expressed, and the recombinant protein was shown to stimulate immune T cells in vitro. The deduced protein was predicted to contain epitopes that bind to human major histocompatibility class II molecules using a TEPITOPE-based algorithm. Synthetic peptides corresponding to the predicted T-cell epitopes induced interferon-gamma production by immune T lymphocytes. The T cell-reactive antigen, which is homologous to secreted fungal aspartyl proteases, protected mice against pulmonary infection with *C. posadasii*. We argue that this immunoproteomic/bioinformatic approach to the identification of candidate vaccines against coccidioidomycosis is both efficient and productive.
INTRODUCTION

_Coccidioides_ is an environmental pathogen and causative agent of a respiratory infection of mammals. The filamentous, saprobic form of the fungus occurs in desert soils of Southwestern United States and parts of Mexico, Central and South American (8). Inhalation of the airborne fungal spores (arthroconidia) can lead to onset of a disease (coccidioidomycosis, San Joaquin Valley fever) which is rarely life-threatening, but causes significant morbidity in more than 40% of infected individuals. The disseminated form of this mycosis is difficult to treat; it usually necessitates long courses of antifungal drug administration, and relapse frequently occurs following the therapeutic regimen (2). The incidence of coccidioidomycosis in California and Arizona has been correlated with seasonal climatic changes, and recurrent outbreaks of the respiratory disease within populations which reside in the endemic regions have been reported (33). The increased numbers of visitors, retirees, military recruits, etc. from non-endemic regions who have moved to the Southwest over the past 10 years has resulted in a large immunonäive population at risk of infection (8). Coccidioidomycosis in Arizona is currently the fourth most commonly reported infectious disease; only gonorrhea, chlamydial and hepatitis C infections are reported more frequently (33). Examinations of the incidence of coccidioidal infections in Arizona between 1998 and 2001 have indicated that persons 65 or older showed the highest rate of disease and hospitalization. However, it was also determined that the incidence of coccidioidomycosis in younger populations had increased, particularly those 0-18 years old (33).
Recovery from infection with either of the two recognized species of *Coccidioides, C. immitis* or *C. posadasii* (15), usually confers lifelong immunity to reinfection (41). Immunization of mice with an attenuated strain of the pathogen has been shown to protect the animals against infection following a lethal, intranasal challenge with *Coccidioides* (32). On the basis of these observations, it has been argued that generation of a vaccine against coccidioidomycosis is feasible and would be cost effective (4, 8). Both clinical and experimental evidence have demonstrated that T-cell immunity is pivotal for defense against this respiratory disease (9). The ability of the host to elicit a strong delayed-type hypersensitivity response to the pathogen is essential. On the other hand, rising titers of antibody to *Coccidioides* antigen typically signals a poor prognosis. Our search for candidate vaccines against coccidioidomycosis using a murine model of the pulmonary disease has focused on T-cell-reactive proteins. Criteria used in this study for evaluation of protection include evidence that the vaccine candidate stimulates a T helper 1 (Th1) pathway of immune response as measured by T lymphocyte secretion of appropriate cytokines (28, 48), significant increase in survival of vaccinated mice compared to non-vaccinated controls after intranasal infection with a potentially lethal inoculum of *Coccidioides*, and demonstration that the majority of vaccinated survivors have cleared the organism from their lungs. In this paper we report a new bacterial-expressed recombinant vaccine candidate derived from a cell wall extract of *C. posadasii* and identified as an aspartyl protease homolog.
Fungal growth conditions. The saprobic and parasitic phases of *C. posadasii* (strain C735) were grown in vitro as previously described (17). Arthroconidia were isolated from saprobic phase cultures after 4 wks of incubation as reported (28), and used to inoculate flasks of defined glucose/salts medium for growth of the parasitic (spherule-endospore) phase (19).

Isolation and protein extraction of the parasitic cell wall fraction. Spherules were isolated from pooled parasitic phase cultures after incubation for 96 h (pre-endosporulation) or 132 h (post-endosporulation) and mixed (1:1; v/v). The same number of arthroconidia (1 x 10^7) were used to inoculate each culture, which resulted in production of approximately equal numbers of first generation, segmented and endosporulating spherules (19). The cell pellet collected by centrifugation (3,000 xg) was washed 4 times with ice-cold disruption buffer (20 mM Tris-HCL, pH 7.4) which contained 2X Protease Inhibitor Cocktail, Set IV (Calbiochem, San Diego, Calif.). The parasitic cells suspended in buffer were disrupted in a BeadBeater™ (BioSpec Products, Inc., Bartlesville, Okla.) using pre-cooled 0.5 mm diam. Zirconia/Silica beads (Biospec). The homogenate was centrifuged (5,000 xg), the pellet washed five times with cold disruption buffer as above, and then frozen and stored at -80º C until ready for use. The cell wall isolate was incubated with 2% Triton X-114 (TX114; Sigma Chemical Co., St. Louis, Mo.) in extraction buffer (50 mM Tris-HCL [pH 6.8], 100 mM NaCl, and protease inhibitor cocktail) for 1 h at 4ºC with vigorous shaking as previously described (17). The supernatant obtained after centrifugation (27,000 xg) was allowed to separate
into the aqueous and detergent phases upon incubation at 30º C for 30 min without agitation. The detergent phase fraction (TX114-DF) was collected, and the protein components precipitated with ice-cold, absolute acetone as reported (17). The acetone-precipitated proteins were washed once with 80% acetone, resuspended in ultrapure MilliQ water (Millipore Corp., Bedford, Mass.), and then reprecipitated in absolute ethanol to remove any residual detergent. The protein fraction was resuspended in MilliQ water and used for vaccination studies described below.

Vaccination, animal challenge and evaluation of protection.

Immunoprotection experiments were conducted with C57BL/6 mice (females, 8 wks old) supplied by the National Cancer Institute (Bethesda, Md.). Mice were immunized subcutaneously (s.c.) with either the total protein fraction extracted from the parasitic cell wall described above (TX114-DF; 14 µg [dry wt] per dose), or a purified, bacterial-expressed recombinant aspartyl protease (rPep1; 1µg or 5 µg per dose) which is described below. The vaccination protocol was the same as previously reported (28). Mice were also immunized with a synthetic oligodeoxynucleotide (ODN) preparation containing unmethylated CpG dinucleotides that was used as an adjuvant (CpG ODN; Integrated DNA Technologies, Inc., Coralville, Iowa) (25). The CpG ODN sequence employed in this study was the same as we have previously reported (13). The oligonucleotides were dissolved in PBS (1 mg/ml) and used as a stock solution for the vaccination experiments. Mice were immunized two times (2 wks apart) with either adjuvant alone (10 µg of CpG prepared in 50 µl of PBS plus 50 µl of incomplete Freund’s adjuvant) as described (28), or selected concentrations of the test reagent (TX114-DF or rPep1) plus adjuvant. Mice were challenged by the intranasal (i.n.) route.
at 4 wks after the second immunization with 80-90 viable arthroconidia obtained from 30-day-old saprobic phase cultures as reported (13). Mice were scored for survival over a 50-90 day period post-challenge, or evaluated for fungal burden in homogenates of their lungs obtained at 15, 30, 60 and 90 days after challenge as previously described (28). Survival differences between groups of i.n.-infected mice (20 animals per group) were analyzed for statistical significance by the Kaplan-Meier method as reported (28). The fungal burden (colony-forming units [CFU]) in the lungs of vaccinated and non-vaccinated, infected mice was expressed on a log scale for individual animals, and the Mann-Whitney U test was used to compare the numbers of CFUs in each group of mice (15 animals per group) as described (13). The limit of detection of the pathogen in organ homogenates is $10^2$ CFU.

**Internal amino acid sequence analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS).** The protein components of the TX114-DF extract of the isolated parasitic cell wall fraction were initially separated by one dimensional-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblot analysis of sister gel separations of the TX114-DF were conducted by incubation with either pooled sera from surviving mice which had been vaccinated with the wall extract and challenged as described above, or with pooled human sera from patients with confirmed coccidioidal infection. The latter was conducted using goat anti-human IgG-specific secondary antibody (Southern Biotechnology Associates, Inc., Birmingham, Ala.) as reported (17). A 43-kilodalton (kDa), Coomassie blue-stained gel band was selected for further examination on the basis of its seroreactivity, prominence and apparent electrophoretic separation from other protein components in the SDS-PAGE gel.
The selected band was excised, destained, and subjected to in-gel digestion with sequencing grade trypsin (Promega, Madison, Wis.) at 37ºC overnight as reported (18). Peptides were extracted from the gel with 60% acetonitrile: 0.1% trifluoroacetic acid and concentrated using a SPD1010 SpeedVac system (ThermoSavant, Holbrook, N.Y.). The peptides were applied to a reverse phase HPLC column (Aquasil C18 Picofit column, 75 µm i.d. X 5 cm, tapered to 15 µm i.d.; New Objective, Woburn, Mass.), eluted using a binary gradient of 1% acetic acid/acetonitrile (5-95% acetonitrile in 35 min), and then introduced into an ion-trap mass spectrometer equipped with a nanospray source (LCQ Deca XP plus; Finnigan Corp., San Jose, Calif.). The tandem mass spectrometer was operated in the double play mode in which the instrument was set to acquire a full MS scan (400-2000 m/z), and a MS/MS spectrum of the most intense ion. Collision-induced dissociation (CID) spectra were obtained that yielded amino acid (aa) sequences of the peptides. A search for matching sequences in the translated *C. posadasii* (strain C735) genome database (18) ([www.tigr.org](http://www.tigr.org)) was conducted using the TurboSEQUEST software package, version 3.0 (Finnigan). Details of the computational method used to match the nascent CID mass spectra of peptides to database sequences have been described (49). Sequence matches were also manually verified. On this basis of peptide sequence matches we identified an open reading frame (ORF) of a 1.4 kilobase (kb) gene in the *C. posadasii* genome database which revealed 99.8% nucleotide sequence identity to the cDNA of a previously reported gene that encodes an aspartyl protease of *Coccidioides* (23). The genomic and cDNA sequences of the gene identified in the *C. posadasii* database, which were confirmed by cloning and nucleotide sequence analysis as
described below, are designated in this paper as *PEP1* and have been deposited in GenBank (accession no. DQ164306).

The basic local alignment search tool (BLAST) (1) was used to search the Swiss-Prot/TrEMBL database ([www.us.expasy.org/tools/blast](http://www.us.expasy.org/tools/blast)) and the National Center for Biotechnology Information (NCBI) non-redundant protein database ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) for proteins with sequence similarities to the translated, full-length *PEP1* gene. Analysis of the predicted hydropathicity profile of Pep1 was performed as reported (26). The GPI-SOM algorithm (14) was used to examine the translated sequence of *PEP1* for a putative glycosylophosphatidylinositol (GPI) anchor site, while the WoLF PSORT II algorithm was employed for prediction of a signal peptide and cellular localization ([www.wolfpsort.seq.cbrc.jp/](http://www.wolfpsort.seq.cbrc.jp/)). The PROSITE algorithm was used to identify conserved motifs in the translated polypeptide with homology to reported proteins (16).

**Two dimensional SDS-PAGE and immunoblot analysis.** Approximately 150 µg aliquots of either the concentrated protein fraction of the TX114-DF extract described above, or the protein fraction of the combined 96-h and 132-h parasitic phase culture supernatants (CS) were subjected to 2D-PAGE separation. The CS fraction was obtained after centrifugation (27,000 xg, 10 min, 4º C), dialysis against distilled water, and lyophilization as previously reported (51). The TX114-DF or CS preparations were solubilized in 2D-PAGE sample buffer which contained 8M urea, 2M thiourea, 4% CHAPS (a zwitterionic detergent; Amersham Biosciences, Piscataway, N.J.), 40 mM Tris, 65 mM dithiothreitol (DTT; Sigma) and 0.5% carrier ampholytes (Amersham). Each sample was subjected to vortexing (30 sec, three times) and insoluble material was
removed by centrifugation (15,000 xg, 30 min, at 4º C). The supernatant was applied to an 18 cm Immobiline™ Drystrip gel (Amersham) overnight at rm. temp. The gel was then subjected to isoelectric focusing (IEF) using an Ettan™ IPGphorII IEF System (Amersham). The IEF gel strips were first electrophoresed at 500 V for 2,500 Vh to remove interfering low molecular weight substances. IEF was performed at a maximum of 200 µA per strip in a two-step process; 3,500 V for 15,000 Vh followed by 5,500 V for 52,500 Vh. The gel strips were subsequently exposed to a reducing buffer at rm. temp. (50 mM Tris-HCl [pH 6.8], 6 M urea, 30% glycerol, 2% SDS, 0.5% DTT, and a trace of bromophenol blue) for 10 min, followed by alkylation with 2.5% w/v iodoacetamide for an additional 10 min. Electrophoresis in the second dimension was conducted in a 10% SDS-PAGE gel at 50 mA using an Ettan Daltsix System (Amersham). Protein spots were visualized by Coomassie blue stain, and gel images were digitally recorded using a GS-700 Imaging Densitometer (Bio-Rad, Hercules, Calif.). Coomassie-stained protein spots in the 2D-PAGE gels of the TX114-DF and CS fractions were selected for excision and LC-MS/MS analysis as described above on the basis of their seroreactivity in immunoblots of the respective 2D-PAGE sister gels. The latter were incubated with pooled sera from 10 human patients with confirmed coccidioidal infection as described above.

**ProPred prediction of promiscuous MHC class II-restricted epitopes.** The web-accessible ProPred algorithm ([www.imtech.res.in/raghava/propred/](http://www.imtech.res.in/raghava/propred/)) (40), which was developed on the basis of the TEPITOPE program (43), was used to predict the presence of promiscuous, human-MHC II-restricted epitopes in the proteins identified by immunoblots of the 2D-PAGE gels. This algorithm has been previously employed to
predict MHC class II epitopes in microbial and tumor antigens (5, 20). The ProPred algorithm contains matrix-based motifs of 51 human leukocyte antigen (HLA)-subregion DR alleles derived from a MHC class II pocket profile database (40). We used the algorithm to identify epitopes of the deduced proteins which were predicted to bind to each of the 51 HLA-DR molecules. The threshold for the ProPred analyses was set at a relatively high stringency of 5%. Under these conditions, promiscuous epitopes were defined as peptides that were predicted to bind to at least 80% of the MHC class II molecules expressed by the 51 HLA-DR alleles.

**Real-time PCR.** Levels of expression of *PEP1* during different stages of in vitro development of first generation parasitic cells were determined by quantitative real time-polymerase chain reaction (QRT-PCR) as reported (18). Parasitic cells were isolated from cultures after 36 h, 96 h and 132 h of incubation, and the majority of the fungal cells showed near synchronous development as presegmented, segmented, or endosporulating spherules, respectively (19). *PEP1*-specific primers were designed using the LightCycler Probe Design software (version 1.0; Roche Diagnostics, Indianapolis, Ind.). The sequences of the sense and antisense primers were 5′-AAATCCTGGAACGGTCAATAC-3′, and 5′-GAAAGCGTCTCCAAAGAATGG-3′, respectively. This primer pair amplified a 221-base pair (bp) PCR product using single-stranded template cDNA generated by reverse transcription of total RNA as reported (13). RNA was isolated separately from the three developmental stages of *C. posadasii* as described (18). A 191-bp amplicon used for normalization of the assay was derived from the constitutively-expressed glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene of *C. posadasii* (GenBank accession no. AF288134) as previously reported (13).
Confirmation that the GAPDH gene of this pathogen is constitutively expressed has been reported (Data analysis was performed as described (18), and the results presented as the ratio of \textit{PEP}1 to \textit{GAPDH} transcripts in each sample.

**Expression of \textit{PEP}1 by \textit{Escherichia coli}**. Oligonucleotide primers were designed to amplify the cDNA of \textit{PEP}1 (1.2-kilobases [kb]), which encodes amino acids 1 to 399 (predicted full-length protein). The nucleotide sequences of the sense and antisense primers were 5'-

\texttt{GGCAGCCATATGGCTAGCATGAGGAACTCCATCTGCTCGCAG-3'},  and 5'-

\texttt{GAATTCGGATCCTTACTGAGTTATGGGCTTTGGCAAGGCC-3'}, which contained engineered \textit{NdeI} and \textit{BamHI} restriction sites, respectively (underlined nucleotide sequences). The amplification parameters were as follows: an initial denaturation step at 94º C for 2 min, followed by 30 cycles which consisted of denaturation at 94º C for 30 sec, annealing at 56º C for 30 sec, and extension at 72º C for 2 min. The 1.2-kb amplicon was subcloned into the pGEM-TE cloning vector (Promega Corp., Madison, Wis.), and the nucleotide sequence of the insert was determined as previously reported (18). The pGEM-TE-\textit{PEP}1 plasmid was digested with \textit{NdeI} and \textit{BamHI} to release the 1.2-kb insert, which was then subcloned into the pET28b expression vector (Novagen) and used to transform \textit{E. coli} strain BL21 (DE3) as described (19). Purification of the recombinant protein (rPep1) was conducted as reported (28). Confirmation of the identity of the protein was performed by LC-MS/MS sequence analysis of peptides generated by trypsin digestion of the purified rPep1 as described above. The endotoxin content of the stock solution that contained the purified recombinant protein (0.22 mg/ml in PBS [0.1M, pH 7.4]) was determined by use of a
Limulus amebocyte lyase kit (QCL-1000; BioWhittaker, Walkersville, Md.) as previously reported (28). The stock solution contained 3.0 to 3.3 endotoxin units per µg of protein.

In vitro assays of murine immune T cell proliferation and cytokine production. Purified rPep1 (5 µg) plus the CpG ODN adjuvant were used to immunize five 8-week-old, female C57BL/6 mice as described above. Two weeks after the second immunization, the spleens were harvested, pooled and macerated as reported (28). Separation of CD90\(^+\) T cells from the cell suspension was conducted using mouse CD90 (Thy 1.2) MicroBeads (Miltenyi Biotec. Inc., Auburn, Calif.) as previously described (48). Antigen presenting cells (APCs) were isolated from pooled splenocytes obtained from five naïve (untreated), age- and gender-matched C57BL/6 mice. This splenocyte suspension was subjected to antibody depletion of the CD90\(^+\) T cells followed by irradiation as previously described (6). All cells were incubated in RPMI 1640 medium containing 10% (v/v) fetal calf serum (FCS), β-mercaptoethanol, penicillin and streptomycin as previously reported (48). Isolated CD90\(^+\) T cells plus APCs were transferred to 96-well flat bottom plates (Costar, Cambridge, Mass.) for proliferation assays (2.5 x 10\(^5\) T cells plus 5 x 10\(^5\) APCs per well). Alternatively, the isolated T cells (1 x 10\(^6\) cells per well) plus APCs (2.5 x 10\(^6\) cells per well) were transferred to 48-well flat bottom plates (Costar) for assays of cytokine production. For proliferation assays, the cells were cultured in medium alone, medium plus 2 µg/ml of mitogen (concanavalin A [Con A]; Sigma), or medium plus a range of concentrations of purified rPep1 (0.1-10 µg/ml), and then pulsed with \(^{3}H\) thymidine after 54 h of incubation and harvested 18 h later for determination of levels of radioisotope uptake as reported (28). For determination of levels of cytokine production, CD90\(^+\) T cells plus APCs were incubated
for 48 h or 120 h in medium alone, or medium to which Con A (2 µg/ml) or rPep1 (1 µg/ml or 5µg/ml) had been added. The cytokine assays were conducted in the presence or absence of polymyxin B (10 µg/ml; Sigma) to assess the influence of endotoxin contamination on production of the selected cytokines. Concentrations of secreted interferon-gamma (IFN-γ), interleukin (IL)-4, IL-5 and IL-10 in the culture supernatants were determined using the OptEIA™ mouse cytokine assay kits (Pharmingen, San Diego, Calif.) as reported (48).

**IFN-γ ELISPOT assays.** Purified rPep1 (5 µg) plus the CpG ODN adjuvant were used to immunize four 8-week-old female C57BL/6 mice, or four 12-week-old HLA-DR4 (DRB1* 0404) transgenic mice (a gift from Dr. Thomas Forsthuber, University of Texas at San Antonio). The immunization protocol was the same as described above. Two weeks after the second immunization, the spleens of the two groups of mice were separately harvested, pooled and macerated as above. Isolation of CD90⁺ T cells from the total splenocytes, collection of APCs, and cell culturing were performed as above. IFN-γ enzyme-linked immunospot (ELISPOT) assays were performed according to instructions of the kit manufacturer (MABTECH, Inc., Mariemont, Oh). In brief, 96-well filtration plates (ELIIP 10SSP; Millipore) were coated with monoclonal anti-murine IFN-γ antibody, washed with PBS and blocked with RPMI containing 2% FCS. CD90⁺ immune T cells (4 x 10⁵) plus APCs (2.5 x 10⁵) were then added to each well and incubated with or without stimulatory reagents (Con A, rPep1, synthetic peptides or medium alone) as described (48). Synthetic peptides spanning each of the 5 ProPred-predicted, promiscuous MHC II-restricted epitopes of Pep1 were constructed using 9-fluorenlymethoxy carbonyl chemistry and supplied by Mimitopes,
A control peptide (18-mer) which corresponds to a region of Pep1 not predicted to contain a T-cell epitope was synthesized and included as a control. The identity and purity of the peptides were confirmed by the manufacturer using mass spectrometry and high pressure liquid chromatography, respectively. The peptides were solubilized in 80% tissue culture grade dimethyl sulfoxide (ATCC, Rockville, Md.) at stock concentrations of 10 mg/ml. The peptide solutions were stored at -80º C in 0.1 ml aliquots until used for the assays.

After incubation with stimulating reagents, the cells were removed from the filtration membrane of each well, the plates were washed, and biotin-labeled anti-IFN-γ monoclonal detection antibody (R4-6A2-biotin) was added to the wells followed by streptavidin-alkaline phosphatase according to the manufacture’s instructions. After incubation, the plates were washed and the substrate, 1-Step™ NBT-BCIP (Pierce, Rockford, Ill.), was added for color development. Each membrane was analyzed using an automated ELISPOT reader system by Zellnet Consulting Inc. (Fort Lee, N.J.). The frequency of IFN-γ secreting antigen-specific CD90^+ T-cells was calculated as the number of spots per 4 x 10^5 CD90^+ T-cells seeded in the presence of antigen minus the number of spots per equal number of CD90^+ T-cells in medium alone.

RESULTS

**Immunization with a detergent extract of the parasitic cell wall (TX114-DF) confers protection against pulmonary coccidioidomycosis.** C57BL/6 mice were vaccinated subcutaneously with a complex mixture of proteins extracted from isolated walls of two separate cultures of parasitic cells which had developed to near synchronous stages of segmentation and endosporulation (13, 19). Our rationale for combining these
cell types was to account for the recognized quantitative and qualitative differences in antigen production during the parasitic cycle (12), which could be important in our search for candidate vaccines. Mice immunized with two doses of the detergent-extracted cell wall protein fraction (TX114-DF; 14 µg each) plus CpG ODN were well protected (90% survival) against a potentially lethal inoculum of *C. posadasii* (80 viable arthroconidia) delivered via the i.n. route, compared to no survivors among the control mice immunized with adjuvant alone (Fig. 1). The latter group of animals began to die at 11 days post-challenge, followed by a sharp decrease in the number of survivors during the subsequent 5 days, and then a short lag period before the rest of the mice died. In the case of the vaccinated mice, 2 animals died at 13 days post-challenge, and the remaining 18 mice appeared to be healthy throughout the subsequent 37 days of the experiment. The same vaccination protocol was repeated twice using separate detergent extracts of cell wall proteins prepared in the same manner. The percent of vaccinated mice which survived was not significantly different between the three experiments, suggesting that the TX114-DF extract of the cell wall contained immunogenic components which confer protective immunity against pulmonary challenge with *C. posadasii*. Compositional analyses of the detergent-soluble fraction were conducted by SDS-PAGE as described below.

A 43-kDa polypeptide identified as an aspartyl protease homolog is a major protein component of TX114-DF. The Coomassie blue-stained SDS-PAGE separation of the TX114-DF preparation revealed a prominent band at 43 kDa, as well as multiple lightly stained bands in the range of 34 kDa to >200 kDa (Fig. 2A). Immunoblot analysis of sister gel separations of this extract incubated either with pooled mouse sera from
survivors reported in Fig. 1, or pooled sera from patients with confirmed coccidioidal infection both showed strong reactivity with the 43-kDa component (not shown). The seroreactive 43-kDa gel band appeared to be electrophoretically separated from other protein components in the SDS-PAGE gel, and was selected for excision and trypsin digestion. The acquired tryptic peptides were subjected to amino acid sequence analysis by tandem mass spectrometry. The sequences were deduced by the mass differences between y- or b-ion “ladder” series resulting from the CID fragmentation spectra of the selected tryptic peptides as reported (34). A representative CID spectrum for a 1,711.88 dalton (Da) peptide is shown in Fig. 2B. Five good CID spectra were obtained, each of which yielded amino acid sequences of 16 to 26 residues. TurboSEQUEST searches of the translated C. posadasii genome database revealed that all five peptides matched a 1.2-kb translated ORF of a 1.4-kb gene (Fig. 2C). A BLAST search of the Swiss-Prot/TrEMBL and non-redundant NCBI protein databases using the translated ORF identified an aspartyl protease of C. posadasii which had previously been described (GenBank accession no. AF162132) (23). The earlier reported gene (PEP) was isolated from the Silveira strain of C. posadasii. The authors estimated that the molecular size of the mature protein in SDS-PAGE gels was 45 kDa, and determined on the basis of its N-terminal sequence that two processing events occurred which resulted in cleavage of an 18-aa signal peptide and a 52-aa propeptide. Two N-glycosylation sites were predicted. The cDNA sequence of the PEP gene of the Silveira strain showed three nucleotide substitutions when aligned with the genomic sequence of the homolog in the database of strain C735. The 1.2-kb cDNA (ORF) of the C735 homolog was PCR-amplified using primers described above in the Materials and Methods, and subjected to nucleotide
sequence analysis. Its sequence matched that of the *C. posadasii* database. The nucleotide substitutions identified in the C735 cDNA homolog did not influence its translated amino acid sequence, which was identical to that of the Silveira strain. The isolated gene of *C. posadasii* strain C735 has been designated *PEP1*.

The WoLF PSORT algorithm predicted Pep1 to be cell wall-associated, secreted into the culture media, or both. The protein sequence contains two conserved aspartyl protease active sites identified by the PROSITE algorithm at D\textsuperscript{103}XG\textsuperscript{105}X S\textsuperscript{107} XXW\textsuperscript{110}, and D\textsuperscript{287}T\textsuperscript{288}G\textsuperscript{289} (D is the active site residue) in accordance with the sequence analysis performed by Johnson and coworkers (37). The hydrophobicity profile of Pep1 predicted three hydrophobic domains (aa 180-222, 275-305, and 345-380). On the basis of the GPI-SOM algorithm (14), the C-terminal hydrophobic region is predicted to contain a GPI anchor signal sequence with a putative anchor site at G\textsuperscript{374}.

**2D-PAGE/immunoblot analyses reveal that Pep1 is one of several seroactive proteins in the parasitic cell wall extract and culture supernatant.** 2D-PAGE gel separations of the TX114-DF wall extract and culture supernatant (CS) were blotted and incubated with pooled patient sera. Prominent, seroactive protein spots with an estimated molecular size of 43 kDa (Fig. 3A, B) were identified as Pep1 by LC-MS/MS in both preparations. These data support the prediction that Pep1 is both wall-bound and released into the culture media (37). Portions of the two immunoblots of the 2D-PAGE gels in the pI range of 3 to 6 revealed additional seroreactive protein components of the TX114-DF and CS preparations. Each protein in the corresponding Coomassie-stained 2D-PAGE gel was excised and sequenced by LC-MS/MS. Seven deduced proteins in the combined wall extract plus culture supernatant were identified in addition to Pep1, and their
sequences have been deposited in the GenBank database (Table 1). One of the excised gel components (gel spot no. 6) failed to yield interpretable CID spectra, and two of the excised proteins in the CS preparation were components of the same polypeptide (gel spot nos. 8 and 9). The latter was identified as a spherule outer wall glycoprotein (SOWgp), which has been previously reported in *C. posadasii* strain C735, and is characterized by prominent, seroreactive 82-kDa and 60-kDa bands (17). Recombinant SOWgp has proved not to be a candidate vaccine against coccidioidomycosis (18).

SOWgp as well as another deduced protein listed in Table 1 (gel spot no. 5) are rich in proline, which may account for their contradictory molecular size estimates in the 2D-PAGE gels compared to the predicted sizes based on the respective amino acid sequences (36). All but one of the deduced proteins were predicted to be cell wall-associated or extracellular, and 3 of these were suggested to be GPI-anchored. All of the deduced proteins were characterized by hydropathicity profiles with well-defined hydrophobic domains (not shown), as revealed by Pep1 and described above. Each of the deduced proteins in Table 1 were subjected to sequence analysis using the ProPred algorithm for prediction of promiscuous epitopes which bind to human MHC class II molecules. Four of the deduced proteins contained 5-7 predicted promiscuous T-cell epitopes, and one of these was Pep1.

**PEP1 is constitutively expressed during the parasitic cycle.** We have previously demonstrated that spherule development during the first generation of the parasitic cycle of *C. posadasii* strain C735 is fairly well synchronized (18). Total RNA was isolated either from spherules prior to endospore release at incubation times of 36 h and 96 h, or after
endospore differentiation and the initiation of endospore release at 132 h. Results of QRT-PCR analysis of *PEP1* gene expression, compared to temporal expression of the constitutive *GAPDH* gene, revealed approximately equal amounts of transcript for each developmental stage examined (data not shown). However, the peak ratios of transcript levels of *PEP1/GAPDH* are about 10-fold lower than that of another *C. posadasii* gene (*MEP1*) which was recently described (18). However, in spite of the comparatively low amount of *PEP1* transcript we have observed that the 43-kDa antigen is an abundant protein in SDS-PAGE separations of both the wall extracts and culture supernatants of parasitic cells. This suggests that the *PEP1* transcript is stable and persistent during the parasitic cycle, and/or Pep1 accumulates both in the wall and culture supernatant during spherule development.

**Bacterial-expressed recombinant Pep1 is seroreactive and stimulates immune T cell response.** *E. coli* transformed with the pET 28b-*PEP1* plasmid produced high levels of recombinant protein either in the presence or absence of IPTG (isopropyl-β-D-thiogalactopyranoside; Fig. 4), albeit the amounts of total protein loaded onto the gel were not equal. The predicted and observed molecular size of the recombinant protein in the SDS-PAGE gel is 47 kDa, which includes the vector-encoded fusion peptide that contained the His-tag at its N-terminus. The 47-kDa rPep1 was isolated by nickel-affinity chromatography, subjected to electrophoresis, excised from the SDS-PAGE gel, trypsin-digested, and processed for LC-MS/MS sequence analysis as described above. Five peptide sequences were obtained by this procedure and each matched the translated sequence of the *PEP1* gene. The recombinant protein was reactive with randomly selected sera from patients with confirmed coccidioidal infection, as demonstrated by the
representative immunoblot in Fig. 4. On the other hand, pooled sera from healthy individuals (i.e., hospital admissions with no indication of fungal infection) did not recognize the purified rPep1.

CD90^+^ T cells isolated from mice immunized with rPep1 plus the CpG ODN adjuvant were stimulated to proliferate in vitro in response to the presence of the purified homologous recombinant protein at concentrations of 1-10 µg/ml (Fig. 5A). Immune T-cells incubated with rPep1 at a concentration of 0.1 µg/ml showed markedly lower response, but significantly greater proliferation than immune T lymphocytes incubated in culture medium alone ($P<0.05$). Immune T cells incubated with the mitogen, Con A, responded in an expected manner.

In vitro T cell immunoassays were also performed to evaluate the amount and type of cytokines secreted by the immune cells during their proliferative response to rPep1 (Fig. 5 B-D). The two concentrations of recombinant protein tested (5 µg/ml and 1 µg/ml) were chosen on the basis that they stimulated comparable levels of immune T cell proliferation (Fig. 5A). Polymyxin B was added to replicate assays to neutralize potential effects of endotoxin contamination on T lymphocyte response. Immune T cells incubated with 5 µg/ml of rPep1 for 48 h and 120 h (+/- polymyxin B) showed levels of IFN-γ production (Th1-type cytokine) which were equal to or higher than the concentrations of the same cytokine detected in the supernatant of immune T cell cultures that had been incubated with the mitogen, Con A (Fig. 5B). Incubation with 1 µg/ml of rPep1 under identical conditions resulted in significantly lower levels of IFN-γ production compared to 5 µg/ml ($P<0.05$), but still at concentrations which were above background. In contrast, T cell stimulation with either 5 µg/ml or 1 µg/ml of the recombinant protein...
resulted in secretion of significantly lower concentrations of both IL-5 and IL-10 (Th2-type cytokines) compared to Con A stimulation under identical incubation conditions (Fig. 5C, D). Even lower concentrations of IL-4 were detected in the T-cell culture supernatants after 48 h and 120 h of incubation in the presence of rPep1 (not shown). On the basis of the results presented in Fig. 5 it appears that rPep1 is a T-cell-reactive antigen, and immune T lymphocytes respond to the presence of the recombinant protein by secretion of high levels of a Th1-type cytokine. The addition of polymyxin B to the reaction mixtures containing rPep1 had no significant effect on levels of cytokine production.

Identification of T-cell epitopes of Pep1. Pep1 sequence analysis using the ProPred algorithm identified five regions of the full-length polypeptide (range of 21-32 amino acids) which were predicted to contain ligands that can bind to human MHC class II molecules. Each of these five regions, which may include more than one T-cell epitope, were synthesized (P1-P5; Table 2) and used separately to test the in vitro response of immune T cells obtained from rPep1-immunized C57BL/6 and HLA-DR4 transgenic mice (Fig. 5A and B, respectively). A synthetic peptide (18-mer; P6 in Table 2) not predicted to bind to any of the 51 HLA-DR molecules examined in the ProPred algorithm was included as a negative control. T cell response to each of the synthetic peptides (5 µg/ml) was compared to that of the recombinant Pep1 (2.5 µg/ml) by IFN-γ ELISPOT assays of the relative numbers of cytokine-producing CD90+ immune T lymphocytes. In both C57BL/6 and HLA-DR4 transgenic mice, significant responses were observed in the presence of synthetic peptides P1 and P2 compared to cells incubated in medium alone ($P<0.05$ and $<0.01$, respectively). On the other hand, the
numbers of IFN-γ-producing cells was much greater in the presence of the recombinant protein (Fig. 6A, B). Immune T cells from the C57BL/6 and transgenic mice showed no significant response to the other synthetic peptides (P3-P5) representing putative epitopes, or to the control peptide, P6. It is possible that induction of significant response would have been observed in the presence of P3-P5 at higher concentrations of the synthetic peptides. These assays are planned for future studies. Nevertheless, results of the IFN-γ ELISPOT assays confirm the ProPred prediction that P1 and P2 are promiscuous epitopes which can activate both murine and HLA-DR4 immune T cells.

**Vaccination with rPep1 enhances survival and results in significant reduction of fungal burden in Coccidioides-challenged mice.** Control C57BL/6 mice immunized with adjuvant alone and then challenged via the intranasal route with a lethal inoculum (90 viable arthroconidia) began to die at about 13 days post-challenge (Fig. 7A). Approximately 7 days later all the control animals had died. Groups of mice (20 per group) vaccinated with rPep1 (1 µg or 5 µg per dose) plus adjuvant and challenged with the same intranasal inoculum, began to die at about 14 to 16 days, but then showed significantly greater survival when compared to the control mice (P values indicated in Fig. 7A). Comparison of the survival plots of TX114-DF-vaccinated mice in Fig. 1 versus rPep1-vaccinated mice in Fig. 7A (5 µg dose) by Kaplan-Meier analysis revealed no statistically significant difference (P=0.79). This survival plot is representative of three separate experiments using the same vaccination and challenge protocols. A trend was evident that the 5µg dose provides better protection than the 1 µg dose, although the difference in percent survival between these two groups of mice was not statistically significant. However, on the basis of this apparent trend we decided to examine the
fungal burden in the lungs and spleen of C57BL/6 mice vaccinated with the 5μg dose of rPep1, inoculated by the i.n. route (90 viable arthroconidia), and sacrificed at 15, 30, 60 and 90 days post-challenge (10 animals randomly selected from survivors of 15 vaccinated/challenged mice per group). Control mice sacrificed at 15 days post-challenge showed high numbers of CFUs in their lungs ($10^5$-$10^7.8$ Fig 7B) and spleen ($10^3$; not shown). None of the vaccinated and challenged mice showed detectable organisms in their spleen between 15 and 90 days after inoculation. The vaccinated mice also revealed progressive pulmonary clearance of *C. posadasii*, resulting in significant reduction of the fungal burden between 15 and 90 days post-challenge as indicated in Fig. 7B.

**CONCLUSIONS**

The majority of *Coccidioides* antigens which have so far been shown to induce a protective immune response in mice against coccidioidomycosis are derived from the parasitic cell wall (13, 27, 31, 39). This same observation has been reported for several other fungal pathogens (35, 47). The rationale for extraction of the isolated spherule wall with Triton X-114 was to release noncovalently linked proteins. Our reasons not to focus on proteins that are covalently bound to the glycan network of the parasitic cell wall (50) are that the majority of these polypeptides are highly glycosylated and, because of their tight association with structural wall components, may not be readily accessible to antigen presenting cells for processing and subsequent T cell activation. TX114 extraction was also selected because the detergent has been shown to at least partially solubilize the lipid-rich spherule outer wall (SOW) layer which coats the parasitic cells of
Finally, we chose to isolate, fractionate and test the protective properties of the TX114-extracted proteins because we wanted to focus our analyses on polypeptides with hydrophobic domains. Temperature dependent phase partitioning with Triton X-114 has proved to be a useful method for enrichment of hydrophobic proteins (52). Since hydrophobicity is considered to play an important role in binding of antigenic peptides to class II major histocompatibility complexes of CD4\(^+\) T lymphocytes (42), we argue that this extraction procedure enhanced our ability to identify candidate T-cell reactive proteins.

The TX-114 detergent extract of the isolated spherule wall material, together with the CpG ODN adjuvant preparation, induced a robust protective immune response in C57BL/6 mice against a lethal pulmonary challenge with *C. posadasii* arthroconidia. We base this conclusion on survival of 90% of the animals at 50 days post-challenge. In addition, we were able to reproduce this level of murine protection using the same vaccination protocol with separate preparations of the detergent extract of the isolated parasitic cell wall, suggesting that the TX114 fraction is a good source of vaccine candidates. A large body of evidence supports the concept that synthetic oligodeoxynucleotides (ODNs) with CpG motifs provide enhanced immune response to co-delivered antigens (25). CpG is suggested to directly activate dendritic cells and macrophages to enhance production of cytokines that create a Th1-like milieu in lymphoid tissue. The choice of adjuvant is a pivotal element in the evaluation of candidate vaccines (29).

The total protein subfraction of the solubilized parasitic cell wall material which was separated by SDS-PAGE and subjected to immunoblot analysis using pooled sera
from patients with confirmed *Coccidioides* infection revealed multiple seroreactive bands. Although seroreactivity does not necessarily indicate T-cell reactivity of an antigen, the fact that the secondary antibody used for our immunoblot assays was anti-IgG-specific argues that the seroreactive proteins contain T-cell epitopes (21). We initially selected the 43-kDa protein component of the parasitic cell wall extract for sequence analysis by LC-MS/MS based on its patient seroreactivity and abundance in Coomassie-stained SDS-PAGE gel separations of the TX114 fraction. Mass spectrometry is a valuable tool for studies of protein structure. Interfacing reverse-phase high performance liquid chromatography with electrospray ionization has permitted efficient and accurate determination of the amino acid sequences of complex starting mixtures of tryptic peptides (49). With near completion of the genome sequence and annotation of *C. posadasii* strain C735, we were able to unambiguously match peptide sequences obtained by LC-MS/MS with a specific gene in the *Coccidioides* database. Through bioinformatic analyses of the translated full-length gene sequence, conserved motifs were identified and a putative function was assigned to the deduced protein. The amino acid sequence of the 43-kDa polypeptide identified in immunoblots of both one-dimensional and 2D-PAGE gel separations of the TX114-DF extract showed high homology to previously reported aspartyl proteases of other filamentous fungi. Sequence analysis of the *C. posadasii* protein (Pep1) revealed three defined hydrophobic domains, including a C-terminal GPI anchor signal sequence. The native Pep1 was also detected in the parasitic culture supernatant, demonstrating that the protein is both cell wall-associated and extracellular. This contrasts with an *A. fumigatus* homolog (Pep2) which has been suggested to be bound to the fungal cell wall and not released into the
culture supernatant (38). The reason for this apparent difference in localization of these structurally-related proteins (93% sequence similarity) is unknown.

Two-dimensional electrophoresis, combined with immunoblot analysis and bioinformatics (10) have been applied for the first time in this report to the characterization of *C. posadasii* antigens. Incubation of 2D-PAGE gel separations of the detergent extract and culture supernatant with patient sera revealed multiple seroreactive proteins in the respective immunoblots. For comparative purposes in this study, we examined a narrow pH range in the 2D gels that included the estimated pI of the native Pep1 (approx. 4.4 to 4.5). All seroreactive proteins were excised from the Coomassie-stained sister gel, trypsin-digested, and the fractionated peptides were subjected to LC-MS/MS sequence analysis. Each of the deduced proteins examined in the TX114-DF extract and culture supernatant, except the spherule outer wall glycoprotein (SOWgp), revealed multiple hydrophobic domains, which may account for their partitioning into the detergent phase during the isolation procedure. Although SOWgp was not expected to be isolated in the detergent fraction (17), its association with the lipid layer at the surface of parasitic cells (7, 18) may account for its presence in the TX114-DF extract. The WoLF PSORT algorithm predicted that all but one of the deduced proteins were cell wall-associated and/or extracellular. Patient seroreactivity, hydrophobicity, and parasitic cell wall association are features which suggested that these proteins were worthy of further examination as candidate T-cell-reactive antigens. An additional criterion for selection of antigens as vaccine candidates is the presence of epitopes which bind to MHC class II molecules (8). The ProPred algorithm has proved to be a valuable bioinformatics tool for identification of putative T cell epitopes in microbial antigens, and has permitted
researchers to successfully progress from genome sequences to epitope-derived vaccine design (11). On the basis of ProPred analyses of the deduced proteins identified in the 2D-PAGE gels, four cell wall-associated/extracellular antigens and one cytoplasmic antigen were shown to contain multiple promiscuous epitopes predicted to bind to at least 80% of the representative HLA-DR molecules in the algorithm. HLA-DR molecules account for more than 90% of the HLA class II isotypes expressed on APCs (43). The full-length sequence of Pep1 was predicted to contain five promiscuous epitopes.

The aspartyl protease (Pep1) of \textit{C. posadasii} was originally isolated by Johnson and coworkers (23) from disrupted, formaldehyde-killed spherules (FKS). A FKS vaccine has been shown to induce a protective response in mice and primates against coccidioidal infection, but also revealed serious irritant properties which prevented its successful application as a human vaccine (30). Our interest in Pep1 was piqued by its abundance in SDS-PAGE gel separations of both parasitic cell wall extracts and culture supernatants of \textit{C. posadasii}. In support of this observation, we found that expression of the \textit{PEP1} gene is constitutive during the parasitic cycle. Aspartyl proteases produced by other microbial pathogens have been proposed to be vaccine candidates. Immunization of mice with a secreted aspartyl protease of \textit{Candida albicans} (Sap2) has been shown to significantly decrease severity of systemic candidiasis (46). Alum was used as the adjuvant. The authors proposed that the protective response was antibody-mediated. An aspartyl protease isolated from \textit{Schistosoma japonicum}, causative agent of schistosomiasis, has also been evaluated as a vaccine in a murine model of this disease (44). Immunization with the recombinant protein resulted in reduced worm burden in challenged mice, but little to no effect in reducing the fecundity of the pathogen. The
authors showed that the schistosome protease induced a mixed Th1/Th2 cytokine response (45). Immunization of C57BL/6 mice with recombinant Pep1 of C. posadasii induced a moderate in vitro proliferative response of isolated immune T cells in a recall experiment. Of particular interest, however, was that IFN-γ was the most abundant secreted cytokine in the supernatants of the activated T lymphocytes. Only after incubation of the CD90+ T cells with antigen for 120 h was it possible to detect significant amounts of secreted IL-5 and IL-10. Results of clinical and animal model studies of coccidioidomycosis have supported the argument that Coccidioides antigens which stimulate a Th1 pathway of host response are essential components of a vaccine against this respiratory disease (9). Our data obtained from IFN-γ ELISPOT assays of immune CD90+ T cells of both C57BL/6 and HLA-DR4 transgenic mice in the presence of the recombinant Pep1 provide additional support that this antigen stimulates a potent Th1 pathway of immune response. Two synthetic peptides (P1, P2), which represent N-terminal regions of Pep1 that contain predicted MHC II-binding epitopes, were shown to induce IFN-γ production by immune T cells isolated from both strains of mice. These data suggest that promiscuous epitopes predicted to bind to human HLA-DR molecules also bind to murine MCH II molecules, and vice versa. P1 includes the signal peptide of the aspartyl protease. Protective epitopes of another vaccine candidate of C. posadasii, referred to as Ag2/Pra, have been demonstrated to be present within the N-terminal region of the protein, including the 18-residue signal peptide (22).

Results of rPep1 vaccination of C57BL/6 mice against a potentially lethal pulmonary infection of C. posadasii suggested that the animals had mounted a potent and durable cellular immune response against the pathogen. The majority of survivors
cleared the fungus from their lungs over a 90 day period post-challenge. It is possible that immunization with rPep1 activates both antibody- and cell-mediated immune defenses during the protective response against *Coccidioides*. Anti-rPep1 antibody (both IgG2a and IgG1 isotypes) were detected by ELISA in vaccinated mice just prior to infection, and at 7 and 12 days post-challenge (data not shown). Humoral immunity may play a significant role in defense against coccidioidomycosis, particularly by opsonization of endospores which are small enough to be engulfed by host phagocytes (18). Since Pep1 is cell wall-associated, and produced throughout the parasitic cycle, this cell surface antigen may be involved in opsonization. Opsonins have been shown to contribute to the activation and binding of dendritic cells to *Cryptococcus neoformans* yeast, which results in enhanced antifungal activity (24). Evidence has been presented that dendritic cell activation is pivotal to defense against coccidioidal infection (3). Although the mechanisms by which vaccination with rPep1 influences innate and acquired immune response to *C. posadasii* infection are essentially unknown, the level of protection afforded by this single antigenic protein plus CpG ODN adjuvant engenders confidence that development of a recombinant vaccine against human coccidioidomycosis is feasible (8). This study has also demonstrated that combined applications of immunoproteomics and bioinformatics to compositional analyses of crude, protective cell wall extracts of *Coccidioides* represent an efficient method to screen for T-cell-reactive antigens and vaccine candidates.
ACKNOWLEDGEMENTS

Support for this study was provided by Public Health Service grants AI19149 and UO1 AI50910 (Coccidioides genome sequencing project) from the National Institute of Allergy and Infectious Diseases, National Institutes of Health.
REFERENCES


FIG. 1. Representative comparison of the protective efficacy of a Triton X-114 extract of the isolated parasitic cell wall of *C. posadasii* (TX114-DF plus immunoadjuvant [Adj], CpG ODN) versus adjuvant alone both delivered subcutaneously to C57BL/6 mice. The animals were subsequently challenged with a potentially lethal inoculum (80 viable arthroconidia) of *C. posadasii* via the i.n. route.
FIGURE 1

$P = 0.0004$

- ○ TX114-DF + Adj.
- ● PBS + Adj.

% Survival

Days post-challenge

100% Survival
FIG. 2.  

(A-C) Application of LC-MS/MS to the identification of a major protein component of the parasitic cell wall extract, TX114-DF. (A) Coomassie-stained SDS-PAGE gel separation of TX114-DF. (B) CID mass spectrum of 1711.88 Da peptide \([(M + 2H)^{2+} = 856.94\text{ Da}]\) derived from trypsin digest of excised 43-kDa band in (A). (C) Complete sequence of the putative \(C.\ posadasii\) aspartyl protease (Pep1). Five underlined peptide sequences are those identified by LC-MS/MS analysis and matched with the translated \(C.\ posadasii\) genome database using the TurboSEQUEST software package.
FIGURE 2

A

MW (kDa)

Std. TX114-DF

200
116
66
45
31
22
14
6

B

LC-MS/MS

Intensity (x10^4)

m/z

0
200
400
600
800
1000
1200
1400
1600

1000
1100
1200
1300
1400
1500
1600

C

MRNSILLAATVLLGCSTASKVHKLKLLKLPLTEQLEYG
DIETHVRALGQKYFGSLPSSQQTVLSDSEYYTSSTGHHNV
LVDNFLNAQYFSEISIGNPPQNFKVVLDTGSSNLWVPSS
ECGISACYLHKNKDSSATSTYKKNTEFAIR/YSQSLS
GFVSQDTLR/IGDLTIEGQDFEAETNEPGLAFAFGR/F
DGLGLGYDTISYNK/IVPYPYNMINELIDEPYFGFY
LDGTKN/EGDDSAYATFGGVSSLLFSGEMIKPLRRKAY
VEVFDAIAFGERAELDGIHTGSLIAILPSTELAE
LNRGAKKWSWQYTVDCNKRPDPDLFTLSGHNFTR
IGPYDYLEVQGSCISSEFMDFPEPVGPIALGDAFLR
R/FYTMDFLNNLAVGALK/AGN
FIG. 3. (A, B). Immunoblots of 2D-PAGE-separated TX114-DF (A) and culture supernatant (CS) proteins (B). Numbers correspond to protein spots identified by LC-MS/MS (see Table 1).
FIGURE 3

A

MW
(kDa)

TX114-DF

B

MW
(kDa)

CS

Pep1

Pep1

31

31

45

45

66

66

97

97

4.5

pH

6

3

pH

5
FIG. 4. SDS-PAGE gel separation and immunoblot (Iblt.) of *E. coli*-expressed rPep1. Shown are standards (Std.), lysates of bacteria transformed with the pET28b-*PEPL* plasmid vector in the presence (+) or absence (-) of IPTG, and the nickel-affinity isolated rPep1 which was subsequently purified by electroelution from an SDS-PAGE gel as previously reported (13). The immunoblot of the rPep1 isolated by nickel-affinity chromatography was incubated either with serum from a patient with confirmed coccidioidal infection, or serum from a control patient.
FIG. 5.  

(A) In vitro proliferative response of isolated CD90^+ T cells derived from rPep1 + CpG ODN-immunized C57BL/6 mice stimulated with different concentrations of the homologous antigen.  

(B-D) ELISAs of secreted cytokines (IFN-\(\alpha\), IL-5 and IL-10) produced by immune T cells stimulated with Con A (positive control) or rPep1 (1 \(\mu\)g or 5 \(\mu\)g) in the presence (+) or absence (-) of polymyxin B.  

Assays were conducted with culture supernatants obtained after incubation for 48 h or 120 h.  

Immune T cells grown in medium alone served as a negative control.  

Mean values plus standard deviations (S.D.) for three separate determinations of both the proliferative response and ELISAs of cytokine concentrations (pg/ml) are reported.
FIGURE 5

A

![Graph A showing CPM (x10^3) vs rPep1 concentration (µg/ml).]

B

![Graph B showing IFN-γ concentration (pg/ml) vs PMB and rPep1 concentration (µg/ml).]

- Medium
- Con A
- 10 µg rPep1
- 5 µg rPep1
- 1 µg rPep1
- 0.1 µg rPep1

- 48 h
- 120 h
FIGURE 5

C

![Graph showing IL-10 concentration (pg/ml) over time for different conditions.]

D

![Graph showing IL-5 concentration (pg/ml) over time for different conditions.]

<table>
<thead>
<tr>
<th>Condition</th>
<th>48 h</th>
<th>120 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PMB</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Con A</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>5µg rPep1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>1µg rPep1</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
FIG. 6. Assessment of IFN-γ production by immune CD90+ T cells derived from C57BL/6 (A) or HLA-DR4 (DRB1*0404) transgenic mice conducted by IFN-γ ELISPOT assays. Asterisks indicate statistically significant differences between response of T cells in presence of peptides versus medium alone. A representative result of three separate experiments is shown here.
FIGURE 6

C57BL/6 mice

IFN-γ spots/well

ex vivo stimulus

Media Pep1 P1 P2 P3 P4 P5 P6

2.5 µg/ml 5 µg/ml

* P < 0.05 compared to media alone
FIGURE 6

B

HLA-DR4 transgenic mice

* *  P < 0.01 compared to media alone
FIG. 7. (A) Representative comparison of the protective efficacy of rPep1 (1 µg or 5 µg) plus CpG ODN adjuvant used to immunize C57BL/6 mice, which were subsequently challenged by the i.n. route with a lethal inoculum of *C. posadasii*. Control mice were immunized with adjuvant alone. Statistical significance (*P* values) of the difference in survival plots of the vaccinated versus non-vaccinated mice is shown. The results are representative of three separate vaccination/survival experiments using the same immunization and challenge protocols. (B) Plot of CFU of *C. posadasii* detected in dilution plate cultures of lung homogenates obtained from non-vaccinated (PBS + Adj.) or vaccinated C57BL/6 mice (5 µg rPep1 + Adj.). The animals were inoculated intranasally with *C. posadasii* as above and sacrificed at different times post-challenge as indicated. The *P* value indicates a significant difference in the fungal burden of vaccinated mice at 15 versus 90 days post-challenge.
FIGURE 7

A

% Survival vs Days post-challenge

- PBS + Adj.
- rPep1 (1 µg) + Adj.
- rPep1 (5 µg) + Adj.

P = 0.0001
P = 0.01
FIGURE 7

B

CFU in lungs ($\log_{10}$)

$\begin{array}{ccccccc}
15 & 15 & 30 & 60 & 90 \\
\hline
\text{PBS + Adj.} & \text{5 $\mu$g rPep1 + Adj.}
\end{array}$

Days post-challenge

$P = 0.002$
<table>
<thead>
<tr>
<th>Gel spot no.</th>
<th>Putative protein identification&lt;sup&gt;a&lt;/sup&gt;</th>
<th>GenBank accession no.</th>
<th>Predicted MW (kDa) and pI&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Observed MW (kDa) and pI (approx.)</th>
<th>Predicted localization and GPI-anchor&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Number of predicted promiscuous T-cell reactive epitopes&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Aspartyl protease (Pep1)</td>
<td>DQ164306</td>
<td>43.0 / 4.4</td>
<td>43.0 / 4.4</td>
<td>Cw / extracellular/GPI</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>Carboxypeptidase Y</td>
<td>DQ176864</td>
<td>60.3 / 5.5</td>
<td>56 / 5.5</td>
<td>Cw / extracellular</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>Proline-rich antigen 2 (Pra2)</td>
<td>AAM66748</td>
<td>12.9 / 8.6</td>
<td>56 / 5.2</td>
<td>Cw / extracellular</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>Phospholipase B (Plb)</td>
<td>DQ188099</td>
<td>68.6 / 6.4</td>
<td>60 / 5.0</td>
<td>Cw / extracellular/GPI</td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td>Proline threonine-rich protein</td>
<td>DQ176865</td>
<td>29.0 / 5.3</td>
<td>66 / 5.3</td>
<td>Cw / extracellular</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>No sequence obtained</td>
<td>____</td>
<td>____</td>
<td>____</td>
<td>____</td>
<td>____</td>
</tr>
<tr>
<td>7</td>
<td>Glycosyl hydrolase</td>
<td>DQ176866</td>
<td>86.2 / 5.5</td>
<td>87 / 5.5</td>
<td>Cytoplasmic</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>Spherule outer wall glycoprotein (SOWgp)</td>
<td>AAL09436</td>
<td>46.4 / 4.4</td>
<td>82 / 4.6</td>
<td>Cw / extracellular/GPI</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>Spherule outer wall glycoprotein (SOWgp)</td>
<td>AAL09436</td>
<td>46.4 / 4.4</td>
<td>82 + 60 / 4.6</td>
<td>Cw / extracellular/GPI</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>Alphamannosidase (Amm1)</td>
<td>DQ176863</td>
<td>56.9 / 4.8</td>
<td>57 / 4.8</td>
<td>Cw / extracellular</td>
<td>6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Protein identity based on homology to reported fungal proteins determined by BLAST searches (1).

<sup>b</sup> Based on amino acid sequence analysis using ExPASy Proteomic Server (http://us.expasy.org/).

<sup>c</sup> Based on amino acid sequence analysis using WoLF PSORT II and GPI-SOM algorithms; Cw, cell wall-associated.

<sup>d</sup> Based on amino acid sequence using the ProPred algorithm. A promiscuous epitope is predicted to bind to at least 80% of the HLA-DR molecules in the ProPred algorithm (40, 43).
TABLE 2. Amino acid sequences of synthetic peptides selected from ProPred prediction of Pep1 ligands which bind to human MHC class II molecules

<table>
<thead>
<tr>
<th>Peptide reference</th>
<th>Peptide sequence</th>
<th>Amino acid sequence nos. of Pep1</th>
<th>% of HLA-DR molecules predicted to bind to epitope(s) in P1-P6&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>MRNSILLAATVLLGCTSAKVKHL</td>
<td>1-23</td>
<td>100</td>
</tr>
<tr>
<td>P2</td>
<td>HVRALGQKYFGSLPSSQQTV</td>
<td>41-62</td>
<td>80</td>
</tr>
<tr>
<td>P3</td>
<td>FAIRYGSGSLGFVSQDTLRIGDL</td>
<td>142-165</td>
<td>98</td>
</tr>
<tr>
<td>P4</td>
<td>YILEVQGSCISSFMGMDFPEPVGPLAILGDAF</td>
<td>345-377</td>
<td>80</td>
</tr>
<tr>
<td>P5</td>
<td>DAFLRRFYTYDLGNVLVGLAKAGN</td>
<td>375-399</td>
<td>88</td>
</tr>
<tr>
<td>P6</td>
<td>GSIACYLHNKYDSSASST</td>
<td>117-134</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percentage of 51 human HLA-DR molecules included in the ProPred algorithm to which peptides (P1-P6) are predicted to bind at a threshold of 5%.

<sup>b</sup> Peptide predicted by ProPred algorithm not to bind to human MHC class II molecules served as a control in the IFN-γ ELISPOT assay of immune T cells obtained from C57BL/6 and HLA-DR4 transgenic mice (see Fig. 6).
An Immunoproteomics and Bioinformatics Approach to Identification
of *Coccidioides posadasii* Cell Wall-associated and T Cell Reactive
Antigens Yields a Protective Multivalent Recombinant Vaccine

Eric J. Tarcha\(^1\), Venkatesha Basrur\(^1\), Chiung-Yu Hung\(^2\),
Malcolm J. Gardner\(^3\), and Garry T. Cole\(^2*\)

Department of Medical Microbiology and Immunology
Medical University of Ohio, Toledo, OH 43614 \(^1\)
Department of Biology, University of Texas at San Antonio
San Antonio, Texas 78249 \(^2\)
and The Institute for Genomic Research, Rockville, MD 20850 \(^3\)

Prepared for submission
102
**ABSTRACT**

*Coccidioides* spp are primary fungal pathogens that are endemic to the alkaline desert soil of the Southwestern United States and the etiological agents of coccidioidomycosis (Valley fever), a respiratory disease in humans. *Coccidioides* represents the only fungal pathogens on the Center for Disease Control’s select agent list of possible weapons of bioterrorism, and cause significant morbidity in infected individuals. Thus the need for a human vaccine against coccidioidomycosis has come to the forefront. Work has centered on the identification and characterization of recombinant T cell-reactive antigens, because clinical and experimental data suggest that activation of a durable MHC II restricted T cell Th1 immune response is of principal importance in establishing durable protective immunity. In this study we describe an immunoproteomic and bioinformatic approach for profiling a diverse immunogenic protein component of the coccidioidal parasitic cell wall. A phospholipase B (Plb) and alpha-mannosidase (Amn1), as well as a previously described aspartyl protease (Pep1), were selected as candidate vaccines on the basis of their immunogenicity, cellular localization, predicted promiscuous T cell epitope content, and T cell reactivity. These antigens were evaluated individually, and in combination, for their protective efficacy in a pulmonary murine model of infection. Each individual protein showed significant protection in *C. posadasii* infected mice as evaluated by survival after lethal challenge (53%-61% survival). A combinatorial vaccine composed of all three of the protective antigens enhanced survival in infected mice (86% survival) and significantly improved clearance of the pathogen from lungs of the surviving mice by 90 days post-challenge. This strategy has been successful in producing the most comprehensive profile of immunogenic coccidioidal cell
wall antigens to date, and lays the groundwork for the development of an epitope-driven, multivalent human vaccine against coccidioidomycosis.
INTRODUCTION

*Coccidioides posadasii* and *C. immitis*, the two recognized species of the human respiratory pathogen, are the dimorphic etiological agents of coccidioidomycosis (15, 18). Coccidioidomycosis (also known as San Joaquin Valley Fever) is a respiratory fungal infection that often presents as a self-limiting acute or subacute pneumonia in approximately 40% of infected individuals, whose symptoms can persist for months (19). The saprobic mold form of the fungus is found in the alkaline desert soils of the Southwestern United States and parts of Mexico, Central and South American, resulting in endemicity of disease in these areas (4, 5). Natural aerosolization and subsequent inhalation of the fungal spores by a human host can lead to onset of disease that is rarely life threatening, but causes significant morbidity in infected individuals. Dissemination of the parasitic phase of the fungus occurs primarily in immunocompromised individuals, but a small percentage of immunocompetent people who have been infected will develop the disseminated form of disease (19). Disseminated coccidioidomycosis is often extremely difficult to treat; with long courses of possibly toxic antifungal pharmaceuticals necessary to control infection and frequent relapse of infections occur post-treatment (2). In the past decade, there has been a dramatic increase in the number of people from non-endemic regions who have visited or relocated to the Southwestern U.S resulting in approximately 150,000 new infections each year, and a large population of immunonaive individuals at risk of infection (4, 19). *Coccidioides* also represents the only fungal pathogen listed as potential weapon of bioterrorism by the Centers for Disease Control and Prevention (12). The status of the fungus as a select agent and the
reemergence of this pulmonary disease supports the need for a human coccidioidal vaccine.

Immunization of mice with formaldehyde-killed parasitic spherules or vaccination with an avirulent strain of *Coccidioides* has been shown to confer protective immunity in the animals against infection following a lethal, pulmonary challenge with *Coccidioides* arthroconidia (38, 39). These data, along with the observation that resolution of natural infection with *Coccidioides* often results in life-long immunity (46), suggests that development of a vaccine against coccidioidomycosis is possible. The importance of T-cell mediated immunity for effective defense against *Coccidioides* has been indicated by clinical as well as experimental data (6), while the role of B cells and antibody in protective immunity to this pathogen has been disputed (25, 35). Therefore, our previous studies in search of candidate vaccines against coccidioidomycosis have focused on individual T cell and immunoglobulin G (IgG)-specific seroreactive proteins that confer protection to mice in a murine pulmonary model of infection (47). Such a vaccine is suggested to stimulate both the humoral and cellular (Th1 and Th2) compartments of the immune system. The literature suggests that an ideal coccidioidal vaccine should be cost effective to produce, physically stable and contain an adequate number of essential epitopes to induce long-lasting immunity against infection (4, 5). We argue in this study, that an effective vaccine against *Coccidioides* should include promiscuous protective T cell epitopes and be polymeric in nature.

This present study was aimed at identifying immunoreactive, parasitic cell wall-associated antigens that could be used as components of a polyvalent coccidioidal
vaccine. We first used an immunoproteomics and bioinformatics strategy to identify seroreactive proteins in a cell wall preparation that were predicted to contain promiscuous T cell epitopes. This approach resulted in the identification of 34 immunogenic proteins associated with the wall of *C. posadasii* spherules. This is, to date, the most comprehensive analysis of the cell wall protein components of this pathogen. Three proteins, phospholipase B (Plb), alpha-mannosidase 1 (Amn1), and a previously described aspartyl protease (Pep1) were selected for further evaluation based on their relative predicted promiscuous T cell epitope content. The T cell reactivity of recombinantly expressed Plb and Amn1, as well as the predictive value of the ProPred algorithm, was demonstrated by in vitro validation of selected promiscuous epitopes and full-length proteins by IFN-γ ELISPOT assays. Finally, we showed that a combinatorial vaccine composed of recombinant Pep1, Amn1 and Plb conferred greater protective immunity to infected mice compared to vaccines composed of the single antigens alone. Based on previous data and the observations reported in this study, we provide a novel strategy for identification of coccidioidal vaccine candidates and the foundation for development of a polyvalent recombinant protein vaccine.

**MATERIALS AND METHODS**

**Fungal culture conditions and isolation of parasitic cell wall fraction.** Both the saproic and parasitic phases of *C. posadasii* (strain C735) were grown in vitro as previously described (20). Arthroconidia were harvested from saproic phase cultures after 4 wks of incubation as reported (31). The arthroconidia were used to inoculate
flasks of defined glucose/salts medium (29) for growth of the parasitic (spherule-endospore) phase (21), or to prepare an inoculum for murine infection. Spherules were harvested from parasitic phase cultures after incubation for 96 h (pre-endosporulation) or 132 h (post-endosporulation) and mixed (1:1; v/v). The cell pellet collected by centrifugation (3,000 x g) was washed 4 times with ice-cold disruption buffer [20 mM Tris-HCL, pH 7.4, Protease Inhibitor Cocktail, Set IV (Calbiochem, San Diego, Calif.)]. The parasitic cells suspended in buffer were disrupted in a BeadBeater™ (BioSpec Products, Inc., Bartlesville, Okla.) using pre-cooled 0.5 mm diameter Zirconia/Silica beads (Biospec). Complete disruption of the cells was determined by the lack of fungal growth on glucose-yeast extract media after plating and incubation of the homogenate as previously described (20). The homogenate was centrifuged (5,000 x g), the pellet washed 5 times with cold disruption buffer as above, and stored at -80º C until ready for use.

Two dimensional SDS-PAGE and immunoblot analysis. Approximately 250 mg of packed-cell wall material was suspended in 2D-PAGE sample buffer which contained 8M urea, 2M thiourea, 4% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; a zwitterionic detergent; AmershamBiosciences, Piscataway, N.J.}, 40 mM Tris, 65 mM dithiothreitol (DTT; Sigma, St. Louis, Mo.) and 0.5% carrier ampholytes (Amersham). The cell wall mixture was briefly sonicated (15 sec sonication, 1 min ice, three times) to break-up the cell wall material and enhance the solubilization of released proteins. Insoluble material was removed from the sample buffer by high-speed centrifugation (15000 x g, 30 min, 4°C). Approximately 340 µL of the resulting supernatant was used to hydrate 18 cm pH 3-10 NL Immobiline Drystrips
overnight. The proteins were then subjected 2D-PAGE as previously described (47). Briefly, isoelectric focusing (IEF) was performed at a maximum of 200 µA per strip in a two-step process; 3,500 V for 15,000 Vh followed by 5,500 V for 52,500 Vh. The gel strips were subsequently exposed to a reducing buffer (RT with 0.5% DTT) at for 10 min, followed by alkylation with 2.5% w/v iodoacetamide for an additional 10 min. Electrophoresis in the second dimension was conducted in either a 10% or 12% SDS-PAGE gel at 50 mA using an Ettan Dalt six System (Amersham). Protein spots were visualized by Coomassie blue stain, and gel images were digitally recorded using a GS-700 Imaging Densitometer (Bio-Rad, Hercules, Calif.). Immunoblot analysis of sister gel separations were conducted with pooled human sera from ten patients with confirmed coccidioidal infection, as well as pooled healthy control sera. The blotting was conducted as reported (20), using goat anti-human IgG-specific secondary antibody (Southern Biotechnology Associates, Inc., Birmingham, Ala.). Coomassie-stained protein spots in the 2D-PAGE gels were selected for excision and LC-MS/MS sequence analysis based on their seroreactivity in the immunoblots of 2D-PAGE sister gels.

**Internal amino acid sequence analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS).** Proteins spots were selected for sequencing on the basis of their seroreactivity with pooled coccidioidomycosis patient sera. Coomassie blue-stained protein spots corresponding to immunoreactive proteins in the western blot were excised, destained, and subjected to in-gel digestion with sequencing grade trypsin (Promega, Madison, Wis.) at 37°C overnight as reported (22). Peptides were extracted from the gel and subjected to LC-MS/MS analysis as previously described (47). Briefly,
the peptides were applied to a reverse phase HPLC column (Aquasil C18 Picofrit column, 75 µm i.d. X 5 cm, tapered to 15 µm i.d.; New Objective, Woburn, Mass.), eluted using a binary gradient of 1% acetic acid/acetonitrile (5-95% acetonitrile in 35 min), and then introduced into an ion-trap mass spectrometer equipped with a nanospray source (LCQ Deca XP plus; Finnigan Corp., San Jose, Calif.). Collision-induced dissociation (CID) spectra were obtained that yielded amino acid (aa) sequences of the peptides. A search for matching sequences in the translated *C. posadasii* (strain C735) genome database (22) (www.tigr.org) was conducted using the TurboSEQUEST software package, version 3.0 (Finnigan). Peptide hits with cross correlation value (Xcorr) and ΔCn of <2 and <0.2, respectively, were considered positive and manually verified. Putative full-length amino acid sequences were obtained from the genomic database for proteins with matching peptide sequences. The basic local alignment search tool (BLAST)(1) was used to search the Swiss-Prot/TrEMBL database (www.us.expasy.org/tools/blast) for protein sequences with similarities to the translated, full-length protein sequences in order to determine the identity of the *C. posadasii* proteins based on sequence homology. The Wolf PSORT II algorithm was used for prediction of a signal peptide (www.wolfpsort.seq.cbrc.jp/) and prediction of cellular localization, while the GPI-SOM algorithm (14) was used to examine the translated protein sequences for a putative glycophosphatidylinositol (GPI) anchor site.

**ProPred prediction of promiscuous MHC II restricted epitopes and peptide synthesis.** Prediction of promiscuous human major histocompatibility complex (MHC) class II-restricted epitopes was performed using the ProPred algorithm (45) as previously described (47). Amino acid sequences of seroreactive proteins that were predicted by the
Wolf PSORT II algorithm to be extracellular/cell wall-associated were selected for epitope analysis. The threshold setting for the analyses was set at a relatively high stringency threshold of 5%. Promiscuous epitopes were defined as peptides that will bind at least 80% of the MHC class II molecules expressed by the 51 HLA-DR alleles available in the algorithm.

Synthetic peptides spanning each of the ProPred-predicted, promiscuous MHC II-restricted epitopes of Amn1 and Plb (Table 3) were constructed using 9-fluorenylmethyloxycarbonyl chemistry and supplied by Mimotopes, Ltd. (Morris Plains, N.J.). Two control peptides (20-mers) that correspond to regions of Plb and Amn1 not predicted to contain a T-cell epitope were synthesized. The identity and purity of the peptides were confirmed by the manufacturer using mass spectrometry and high pressure liquid chromatography, respectively. The peptides were solubilized in 80% tissue culture grade dimethyl sulfoxide (ATCC, Rockville, Md.) at stock concentrations of 10 mg/ml. The peptide solutions were stored at -80º C in 0.1 ml aliquots until used for the assays.

**Expression of AMN1 and PLB by Escherichia coli.** Oligonucleotide primers were designed to amplify the cDNAs of AMN1 and PLB, which encode amino acids 1 to 520 and 1 to 646 respectively (predicted full-length protein). The nucleotide sequences of the sense and antisense primers to amplify the AMN1 cDNA were 5’-

GAGCTCCCATATGAAGGGATCCCCGTACTC-3’,

and 5’-

CTCGAGAAGCTTCTATGACAGAAGCGAGATCA-3’,

which contained engineered Ndel and HindIII restriction sites, respectively (underlined nucleotides). The amplification parameters for AMN1 cDNA were as follows: an initial denaturation step at 94º C for 2 min, followed by 30 cycles which consisted of denaturation at 94º C for 30
sec, annealing at 56º C for 30 sec, and extension at 72º C for 2.5 min. The 1.6-kb amplicon was subcloned into the pGEM-TE cloning vector (Promega Corp., Madison, Wis.), and the nucleotide sequence of the insert was determined as previously reported (21). The pGEM-TE-AMN1 plasmid was digested with NdeI and HindIII to release the 1.6-kb insert, which was then subcloned into the pET32b expression vector (Novagen) and used to transform E. coli strain BL21 (DE3) as described (21).

The nucleotide sequences of the sense and antisense primers to amplify the PLB cDNA were 5'-ATGCTAGCCATATGAGACCTATCGGGGCC-3', and 5'-GCGGCCGCTCGAGTGGGTGGAAATTAGTATACCA-3', which contained engineered NdeI and XhoI restriction sites, respectively (underlined nucleotides). The amplification parameters for PLB cDNA were as follows: an initial denaturation step at 94º C for 2 min, followed by 30 cycles which consisted of denaturation at 94º C for 30 sec, annealing at 56º C for 30 sec, and extension at 72º C for 3 min. The 1.9-kb amplicon was subcloned, sequenced, inserted into the pET32b expression vector and used to transform E. coli strain BL21 (DE3) as described above.

Purification of the recombinant proteins (rAmn1 and rPlb) was conducted as reported (31). The recombinant proteins were expressed with a vector encoded 109 amino acid N-terminal Trx-Tag™ thioredoxin fusion peptide in order to enhance solubility of the purified recombinant protein. The endotoxin content of the stock solutions that contained the purified recombinant proteins was determined by use of a Limulus amebocyte lyase kit (QCL-1000; BioWhittaker, Walkersville, Md.) as previously reported (31). The stock solutions contained 3.0 to 5.0 endotoxin units per µg of protein. The recombinant proteins were separated by one dimensional SDS-PAGE tested for
seroreactivity with pooled patient as well as pooled healthy control sera by immunoblot analysis as described above. The identities of the purified proteins were confirmed by LC-MS/MS sequence analysis as described above.

**IFN-γ ELISPOT assays.** Purified recombinant protein (5 µg of either rAmn1, rPlb) plus the CpG adjuvant were used to immunize four 8-week-old, female C57BL/6 mice or four 12-week-old HLA-DR4 (DRB1*0404) transgenic mice (a gift from Dr. Thomas Forsthuber) as described (32). Two weeks after the second immunization, the spleens of mice were separately harvested, pooled and macerated as previously reported (31). Separation of CD90^+^ T cells from the cell suspension was conducted using mouse CD90 (Thy 1.2) MicroBeads (Miltenyi Biotec. Inc., Auburn, Calif.) as previously described (48). Antigen presenting cells (APCs) were isolated from pooled splenocytes obtained from five naïve (untreated), age- and gender-matched mice. This splenocyte suspension was subjected to antibody depletion of the CD90^+^ T cells.

IFN-γ enzyme-linked immunospot (ELISPOT) assays were performed as previously reported (47). In brief, 96-well filtration plates (ELIIP 10SSP; Millipore, Bedford, Mass) were coated with monoclonal anti-murine IFN-γ antibody, washed with PBS and blocked with RPMI containing 2% FCS. CD90^+^ immune T cells (4 x 10^5^) plus APCs (2.5 x 10^5^) were then added to each well and incubated with or without stimulatory reagents (Con A, rAmn1, rPlb, synthetic peptides or medium alone) as described (48).

After incubation with stimulating reagents, the cells were removed from the filtration membrane of each well, the plates were washed, and biotin-labeled anti-IFN-γ monoclonal detection antibody (R4-6A2-biotin) was added to the wells followed by streptavidin-alkaline phosphatase according to the manufacture’s instructions. Detection
and analysis was performed as previously described (47). The frequency of IFN-γ secreting antigen-specific CD90⁺ T-cells was calculated as the number of spots per 4 x 10⁵ CD90⁺ T-cells seeded in the presence of antigen minus the number of spots per equal number of CD90⁺ T-cells in medium alone.

**Vaccination, animal challenge and evaluation of the protective efficacy of recombinant proteins.** Immunoprotection experiments were conducted with C57BL/6 mice (females, 8 wks old) supplied by the National Cancer Institute (Bethesda, Md.). Mice were immunized subcutaneously (s.c.) separately with either rAmn1 or rPlb (at 1µg or 5 µg dose), rPep1 (1µg dose) (47), or a combination of 1µg of each of the recombinant proteins. The vaccination protocol used is the same as previously reported (31). Synthetic oligodeoxynucleotide (ODN) preparations containing unmethylated CpG dinucleotides was used as adjuvant as previously reported (CpG ODN; Integrated DNA Technologies, Inc., Coralville, Iowa) (32). Mice were immunized and boosted (2 wks apart) with either adjuvant alone (10 µg of CpG prepared in 50 µl of PBS plus 50 µl of incomplete Freund’s adjuvant) as described (31), or with the recombinant proteins plus adjuvant. Mice were challenged by the intranasal (i.n.) route at 4 wks after the boost with 70-80 viable arthroconidia obtained from 30-day-old saprobic phase cultures as reported (10). Survival of the challenged mice was determined over a 90-day period post-challenge. At the end of the 90-day period, homogenates of the lungs of the surviving mice were evaluated for fungal burden as previously described (31). Survival differences between groups of i.n.-infected mice were analyzed for statistical significance by the Kaplan-Meier method as reported (31). The fungal burden (colony-forming units [CFU]) in the lungs of vaccinated and non-vaccinated, infected mice was expressed on a log scale.
for individual animals, and the Mann-Whitney U test was used to compare the numbers of CFUs in each group of mice as described (24). The survival and CFU experiments were performed with 16 mice per group and repeated three times.

RESULTS

Profiling of *C. posadasii* seroreactive cell wall-associated antigens. Cell wall material was isolated and combined from cultures of parasitic cells that had developed to near synchronous stages of segmentation and endosporulation (10). A hydrophobic Triton X-114 detergent phase (TX114-DF) extract of cell-wall proteins from these cell types was previously shown to be a source of patient seroreactive proteins, and shown to confer protective immunity in a murine model of coccidioidomycosis (47). In an effort to profile a large number of proteins associated with the parasitic cell wall, and to identify proteins that could possibly be used as components of a polymeric vaccine, a diverse protein component of the isolated cell wall material was extracted. The extraction was performed using denaturants, a reducing agent, detergent, and mechanical disruption (CWP fraction).

The CWP fraction was separated by 2D-PAGE and visualized by Coomassie blue staining, revealing a complex protein profile containing greater than 400 protein spots (Fig. 1A). Multiple sample repeats of the CWP 2D-PAGE gel separations showed a similar profile. The impracticability of identifying the all of the protein spots observed in the 2D-PAGE separations prompted the employment of immunoblotting with patient sera in an effort to focus our protein identification on immunoreactive antigens. The CWP gel separations were blotted and incubated with pooled coccidioidomycosis patient sera, as
well as pooled healthy control sera. The complexity of the protein profile of the *Coccidioides* specific seroreactive proteins was greatly reduced compared to that observed in the Coomassie-stained gels (Fig. 1). Blots incubated with pooled healthy control sera showed little to no reactivity (not shown). There were approximately 70-80 proteins that were observed to be reactive with patient sera in the immunoblots (Fig. 1B), with 50 of those spots able to be aligned with the sister Coomassie-stained 2D-PAGE gels. The aligned spots were excised from the stained gels and sequenced by LC-MS/MS. Sequence was obtained from 44 of the 50 spots (Fig. 1), with 34 unique seroreactive proteins identified (Table 1). The predicted full-length amino acid sequences of the identified proteins were obtained from the recently available *C. posadasii* predicted protein database. The full-length amino acid sequences of the identified proteins were analyzed using a pI / molecular weight (MW) predictor (www.expasy.org), as well as the WolfPSORT II and GPI-SOM algorithms for prediction of cellular localization (Table 1). Wolf PSORT II predicted sixteen of the 34 proteins to be localized in the cell wall or extracellularly, six proteins to be localized in the cytoplasm and 12 proteins to be mitochondrial of origin. Three of the proteins, [[aspartyl protease 1 (Pep1), spherule outer wall glycoprotein (SOWgp), phospholipase B (Plb)]] were suggested to contain GPI-anchor signal peptides and putative anchor sites. Multiple proteins, such as aconitase, had conflicting predicted and experimentally observed pI / MW, while others, such as malate dehydrogenase, were identified from more than one spot on the gel. These phenomena could be a possible result of proteolytic degradation, and/or post-translational processing of the proteins. Some of the prominent seroreactive CWP fraction proteins have been previously reported to be immunogenic, coccidioidal
cell wall-associated antigens. Proteins such as SOWgp (20), Pep1 (47), the serodiagnostic complement fixation (CF) antigen chitinase 1 protein (Cts1) (7, 42), and the glycosyl-hydrolase, tube precipitin antigen, beta-glucosidase 2 (Bgl2) (7, 31) have been reported to stimulate antibody production in coccidioidomycosis patients. Although the observation of predicted cytoplasmic and mitochondrial proteins in the CWP fraction indicates the possibility of cytoplasm and mitochondria contamination in the cell wall preparation, there has been reports of some of these proteins to be immunogenic and/or located at the cell surface in other fungal systems (Table 1). With these data taken into consideration the deposition of some of these antigens into the cell wall cannot be ruled out.

ProPred analysis of seroreactive, Wolf PSORT II-predicted cell wall antigens for promiscuous MHC class II-restricted epitopes. Though focusing our protein identification efforts on antigens that were reactive with patient sera reduced the number of possible vaccine candidates, another screening procedure was required to select a reasonable number of proteins to further characterize. Consequently the ProPred algorithm was used to screen each of the Wolf PSORT II predicted cell wall associated proteins in Table 1 for the number of MHC class II restricted promiscuous epitopes each one contained. These antigens contained a wide range of predicted promiscuous T-cell epitopes, as high as eight and a few as zero (Table 2). The ratio of promiscuous epitopes per kDa of polypeptide was calculated for each of these proteins to “normalize” the relative number of epitopes in each protein to each protein’s molecular weight. The antigens that had the highest ratio of promiscuous epitopes to MW were Pep1, Plb, and Amn1 (Table 2). While the protective efficacy of Pep1 has already been studied (47), Plb and Amn1 were selected for further characterization due to the fact that they had the next
highest ratios of predicted promiscuous epitopes to molecular weight and were predicted to be targeted for the cell surface.

**Bacterial-expressed recombinant Amn1 and Plb are seroreactive.** E. coli transformed with the pET 32b-AMN1 plasmid produced increased levels of recombinant protein after induction of IPTG (isopropyl-β-D-thiogalactopyranoside; Fig. 2B), while the pET 32b-PLB transformed E. coli produced less protein after induction (Fig. 2A) The observed molecular sizes of the recombinant proteins in the SDS-PAGE gel were accurate to the predicted molecular weights which included the vector-encoded fusion peptide that contained the His-tag and Trx-Tag™ at their N-terminus. The recombinant Amn1 (rAmn1) and recombinant Plb (rPlb) were isolated by nickel-affinity chromatography and the identities of the proteins were confirmed by LC-MS/MS sequencing. The recombinant proteins were reactive with pooled sera from patients with confirmed coccidioidal infection (Fig. 2A and 2B), while pooled sera from healthy individuals (i.e., hospital admissions with no indication of fungal infection) did not recognize the purified recombinant proteins.

**Recombinant Amn1 and Plb along with select predicted promiscuous epitopes of these proteins stimulate immune T-cells from C57BL/6 and human MHC II transgenic mice.** Sequence analysis of the Amn1 and Plb amino acid sequences using the ProPred algorithm identified six and seven regions respectively of the full-length proteins, which were predicted to contain ligands that can bind promiscuously to human MHC class II molecules. For each of these proteins, six 20mers corresponding to these regions and two 20mers that were not predicted to contain any promiscuous epitopes were synthesized (P1-P14; Table 2). The full-length recombinant
proteins and these synthetic peptides were used to test the in vitro response of immune T cells obtained from rAmn1 and rPlb immunized C57BL/6 and HLA-DR4 transgenic mice. T cell response to each of the synthetic peptides (5 µg/ml) and the recombinant antigens (1.0 µg/ml) were assayed by IFN-γ ELISPOT, observing the relative numbers of cytokine-producing CD90+ immune T lymphocytes. In both C57BL/6 and HLA-DR4 transgenic mice, the full-length recombinant antigens stimulated a significant number of immune T cells, suggesting that both rAmn1 and rPlb contained T cell epitopes for both the MHC haplotypes tested (Figs. 3 and 4). The T cells from rAmn1 immunized C57BL/6 and HLA-DR4 transgenic mice produced significant responses in the presence of synthetic peptides P1 and P3 compared to cells incubated in medium alone (P<0.05 for C57BL/6 and <0.01 for HLA-DR4). Peptides P2 and P5 stimulated a significant response (P<0.01) in the HLA-DR4 transgenic mice alone (Fig. 3). On the other hand, peptides P6 and P7 stimulated significant responses (P<0.05) in C57BL/6 mice (Fig. 3). While P7 was not predicted to contain MHC II restricted epitopes for human MHC II haplotypes, it was observed to stimulate C57BL/6 immune T-cells, suggesting that P7 contains an epitope that can be presented by the MHC II of the H2-b haplotype mice. The T cells from rPlb immunized C57BL/6 and HLA-DR4 transgenic mice produced significant responses in the presence of synthetic peptide P12 compared to cells incubated in medium alone (P<0.05). Peptides P11 and P13 stimulated a significant response (P<0.05) in the C57BL/6 mice alone (Fig. 4), while peptide P8 stimulated a significant response (P<0.05) in the HLA-DR4 transgenic mice (Fig. 4). On the basis of the results presented in Figs. 3 and 4, rAmn1 and rPlb are T-cell-reactive antigens that contain multiple MHCII restricted epitopes. These data also suggest that ProPred is viable in
determining some of the promiscuous epitopes which can activate both murine and HLA-DR4 immune T cells.

**Vaccination with rAmn1 or rPlb confers protective immunity to Coccidioides-challenged mice.** Typically control C57BL/6 mice immunized with PBS/adjuvant and then challenged via the intranasal (i.n.) route with a lethal inoculum (70-80 viable arthroconidia) began to die at around 14 days post-challenge (Fig. 5). By 20 days post-challenge all the control animals had died. Groups of mice (16 per group) vaccinated with rAmn1 or rPlb (1 µg or 5 µg per dose) plus adjuvant showed significantly greater survival when compared to the control mice ($P < 0.01$). The 1 µg rAmn1 plus adjuvant group had 66% of the mice surviving to 90 days post challenge, while the 5 µg rAmn1 plus adjuvant group had 61% survival. Vaccinating and boosting with 1 µg of rPlb plus adjuvant resulted in 61% survival while the 5 µg rPlb plus adjuvant group had 73% survival at 90 days. The survival between the 1 µg or 5 µg doses of each antigen were not significant to each other.

**Vaccination with a combination of rAmn1, rPlb, and rPep1 compared to the single antigens, enhances survival and significantly improves clearance of the fungus from the lungs of Coccidioides-challenged mice.**

C57BL/6 mice were vaccinated with a combination of rAmn1, rPlb and rPep1 (1 µg each) with adjuvant, 1 µg of each antigen plus adjuvant independently, or with PBS/adjuvant alone. The mice were challenged via the i.n. route with a similar lethal dose of *C. posadasii* as above (70-80 arthroconidia). While the percentage of surviving mice vaccinated with 1 µg of each antigen ranged from 53% (rPep1) to 61% (rAmn1), the percentage of the surviving mice in the combination vaccinated mice (86%) was
consistently better than the single antigens alone (Fig. 6A), and all groups showed significantly better survival to the PBS/adjuvant vaccinated mice ($P < 0.01$). This trend was observed in three separate experiments using the same vaccination and challenge protocols.

The fungal burden in the lungs of the surviving mice were measured at 90 days post-challenge to determine differences in the clearance of the pathogen in the different vaccinated groups. Control mice sacrificed at 15 days post-challenge showed a median number of $7.07 \log_{10}$ CFU (range, 7.4 to 6.4) in their lungs (Fig 6B). Surviving mice vaccinated with single antigens plus adjuvant showed median CFU counts of $2.17 \log_{10}$, $1.30 \log_{10}$, and $2.62 \log_{10}$ for rPep1, rAmn1, and rPlb respectively. All of the mice showed highly significant clearance to the PBS/adjuvant vaccinated mice at 15 days ($P < 0.01$). The mice vaccinated with the combination of antigens plus adjuvant showed a median number of $0.00 \log_{10}$ CFU (with lung CFUs ranging from 0.0 to 1.9 in individual mice) in their lungs, which was significantly lower than any of the single antigens alone ($P < 0.05$) (Fig. 5B). These CFU data are a composite of the three survival experiments referenced above.

**DISCUSSION**

Coccidioidal vaccines composed of complex mixtures of cell wall protein extracts or whole killed spherules have shown considerable protection in the mouse model of coccidioidomycosis (28, 38, 47, 49), but interest in the development of a defined protein subunit vaccine for human use has grown due to the toxicity of whole cell vaccines and variable composition of the crude protein fractions (4, 37). Recent efforts to discover
protective antigens of *Coccidioides* have resulted in the identification of proteins associated with the cell surface of the fungus (10, 44, 47). The increased accessibility of these proteins to antigen presenting cells, for processing and T cell activation during the course of disease, make these proteins the best candidates (in theory) for an effective vaccine. Current data suggest that, as with most fungal diseases, coccidioidomycosis requires a robust MHC class II-restricted T cell mediated immune response for protection against disease (25). While the role of B-cells in protective immunity to *C. posadasi* has become the subject of debate (35), the majority of data in the literature suggest that MHC II restricted T cell mediated immunity is key to controlling disease. It has been observed that single antigens can provide a degree of immunoprotection against coccidiodal infection, but no single antigen has been able to confer the levels of protective efficacy required for an acceptable human vaccine. We consequently believe that a fully protective vaccine against coccidioidomycosis, a vaccine that will provide sterilizing immunity, will be composed of multiple protective antigens containing promiscuous T cell epitopes.

We have previously demonstrated that an immunoproteomics and bioinformatics approach is an efficient and productive way to screen for T cell reactive vaccine candidates (47). For that reason, we extracted a diverse protein component of immunoreactive antigens in the parasitic cell wall using 2D-PAGE sample buffer containing detergent, a reducing agent, and denaturants along with mechanical disruption to profile proteins that could possibly be included in a polyvalent vaccine. The 2D-PAGE separations and immublot analysis of the CWP fraction revealed a wide variety of immunogenic proteins associated with the parasitic cell wall. The WoLF PSORT
algorithm predicted that approximately half of the deduced proteins were cell wall-associated and/or extracellular, and the other half of cytosolic or mitochondrial origin. While patient seroreactivity and parasitic cell wall association suggested that these proteins were worthy of further examination, another criterion for selection of antigens for further evaluation as vaccine candidates is the presence of MHC class II restricted epitopes in the protein amino acid sequence (6). Detection of IgG specific seroreactive proteins in the western blots of the 2D-PAGE gels suggested that the antigens contained T-cell epitopes (23). We subsequently predicted that the antigens contain multiple promiscuous MHC class II restricted epitopes on the basis of analysis using the ProPred algorithm. The use of proteins containing predicted promiscuous T cell epitopes for a coccidioidal vaccine had not been addressed previous to the identification and subsequent characterization of Pep1 as a vaccine candidate (47). We argue that efforts to develop a human vaccine against coccidioidomycosis should be focused on proteins containing epitopes that can be presented by a wide range of MHC class II alleles, with the expectation that the vaccine will stimulate an adaptive memory immune response in the entire post-vaccinated population. Therefore, an epitope-driven polyvalent vaccine of this type will most likely be composed of multiple protective proteins or a single chimeric protein constructed to contain multiple protective promiscuous epitopes from these antigens. With this in mind, we once again employed the ProPred algorithm to screen the seroreactive CWP proteins to identify the predicted promiscuous MHC class II epitopes in each Wolf PSORT determined cell wall-associated antigen. We limited our epitope screening to the predicted cell wall-associated antigens because of our greater confidence in their surface localization. A ratio of predicted epitopes per kDa of molecular weight
was calculated for the antigens analyzed by the ProPred algorithm. This was done to
normalize the number of epitopes in each protein independent of the size of the protein.
The proteins were then ranked based on this ratio, and interestingly, the antigen with the
highest ratio was the Pep1 protein. Pep1 was previously shown to confer protective
immunity when used as vaccine against pulmonary coccidioidomycosis in C57BL/6 mice
and a number of the ProPred predicted epitopes were found to stimulate immune T cells
in C57BL/6 and human MHC II transgenic mice (HLA-DR4) (47). Furthermore, two of
the antigens with the lowest ratios were SOWgp and Mep1 have been tested as vaccine
candidates and shown not to be protective (unpublished data), further demonstrating that
the prediction of promiscuous epitopes may provide some indication of potential
protective efficacy of candidate proteins. The next two highest ratios belonged to Plb and
Amn1 (Table 2), which were previously found to be components of the protective
TX114DF fraction and parasitic phase CS, respectively (47). These antigens were
selected for further characterization based on their seroreactivity, predicted cell surface
localization and the relatively high ratio of predicted promiscuous T cell epitopes in the
proteins.

Clinical studies of coccidioidal disease as well as data from murine models of
coccidioidomycosis have consistently suggested that *Coccidioides* antigens which
stimulate a Th1 type of host immune response are essential for a vaccine to be effective
against this disease (6). The IFN-γ ELISPOT assays of immune CD90+ T cells of both
C57BL/6 and HLA-DR4 transgenic mice in the presence of rAmn1 and rPlb
demonstrated that these antigens stimulate a Th1 type recall response. Multiple peptides
containing promiscuous Amn1 and Plb epitopes were able to stimulate IFN-γ secretion in
both C57BL/6 and human MHC II transgenic mice. This observation suggests that an antigen containing multiple promiscuous epitopes will have an increased probability of stimulating a memory cell-mediated immune response in a population that expresses many different MHC class II alleles, a key factor in the development of an effective vaccine.

Both rAmn1 and rPlb, in preparations with the CpG ODN adjuvant, induced a significant protective immune response in C57BL/6 mice against a lethal pulmonary challenge with C. posadasii arthroconidia. This conclusion is supported by the observation of significantly increased levels of survival in the vaccinated mice at 90 days post-challenge. Vaccination with a 1 µg dose or 5 µg dose plus adjuvant did not significantly change the percentage of surviving mice at 90 days post challenge. We were able to reproduce this level of murine protection using the same vaccination protocol in three separate survival experiments. The choice of adjuvant appears to be imperative to finding a successful vaccine against coccidioidomycosis. A large number of vaccine studies, involving Coccidioides and other microbial pathogens, have demonstrated the advantage of co-administration of synthetic oligodeoxynucleotides (ODNs) with CpG motifs with recombinant antigens to enhance the levels of Th1-type cytokines in the lymphoid tissue (10, 26). Recent studies suggest that CpG ODN is a pivotal element of the vaccine preparation and induction of INF-γ and IL-12 production is required for the host to develop protective immunity to coccidoidal challenge (25, 33, 34). Therefore, a Th1 biased adjuvant must be further evaluated and licensed for human use if a subunit vaccine against coccidioidomycosis is to be considered.
A number of individual recombinant proteins have been studied as potential vaccine candidates against coccidioidomycosis. Although antigens such as Pep1, Gel1, Ag2/Pra and Ure have shown promise as single immunogens, none of these have conferred the levels of protective immunity killed whole spherule or complex multi-component extract vaccines have demonstrated (7, 10, 30, 31, 44, 47, 49). As a result, we believe that a recombinant subunit vaccine will most likely have to be composed of either multiple protective proteins that contain many promiscuous epitopes, or a recombinant chimeric protein constructed to contain protective epitopes of many antigens. To determine the protective efficacy of a combinatorial recombinant vaccine made up of multiple protective antigens, we tested the effects of a vaccine combination of rPep1, rAmn1 and rPlb plus CpG ODN in C57BL/6 mice against a potentially lethal pulmonary infection with *C. posadasii*. Results of the survival studies suggest that vaccination with the combination of proteins induces a potent and long-lasting cellular immune response against the pathogen. We observed that the percentage of surviving mice in the combination group was consistently higher than the single antigens alone in two separate experiments. Although the percentage of surviving mice in the combination group was improved, it was not statistically significant compared to the single antigens. We therefore examined clearance of the pathogen from lungs of the mice surviving to 90 days post-challenge. The majority of survivors in the triple combination group cleared the fungus from their lungs over the period post-challenge, and the numbers of CFU were significantly less than in the mice vaccinated with the single antigens alone. These data demonstrate that by combining multiple protective antigens in a vaccine, it is possible to improve the protective efficacy of the immunogenic reagent in our system. Although the
mechanisms by which vaccination with these antigens individually or combined influences the immune response to *C. posadasii* infection have yet to be explored, the improvement of protection afforded by combining these protective proteins plus CpG ODN adjuvant provokes the belief that the development of a polyvalent recombinant vaccine against coccidioidomycosis is a viable possibility. Further studies need to be performed to permit development of a polymeric vaccine against coccidioidomycosis, by looking at different concentrations and identities of protective antigens used, as well as to examine the mechanisms of the induced protective immunity. This study has established that an immunoproteomics and bioinformatics strategy is a successful approach to the production of an epitope-driven, polyvalent vaccine against coccidioidomycosis and lays a solid foundation for the future development of an effective human vaccine against coccidioidomycosis
REFERENCES


15. **Fisher, M. C., G. L. Koenig, T. J. White, and J. W. Taylor.** 2002. Molecular and phenotypic description of *Coccidioides posadasii* sp. nov., previously recognized as the non-California population of *Coccidioides immitis*. Mycologia **94**:73-84.


and Therapeutic Candidates for Systemic Candidiasis by Proteomic and Bioinformatic Analyses Mol Cell Proteomics 5:79-96.


FIG. 1. (A, B). Coomassie-stained gel (A) and immunoblot (B) of 2D-PAGE separation of the CWP fraction. Numbers correspond to protein spots identified by LC-MS/MS (see Table 1).
FIGURE 1

A. MW (kDa)

97 66 45 31 22

3 pH 10

138
FIG. 2.  (A, B).  SDS-PAGE gel separation and immunoblot (Iblt.) of *E. coli*-expressed rPlb (A) and rAmn1 (B). Shown are standards (Std.), lysates of bacteria transformed with the pET32b-*PLB* or pET32b-*Amn1* plasmid vector in the presence (+) or absence (-) of IPTG, and the nickel-affinity isolated rPlb or rAmn1. The immunoblots of the rPlb and rAmn1 isolated by nickel-affinity chromatography were incubated either with serum from a patient with confirmed coccidioidal infection, or serum from a healthy control patient.
FIGURE 2

A.  

Bacterial lysate (-IPTG)  Bacterial lysate (+IPTG)  Purified rPlb  Iblt.  Infected patient  Control

B.  

Bacterial lysate (-IPTG)  Bacterial lysate (+IPTG)  Purified rAmn1  Iblt.  Infected patient  Control

Std.  200  116  97  66  45  31  22  14
FIG. 3. Assessment of IFN-γ production by rAmn1 immune CD90\(^+\) T cells derived from C57BL/6 (A) or HLA-DR4 (DRB1\(^*\)0404) transgenic mice stimulated with homologous antigen and synthetic peptides by IFN-γ ELISPOT assays. Asterisks indicate statistically significant differences between response of T cells in presence of peptides versus medium alone. A representative result of three separate experiments is shown here.
FIGURE 3

A.

![Bar graph showing IFN-γ spots/well](image)

- **C57BL/6 mice**

- **Ex vivo stimulus**: Media, rAmn1, P1, P2, P3, P4, P5, P6, P7

- **Concentrations**: 1 µg/mL and 5 µg/mL

* * * P < 0.05 compared to media alone
FIGURE 3

B.

HLA-DR4 transgenic mice

IFN-γ spots/well

ex vivo stimulus*

1 μg/mL

5 μg/mL

* P < 0.05 compared to media alone

** P < 0.01 compared to media alone

* P < 0.05 compared to media alone

** P < 0.01 compared to media alone
FIG. 4. Assessment of IFN-γ production by rPlb immune CD90\(^+\) T cells derived from C57BL/6 (A) or HLA-DR4 (DRB1*0404) transgenic mice stimulated with homologous antigen and synthetic peptides by IFN-γ ELISPOT assays. Asterisks indicate statistically significant differences between response of T cells in presence of peptides versus medium alone. A representative result of three separate experiments is shown here.
FIGURE 4

A. C57BL/6 mice

*P < 0.05 compared to media alone
**FIGURE 4**

B. HLA-DR4 transgenic mice

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Media</th>
<th>P1b</th>
<th>P8</th>
<th>P9</th>
<th>P10</th>
<th>P11</th>
<th>P12</th>
<th>P13</th>
<th>P14</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><img src="image" alt="Bar Graph" /></td>
<td><img src="image" alt="Bar Graph" /></td>
<td><img src="image" alt="Bar Graph" /></td>
<td><img src="image" alt="Bar Graph" /></td>
<td><img src="image" alt="Bar Graph" /></td>
<td><img src="image" alt="Bar Graph" /></td>
<td><img src="image" alt="Bar Graph" /></td>
<td><img src="image" alt="Bar Graph" /></td>
<td><img src="image" alt="Bar Graph" /></td>
</tr>
</tbody>
</table>

*IFN-γ spots/well*

*ex vivo stimulus*

1 µg/mL 5 µg/mL

* P < 0.05 compared to media alone
FIG. 5. Representative comparison of the protective efficacy of rAmn1 (A) or rPlb (B) (1 µg or 5 µg) plus CpG ODN adjuvant used to immunize C57BL/6 mice, which were subsequently challenged by the i.n. route with a lethal inoculum of *C. posadasii*. Control mice were immunized with adjuvant alone. Statistical significance of the difference in survival plots of the vaccinated versus non-vaccinated mice is shown. The results are representative of three separate vaccination/survival experiments using the same immunization and challenge protocols.
FIGURE 5

A. 100
90
80
70
60
50
40
30
20
10
0

% Survival

0 10 20 30 40 50 60 70 80 90

Days post-challenge

* $P < 0.01$ compared to PBS

- rAmn1 (5 µg) + Adj.
- rAmn1 (1 µg) + Adj.
- PBS + Adj.
FIGURE 5

B.  

% Survival

Days post-challenge

* $P < 0.01$ compared to PBS
FIG. 6. (A) Representative comparison of the protective efficacy of rPep1, rPlb, rAmn1 (1 µg) and a polyvalent vaccine (rPep1, rPlb, rAmn1 1 µg each) plus CpG ODN adjuvant used to immunize C57BL/6 mice, which were subsequently challenged by the i.n. route with a lethal inoculum of *C. posadasii*. Control mice were immunized with adjuvant alone. Statistical significance (*P* values) of the difference in survival plots of the vaccinated versus non-vaccinated mice is shown. The results are representative of three separate vaccination/survival experiments using the same immunization and challenge protocols. (B) Plot of CFU of *C. posadasii* detected in dilution plate cultures of lung homogenates obtained from non-vaccinated C57BL/6 mice (PBS + Adj.) at 15 days post-challenge or surviving vaccinated C57BL/6 mice [rPep1, rPlb, or rAmn1 (1 µg) or polyvalent vaccine + Adj.] at 90 days post-challenge. The *P* values indicate a significant difference in the fungal burden of non-vaccinated mice at 15 days post-challenge versus vaccinated mice 90 days post-challenge. The *P* values also indicate a significant difference in the fungal burden of single antigen vaccinated mice at 90 days post-challenge versus polyvalent vaccinated mice 90 days post-challenge.
**FIGURE 6**

A. % Survival over time for different treatments:
- rPep1 (1 µg) + Adj.
- rPlb1 (1 µg) + Adj.
- rAmn1 (1 µg) + Adj.
- rPep1 + rPlb1 + rAmn1 (1 µg ea.) + Adj.
- PBS + Adj.

* * $p < 0.01$ compared to PBS
FIGURE 6

B. 8

<table>
<thead>
<tr>
<th></th>
<th>Days post-challenge</th>
<th>15</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFU in lungs (log_{10})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td></td>
<td>7.38</td>
<td>6.41</td>
</tr>
<tr>
<td>rPep1 (1 µg)</td>
<td></td>
<td>2.98 (2.17)</td>
<td>0.00 (1.30)</td>
</tr>
<tr>
<td>rAmn1 (1 µg)</td>
<td></td>
<td>3.73 *</td>
<td>0.00 (2.62)</td>
</tr>
<tr>
<td>rPlb (1 µg)</td>
<td></td>
<td>3.63 *</td>
<td>0.00 (0.00)</td>
</tr>
<tr>
<td>rPep1 + rAmn1 + rPlb (1 µg ea.)</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

* P < 0.01 compared to PBS

# P < 0.05 compared to single antigens
<table>
<thead>
<tr>
<th>Gel spot no.</th>
<th>Putative protein identification</th>
<th>Homolog-Accession No.</th>
<th>e value</th>
<th>Predicted MW (kDa) and pI</th>
<th>Predicted localization and GPI-anchor</th>
<th>Reported immunogenicity (ref.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Chitinase 1 (Cts1)</td>
<td>P54196</td>
<td>0.0</td>
<td>47.4 / 5.8</td>
<td>Cw / extracellular</td>
<td>+(7, 42)</td>
</tr>
<tr>
<td>2</td>
<td>Aspartyl proteinase (Pep1)</td>
<td>DQ164306</td>
<td>0.0</td>
<td>43.5 / 4.7</td>
<td>Cw / extracellular / GPI</td>
<td>+(47)</td>
</tr>
<tr>
<td>3</td>
<td>Alpha-mannosidase (Amn1)</td>
<td>DQ176863</td>
<td>0.0</td>
<td>56.9 / 4.8</td>
<td>Cw / extracellular</td>
<td>+(47)</td>
</tr>
<tr>
<td>4</td>
<td>Metalloproteinase 1 (Mep1)</td>
<td>AAQ07436</td>
<td>0.0</td>
<td>29.7 / 6.1</td>
<td>Cw / extracellular</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Endo-1,3-beta-glucanase</td>
<td>XP_750173</td>
<td>0.0</td>
<td>96.3 / 7.2</td>
<td>Cw / extracellular</td>
<td>+(16, 27, 43)</td>
</tr>
<tr>
<td>6</td>
<td>Endo-1,3-beta-glucanase</td>
<td>XP_750173</td>
<td>0.0</td>
<td>96.3 / 7.2</td>
<td>Cw / extracellular</td>
<td>+(16, 27, 43)</td>
</tr>
<tr>
<td>7</td>
<td>Carboxypeptidase Y</td>
<td>DQ176864</td>
<td>0.0</td>
<td>60.3 / 5.4</td>
<td>Cw / extracellular</td>
<td>+(47)</td>
</tr>
<tr>
<td>8</td>
<td>Exo-1,3-beta-D-glucanase</td>
<td>CAF32160</td>
<td>0.0</td>
<td>93.1 / 5.1</td>
<td>Cw / extracellular</td>
<td>+(17, 27)</td>
</tr>
<tr>
<td>9</td>
<td>Protein disulfide isomerase</td>
<td>AAL50638</td>
<td>e-130</td>
<td>57.3 / 4.8</td>
<td>Cw / extracellular</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Proline threonine-rich protein</td>
<td>DQ176865</td>
<td>0.0</td>
<td>29.0 / 5.3</td>
<td>Cw / extracellular</td>
<td>+(47)</td>
</tr>
<tr>
<td>11</td>
<td>Beta-glucosidase 5 (Bgl5)</td>
<td>AAL09829</td>
<td>0.0</td>
<td>56.7 / 6.0</td>
<td>Cw / extracellular</td>
<td>+(3)</td>
</tr>
<tr>
<td>12</td>
<td>Beta-glucosidase 2 (Bgl2)</td>
<td>AAF21242</td>
<td>0.0</td>
<td>92.8 / 5.2</td>
<td>Cw / extracellular</td>
<td>+(7, 21, 41)</td>
</tr>
<tr>
<td></td>
<td>Spherule outer wall glycoprotein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>(SOWgp)</td>
<td>AAL09436</td>
<td>0.0</td>
<td>46.3 / 4.4</td>
<td>Cw / extracellular / GPI</td>
<td>+(20)</td>
</tr>
<tr>
<td>14</td>
<td>Phospholipase B (Plb)</td>
<td>DQ188099</td>
<td>0.0</td>
<td>68.6 / 6.4</td>
<td>Cw / extracellular / GPI</td>
<td>+(13, 47)</td>
</tr>
<tr>
<td>15</td>
<td>Beta-glucosidase 4 (Bgl4)</td>
<td>AAL09828</td>
<td>0.0</td>
<td>32.9 / 6.5</td>
<td>Cw / ER</td>
<td>+(3)</td>
</tr>
<tr>
<td>16</td>
<td>Gamma-glutamyltranspeptidase 1</td>
<td>XP_749034</td>
<td>0.0</td>
<td>65.2 / 5.2</td>
<td>Extracellular</td>
<td>+(36)</td>
</tr>
<tr>
<td></td>
<td>Protein Name</td>
<td>Accession</td>
<td>Log2FoldChange</td>
<td>p-value</td>
<td>Location</td>
<td>Fractional Abundance</td>
</tr>
<tr>
<td>---</td>
<td>------------------------------------</td>
<td>---------------</td>
<td>----------------</td>
<td>----------</td>
<td>----------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>17</td>
<td>Porin</td>
<td>XP_662006</td>
<td>e-137</td>
<td>30.0 / 8.8</td>
<td>Cell membrane</td>
<td>+(40)</td>
</tr>
<tr>
<td>18</td>
<td>Elongation factor 2</td>
<td>XP_755686</td>
<td>0.0</td>
<td>93.5 / 6.4</td>
<td>Cytosolic</td>
<td>+(40)</td>
</tr>
<tr>
<td>19</td>
<td>Elongation factor 2</td>
<td>XP_755686</td>
<td>0.0</td>
<td>93.5 / 6.4</td>
<td>Cytosolic</td>
<td>+(40)</td>
</tr>
<tr>
<td>20</td>
<td>Elongation factor 2</td>
<td>XP_755686</td>
<td>0.0</td>
<td>93.5 / 6.4</td>
<td>Cytosolic</td>
<td>+(40)</td>
</tr>
<tr>
<td>21</td>
<td>Alcohol dehydrogenase I</td>
<td>XP_746830</td>
<td>e-134</td>
<td>38.0 / 7.9</td>
<td>Cytosolic</td>
<td>+(8, 40)</td>
</tr>
<tr>
<td>22</td>
<td>Enolase</td>
<td>XP_663350</td>
<td>0.0</td>
<td>47.3 / 5.5</td>
<td>Cytosolic</td>
<td>+(40) (41)</td>
</tr>
<tr>
<td>23</td>
<td>Fructose biphosphate aldolase</td>
<td>XP_754452</td>
<td>0.0</td>
<td>39.7 / 5.6</td>
<td>Cytosolic</td>
<td>+(11, 40, 41)</td>
</tr>
<tr>
<td>24</td>
<td>Citrate synthase</td>
<td>XP_747920</td>
<td>0.0</td>
<td>52.0 / 8.9</td>
<td>Cytosolic</td>
<td>+(40)</td>
</tr>
<tr>
<td>25</td>
<td>Citrate synthase</td>
<td>XP_747920</td>
<td>0.0</td>
<td>52.0 / 8.9</td>
<td>Cytosolic</td>
<td>+(40)</td>
</tr>
<tr>
<td>26</td>
<td>Malate dehydrogenase</td>
<td>AAP37966</td>
<td>e-160</td>
<td>36.0 / 9.2</td>
<td>Cytosolic</td>
<td>+(40)</td>
</tr>
<tr>
<td>27</td>
<td>Malate dehydrogenase</td>
<td>AAP37966</td>
<td>e-160</td>
<td>36.0 / 9.2</td>
<td>Cytosolic</td>
<td>+(40)</td>
</tr>
<tr>
<td>28</td>
<td>Malate dehydrogenase</td>
<td>AAP37966</td>
<td>e-160</td>
<td>36.0 / 9.2</td>
<td>Cytosolic</td>
<td>+(40)</td>
</tr>
<tr>
<td>29</td>
<td>3-Hydroxybutyryl CoA dehydrogenase</td>
<td>XP_746626</td>
<td>e-121</td>
<td>34.1 / 8.9</td>
<td>Mitochondria</td>
<td>+(40)</td>
</tr>
<tr>
<td>30</td>
<td>70 kDa heat shock protein</td>
<td>AAQ83701</td>
<td>0.0</td>
<td>73.2 / 5.7</td>
<td>Mitochondria</td>
<td>+(40)</td>
</tr>
<tr>
<td>31</td>
<td>Aconitase</td>
<td>XP_664125</td>
<td>0.0</td>
<td>84.9 / 7.6</td>
<td>Mitochondria</td>
<td>+(40)</td>
</tr>
<tr>
<td>32</td>
<td>Aconitase</td>
<td>XP_664125</td>
<td>0.0</td>
<td>84.9 / 7.6</td>
<td>Mitochondria</td>
<td>+(40)</td>
</tr>
<tr>
<td>33</td>
<td>Aconitase</td>
<td>XP_664125</td>
<td>0.0</td>
<td>84.9 / 7.6</td>
<td>Mitochondria</td>
<td>+(40)</td>
</tr>
<tr>
<td>34</td>
<td>Aconitase</td>
<td>XP_664125</td>
<td>0.0</td>
<td>84.9 / 7.6</td>
<td>Mitochondria</td>
<td>+(40)</td>
</tr>
<tr>
<td>35</td>
<td>ATP synthase beta chain</td>
<td>XP_753589</td>
<td>0.0</td>
<td>55.9 / 5.2</td>
<td>Mitochondria</td>
<td>+(40)</td>
</tr>
<tr>
<td></td>
<td>Protein Name</td>
<td>Accession</td>
<td>Score</td>
<td>Stdev</td>
<td>Location</td>
<td>Notes</td>
</tr>
<tr>
<td>---</td>
<td>-----------------------------------------------------</td>
<td>-----------</td>
<td>-------</td>
<td>-------</td>
<td>------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>36</td>
<td>Dihydrolipoamide dehydrogenase</td>
<td>XP_749345</td>
<td>0.0</td>
<td>55.2</td>
<td>Mitochondria</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Electron transfer flavoprotein alpha-subunit</td>
<td>XP_748962</td>
<td>e-130</td>
<td>36.6</td>
<td>Mitochondria</td>
<td>-</td>
</tr>
<tr>
<td>37</td>
<td>Electron transfer flavoprotein alpha-subunit</td>
<td>XP_748962</td>
<td>e-130</td>
<td>36.6</td>
<td>Mitochondria</td>
<td>-</td>
</tr>
<tr>
<td>38</td>
<td>Heat shock protein 60 (HSP60)</td>
<td>AAD00521</td>
<td>0.0</td>
<td>62.5</td>
<td>Mitochondria</td>
<td>+(9, 40)-</td>
</tr>
<tr>
<td>40</td>
<td>Ketol-acid reductoisomerase</td>
<td>XP_754177</td>
<td>0.0</td>
<td>44.8</td>
<td>Mitochondria</td>
<td>+(40)</td>
</tr>
<tr>
<td></td>
<td>Mitochondrial processing peptidase</td>
<td>CAE47911</td>
<td>0.0</td>
<td>52.9</td>
<td>Mitochondria</td>
<td>-</td>
</tr>
<tr>
<td>41</td>
<td>NADH-ubiquinone oxidoreductase</td>
<td>CAE47911</td>
<td>e-112</td>
<td>52.9</td>
<td>Mitochondria</td>
<td>-</td>
</tr>
<tr>
<td>42</td>
<td>NADH-ubiquinone oxidoreductase subunit</td>
<td>BAE57062</td>
<td>0.0</td>
<td>33.0</td>
<td>Mitochondria</td>
<td>-</td>
</tr>
<tr>
<td>43</td>
<td>Succinate dehydrogenase</td>
<td>BAE63604</td>
<td>0.0</td>
<td>71.1</td>
<td>Mitochondria</td>
<td>-</td>
</tr>
<tr>
<td>44</td>
<td>Processing enhancing protein precursor</td>
<td>AAA33606</td>
<td>0.0</td>
<td>47.8</td>
<td>Mitochondria</td>
<td>-</td>
</tr>
</tbody>
</table>

*a* Protein identity based on homology to reported fungal proteins determined by BLAST searches (1)

*b* Based on amino acid sequence analysis using ExPASy Proteomic Server (http://us.expasy.org/).

*c* Based on amino acid sequence analysis using WoLF PSORT II and GPI-SOM algorithms; Cw, cell wall-associated.

*d* Based on literature search of *Coccidioides* proteins or homologs in other microbial systems.
<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Seroreactive protein</th>
<th>GenBank accession no</th>
<th>Number of predicted promiscuous T-cell reactive epitopes</th>
<th>Ratio of ProPred predicted promiscuous epitopes to protein molecular weight (KDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Aspartyl proteinase (Pep1)</td>
<td>DQ164306</td>
<td>5</td>
<td>0.115</td>
</tr>
<tr>
<td>14</td>
<td>Phospholipase B (Plb)</td>
<td>DQ188099</td>
<td>7</td>
<td>0.107</td>
</tr>
<tr>
<td>3</td>
<td>Alpha-mannosidase (Amn1)</td>
<td>DQ176863</td>
<td>6</td>
<td>0.105</td>
</tr>
<tr>
<td>1</td>
<td>Chitinase 1 (Cts1)</td>
<td>P54196</td>
<td>5</td>
<td>0.104</td>
</tr>
<tr>
<td>15</td>
<td>Beta-glucosidase 4 (Bgl4)</td>
<td>AAL09828</td>
<td>3</td>
<td>0.091</td>
</tr>
<tr>
<td>8</td>
<td>Exo-1,3-beta-D-glucanase</td>
<td>CAF32160</td>
<td>8</td>
<td>0.085</td>
</tr>
<tr>
<td>5,6</td>
<td>Endo-1,3-beta-glucanase</td>
<td>XP_750173</td>
<td>8</td>
<td>0.083</td>
</tr>
<tr>
<td>7</td>
<td>Carboxypeptidase Y</td>
<td>DQ176864</td>
<td>5</td>
<td>0.083</td>
</tr>
<tr>
<td>12</td>
<td>Beta-glucosidase 2 (Bgl2)</td>
<td>AAF21242</td>
<td>5</td>
<td>0.054</td>
</tr>
<tr>
<td>11</td>
<td>Beta-glucosidase 5 (Bgl5)</td>
<td>AAL09829</td>
<td>3</td>
<td>0.053</td>
</tr>
<tr>
<td>9</td>
<td>Protein disulfide isomerase</td>
<td>AAL50638</td>
<td>2</td>
<td>0.035</td>
</tr>
<tr>
<td>13</td>
<td>Spherule outer wall glycoprotein (SOWgp)</td>
<td>AAL09436</td>
<td>1</td>
<td>0.021</td>
</tr>
<tr>
<td>4</td>
<td>Metalloproteinase 1 (Mep1)</td>
<td>AAQ07436</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>Proline threonine-rich protein</td>
<td>DQ176865</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*a* Protein identity based on homology to reported fungal proteins determined by BLAST searches (1)

*b* Based on amino acid sequence using the ProPred algorithm. A promiscuous epitope is predicted to be presented by at least 80% of the HLA alleles in the ProPred algorithm. (45)

*c* Calculated by dividing the number of predicted promiscuous epitopes by the predicted protein molecular weight (KDa).
TABLE 3. Amino acid sequences of synthetic peptides selected from ProPred prediction of Amn1 and Plb ligands which bind to human MHC class II molecules

<table>
<thead>
<tr>
<th>Peptide no.</th>
<th>Peptide sequence</th>
<th>Amino acid nos.</th>
<th>% of MHCII alleles predicted to bind^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amn1 P1</td>
<td>LFETTIRYLGGMISAYDLLK</td>
<td>125-144</td>
<td>100</td>
</tr>
<tr>
<td>Amn1 P2</td>
<td>PAKVDVLLAQSLKLADVLKF</td>
<td>154-173</td>
<td>100</td>
</tr>
<tr>
<td>Amn1 P3</td>
<td>NGLATTGTLVLWTRLSDIT</td>
<td>199-218</td>
<td>100</td>
</tr>
<tr>
<td>Amn1 P4</td>
<td>DSFYEYLIKMYVYDGRFGK</td>
<td>272-291</td>
<td>82</td>
</tr>
<tr>
<td>Amn1 P5</td>
<td>YYNLPVEVIESIYYAAYMTK</td>
<td>408-427</td>
<td>92</td>
</tr>
<tr>
<td>Amn1 P6</td>
<td>ESFLFAEMKYSYLIHSEPA</td>
<td>471-490</td>
<td>98</td>
</tr>
<tr>
<td>Amn1 P7</td>
<td>FTAIGDVNTPDGRKYDQNE</td>
<td>452-471</td>
<td>0^b</td>
</tr>
<tr>
<td>Plb P8</td>
<td>AIPLDSNVHIRALPNAPNGY</td>
<td>25-44</td>
<td>90</td>
</tr>
<tr>
<td>Plb P9</td>
<td>TFLDVLRYYAQLQSAVAGKQ</td>
<td>208-227</td>
<td>96</td>
</tr>
<tr>
<td>Plb P10</td>
<td>PTIFGFVPLEYLSKFGGV</td>
<td>309-328</td>
<td>88</td>
</tr>
<tr>
<td>Plb P11</td>
<td>PDVNTFVNLGLNTRPTFFGC</td>
<td>480-499</td>
<td>92</td>
</tr>
<tr>
<td>Plb P12</td>
<td>TPLVVYIPNYPYTWSNIST</td>
<td>508-527</td>
<td>90</td>
</tr>
<tr>
<td>Plb P13</td>
<td>ARIRASPSKHSVVVFVVL</td>
<td>621-640</td>
<td>100</td>
</tr>
<tr>
<td>Plb P14</td>
<td>PTCVGCAILSRFSERTGIAM</td>
<td>560-579</td>
<td>2^b</td>
</tr>
</tbody>
</table>

^a Percentage of the 51 human MHCII alleles listed in the ProPred algorithm (http://www.imtech.res.in/raghava/propred/) the given peptide is predicted to bind at a threshold setting of 5%

^b Peptide predicted by ProPred algorithm not to bind to human MHC class II molecules served as a control in the IFN-\(\gamma\) ELISPOT assay of immune T cells obtained from C57BL/6 and HLA-DR4 transgenic mice
SUMMARY AND DISCUSSION

Previous experimental work in the field of *Coccidioides* vaccinology, as well as clinical data from human infections, has suggested that development of a vaccine against coccidioidomycosis is feasible. The literature suggests that an ideal coccidioidal vaccine should be cost effective to produce, physically stable and contain an adequate number of essential protective epitopes to induce long-lasting immunity against infection (Cole et al., 2004; Cox and Magee, 2004). Because of the importance of cell mediated T cell immunity in controlling coccidioidal infection, recent studies in search for candidate vaccines against coccidioidomycosis have focused on individual cell wall or secreted T-cell-reactive proteins that can be recombinantly expressed and purified, and that will confer some degree of immunoprotection in a murine pulmonary model of infection (Delgado et al., 2003; Shubitz et al., 2002). Although many of these individual antigens have shown remarkable protection in the murine model of disease, single protein vaccines have not been able to confer the levels of immunodefense observed by immunization with whole cell or complex protein extracts (Awasthi et al., 2004; Cox and Magee, 2004). We believe the reason for the deficiency of total sterilizing immunity with single antigen vaccines is that they are lacking some of the essential epitopes needed for the host to overcome the high reproductive capacity of this pathogen, which are provided by the heterogeneous antigenic cell-based vaccines (Doolan et al., 2003; Henics and Nagy, 2005; Huppert et al., 1982; Mollenkopf et al., 2004; Resnick et al., 1987). Therefore, it is likely that a protective recombinant protein subunit human vaccine...
against *Coccidioides* must contain genetically unrestricted (“promiscuous”) protective T cell epitopes, and will most likely be multivalent in nature.

This dissertation describes studies that assess the hypothesis that there are immunogenic cell wall and secreted parasitic phase antigens in the immunome of *C. posadasii*, with epitopes that can elicit a protective immune response and can be used to develop a protective multivalent vaccine against coccidioidomycosis. We tested this hypothesis using a combined immunoproteomics and bioinformatics approach to map and identify immunogenic proteins in the cell wall and secreted fractions, as well as to predict which antigens have promiscuous MHC II restricted T-cell epitopes. These analyses were followed by testing recombinant forms of the proteins for T cell reactivity and protective efficacy using in vitro immunoassays and an animal model of *Coccidioides* infection.

In manuscript 1, we identified the major seroreactive proteins in a protective TritonX-114 detergent phase cell wall protein extract as well as parasitic phase culture filtrates of *C. posadasii*, and assess these antigens for the presence of predicted promiscuous MHC II restricted T-cell epitopes using a bioinformatic MHC class II epitope prediction algorithm (ProPred) (Singh and Raghava, 2001). A predominant seroreactive protein in both the detergent cell wall-extract and culture filtrate was sequenced by tandem mass-spectrometry and identified as a homolog of an *Aspergillus fumigatus* aspartyl protease protein. ProPred analysis of the aspartyl protease protein (Pep1) showed that it contained a relatively high number of predicted promiscuous MHC class II epitopes. The Pep1 protein was recombinantly expressed in *E. coli* and purified by nickel affinity chromatography. The recombinant protein was shown to be reactive
with coccidioidomycosis patient sera, reactive with immune T cells from C57BL/6 mice and induce a predominantly Th1 biased recall response in vitro when administered with CpG-ODN and IFA. The full length Pep1 and synthetic peptides containing the predicted promiscuous T-cell epitopes were demonstrated to induce IFN-γ production in immune T cells in human MHC II transgenic mice and C57BL/6 mice by IFN-γ ELISPOT assays. This suggested that the ProPred prediction was able to identify T cell reactive epitopes in the amino acid sequence of Pep1. The protective efficacy of Pep1, a T cell and B cell reactive antigen containing predicted promiscuous epitopes, was tested in a murine (C57BL/6) model of coccidioidomycosis. Both 5 µg and 1 µg doses of rPep1 along with CpG-ODN and IFA provided a significant degree of protection as determined by percent of surviving mice 90 d post i.n. infection, and clearance of the pathogen in the lungs over this same period. This manuscript was the first publication in which proteomics and bioinformatics were used to identify a coccidioidomycosis vaccine candidate.

In manuscript 2, we continued to explore the C. posadasii immunome for possible vaccine candidates with the goal of identifying proteins that could be considered components of a multivalent subunit vaccine. This study centered on the mapping and identification of the major seroreactive proteins in 2D-PAGE separations of extracted parasitic phase cell wall proteins by tandem-mass spectrometry. We were able to identify 34 unique seroreactive proteins, half of which were predicted by the WoLF PSORT algorithm to be extracellular and/or cell wall associated. The predicted extracellular proteins were assessed for the presence of predicted genetically unrestricted MHC class II T-cell epitopes once again using the ProPred algorithm. The analyzed proteins were ranked based on the ratio of their number of predicted promiscuous epitopes to their
predicted molecular weight. This was done to normalize the relative number of epitopes in each protein regardless of their molecular weight. Interestingly, the protective Pep1 was shown to have the highest ratio of epitopes to molecular weight while previously characterized non-protective SOWgp had one of the lowest ratios. The two proteins with the highest predicted promiscuous epitope to molecular weight ratio after Pep1 were a putative phospholipase B protein (Plb) and a predicted alpha-mannosidase protein (Amn1). These proteins were recombinantly expressed in *E. coli* and purified by nickel affinity chromatography and they were shown to be reactive with IgG-specific antibody from patients with confirmed coccidioidomycosis. The full-length rPlb and rAmn1, as well as synthetic peptides containing the predicted promiscuous T-cell epitopes, were demonstrated to induce IFN-γ production in immune T cells in human MHC II transgenic mice and C57BL/6 mice by IFN-γ ELISPOT assays. Based on their predicted cellular localization, seroreactivity, T cell reactivity and high number of predicted genetically unrestricted MHC class II epitopes, we decided to test the protective efficacy of rPlb and rAmn1, in a murine (C57BL/6) model of coccidioidomycosis. Both 5 µg and 1 µg doses of rPlb and rAmn1 along with CpG-ODN and IFA adjuvant provided a significant degree of protection as determined by percent of surviving mice 90 d post i.n. infection. In an effort to determine if a multivalent vaccine composed of multiple recombinant proteins with a number of confirmed T cell epitopes would enhance protection compared to the single antigens alone, we tested a combination of the three proteins with the highest ratio of predicted MHC class II promiscuous epitopes to molecular weight in a the C57BL/6 model of disease. A combination of rPep1, rPlb, and rAmn1 (1 µg each) with CpG-ODN and IFA showed an increased survival compared to each of the single proteins at this
dose, and more noteworthy, showed a significant reduction of fungal burden in the lungs of the survivors at 90 d post-challenge compared to the single antigens alone. This manuscript once again demonstrates the value of an immunoproteomics and bioinformatics approach to vaccine development and also represents the most comprehensive catalog of *C. posadasii* antigenic proteins identified to date, providing researchers a valuable resource for further studies. This manuscript also offers further evidence that use of a defined multivalent vaccine against coccidioidomycosis is a rational approach to optimization of protective efficacy and approaches the sterilizing immunity observed in mice vaccinated with whole cell or undefined subcellular fractionated vaccines.

The studies presented in this dissertation have demonstrated that an immunoproteomics and bioinformatics strategy is an efficient and productive way to identify both B cell and T cell reactive vaccine candidates. The extraction and identification of a diverse protein component of immunoreactive antigens in the parasitic cell wall and parasitic phase culture filtrate provided a wide profile of antigenic proteins that could possible be included in a polyvalent vaccine. While patient seroreactivity and parasitic cell wall association suggested that most of these proteins were worthy of further examination, we used another criterion for selection of antigens for further evaluation as vaccine candidates: the presence of genetically unrestricted MHC class II restricted (promiscuous) epitopes in the protein amino acid sequence (Yoneda and Ellner, 1998). These studies were the first to consider the use of proteins containing predicted promiscuous T cell epitopes for a coccidioidal vaccine. Our argument for using an approach centered around proteins that contain predicted promiscuous T cell epitopes is
that there has been an enormous amount of effort and money spent unsuccessfully on the
development of a human vaccine for coccidioidomycosis focused on proteins that show
protection in a genetically restricted population of inbred mice that have a limited range
of MHC class II allelic expression. The vaccine candidates presented in these studies
have been shown to contain epitopes that can be presented by MHC class II molecules
not only in an inbred mouse population, but also in a transgenic mouse strain expressing a
human MHC class II protein. The transgenic mice used in these studies express the
human MHC class II DRB1*0404 (HLA DR4) allele on their antigen presenting cells,
and lack expression of the endogenous murine MHC II complexes. These candidates
were able to activate antigen specific T cells in both the inbred and HLA DR4 transgenic
mouse strains and are predicted to contain epitopes that can be presented in human
population expressing a wide range of MHC class II alleles. The expectation is a
multivalent vaccine such as this will stimulate a protective adaptive memory immune
response in the entire post-vaccinated population, as a result of having multiple
promiscuous T cell epitopes.

Clinical studies of coccidioidal disease as well as data from murine models of
coccidioidomycosis have consistently suggested that Coccidioides antigens which
stimulate a Th1 type of host immune response are essential for a vaccine to be effective
against disease (Cox and Magee, 1998). The IFN-\(\gamma\) ELISPOT assays of immune CD90\(^+\)
T cells of both C57BL/6 and HLA-DR4 transgenic mice in the presence of rPep1, rAmn1
and rPlb demonstrate that these antigens stimulate a strong Th1 type recall response. We
found that multiple peptides containing predicted promiscuous Pep1, Amn1 and Plb
epitopes were able to stimulate IFN-\(\gamma\) secretion in both C57BL/6 and human MHC II
transgenic mice as well. This suggests that these antigens will have an increased probability of stimulating a protective memory cell-mediated immune response in a population that expresses many different MHC class II alleles, which as suggested above, is a key factor in the development of an robust and durable immune response to infection.

rPep1, rAmn1 and rPlb, in preparation with the CpG-ODN adjuvant and IFA, induced a significant protective immune response in C57BL/6 mice against a lethal pulmonary challenge with *C. posadasii* arthroconidia. Vaccination with a 1 µg dose or 5 µg dose plus adjuvant did not significantly change the percentage of surviving mice at 90 d post-challenge. The demonstration of protection in this murine model of disease, while limited, gives hope that some of the epitopes in these vaccine candidates can stimulate a protective immune response, and also suggests that these antigens are available to the host during critical points of the infectious process. These data reveal that extracellularly expressed proteins have an increased viability as potential vaccine candidates and suggest that cellular localization may be a critical factor in choosing future vaccine proteins.

The choice of adjuvant is imperative to finding a successful vaccine against coccidioidomycosis. Currently, only Th2 biased adjuvants such as alum are approved for human use. Therefore, the *Coccidioides* vaccine research community has to push for further evaluation and licensing of a Th1 biased adjuvant such as CpG-ODN for human use if a subunit vaccine against coccidioidomycosis is to be considered. A large number of vaccine studies, for *Coccidioides* and other microbial pathogens, have demonstrated the advantage of co-administration of synthetic oligodeoxynucleotides (ODNs) with CpG motifs with recombinant antigens to enhance the levels of Th1-type cytokines in the
lymphoid tissue (Delgado et al., 2003; Klinman, 2004; Li et al., 2001). Administration of CpG-ODN with the vaccine preparation has been demonstrated to be pivotal in the induction of INF-γ and IL-12 that is required for the host to develop protective immunity against coccidioidal challenge (Kirkland et al., 2006; Magee and Cox, 1995, 1996). The need for a FDA approved Th1 biased vaccine for human use is desperately needed, and its use with a subunit protein vaccine may be the only way for the vaccine to provide a high degree of protective efficacy in humans.

As previously stated, a number of individual recombinant proteins have been studied as potential vaccine candidates against coccidioidomycosis. Although antigens such as Pep1, Gel1, Ag2/Pra and Ure have shown promise as single immunogens, none of these have conferred the levels of protective immunity killed whole cell or complex multi-component extract vaccines have demonstrated (Cox et al., 1992; Delgado et al., 2003; Levine et al., 1960; Li et al., 2001; Shubitz et al., 2002; Tarcha et al., 2006; Zimmermann et al., 1998). As a result, we believe a recombinant subunit vaccine will most likely have to be composed of either multiple protective proteins that contain many promiscuous epitopes, or a recombinant chimeric protein constructed to contain protective epitopes of many antigens. These experiments suggest that vaccination with the multivalent vaccine induces a potent and long-lasting cellular immune response against the pathogen, and demonstrates that by combining multiple protective antigens in a vaccine, it is possible to improve its protective efficacy. While the majority of survivors in the triple combination group cleared the fungus from their lungs over the period post-challenge, there were some mice that still had residual infection suggesting that there is still improvements that can be made on the vaccine. The lack of total
sterilizing immunity can be attributed to a number of issues. First, although combining multiple protective proteins in the vaccine improves the protective efficacy, there may be important epitopes missing from the profile provided by the three antigens used (Mustafa, 2002; Parra-Lopez et al., 2006). Another possibility is that there we are missing a protective antigen in the vaccine composition that is available to the host at an important point of infection that allows the fungus to escape sterilization. Further, the dosage of the antigens used may not be adequate for stimulation of a protective memory response to overcome the reproductive capacity of the fungus in vivo (Deepe et al., 2005). All these issues, and the mechanisms by which vaccination with these antigens individually or in combination influences the immune response to *C. posadasii* infection have yet to be explored.

Further studies need to be performed to develop a polymeric vaccine against coccidioidomycosis before a vaccine such as this should be considered for clinical trials. Additional studies exploring which protective antigens should be components of the vaccine, as well as examination of the mechanisms of the induced protective immunity will provide a great deal of information that can be applied to the vaccine development process. More immunoreactive proteins need to be identified and tested for protective efficacy. These single antigens as well as the multivalent vaccines need to be tested not only in inbred mouse strains, but also in out-bred mice. This would provide greater insight into how effective these vaccines would be in a diverse human population. We believe that the use of proteomics and bioinformatics in fungal vaccinology offers an opportunity for the efficient discovery of safe, efficacious, and novel subunit vaccines. These studies are the first to explore the immunome of *Coccidioides* for new vaccine
candidates, and have resulted in the identification of proteins which may comprise the backbone of an effective vaccine against coccidioidomycosis. The improvement of protection afforded by combining these protective proteins plus CpG-ODN adjuvant provokes the belief that the development of an epitope-driven multivalent recombinant vaccine against coccidioidomycosis is a viable possibility, and provides a solid foundation for the future development of an effective human vaccine against coccidioidomycosis.
REFERENCES


Converse, J. L. (1957). Effect of surface active agents on endosporulation of


Converse, J. L., and Besemer, A. R. (1959). Nutrition of the parasitic phase of

*Coccidioides immitis* in a chemically defined liquid medium. J Bacteriol 78, 231-239.


Cytokine production by peripheral blood mononuclear cells in human coccidioidomycosis. JID 174, 440-443.


immitis: homology to 4-hydroxyphenylpyruvate dioxygenase and the mammalian F antigen. Gene 161, 107-111.


Coccidioides immitis antigen 2 domain that expresses B-cell-reactive epitopes.
Infect Immun 65, 3376-3380.


characterization of Coccidioides immitis antigen 2 cDNA. Infect Immun 64, 2695-
2699.

Zimmermann, C. R., Johnson, S. M., Martens, G. W., White, A. G., Zimmer, B. L., and
ABSTRACT

*Coccidioides* is a primary fungal pathogen endemic to the alkaline desert soil of the Southwestern United States and the etiological agent of coccidioidomycosis (Valley fever), a respiratory disease in humans. *Coccidioides* represents the only fungal pathogen on the Center for Disease Control’s select agent list of possible weapons of bioterrorism, and can cause significant morbidity in infected individuals. Thus, the need for a human vaccine against coccidioidomycosis has come to the forefront. Work has centered on identification and characterization of recombinant T cell-reactive antigens, because clinical and experimental data suggest that activation of a durable MHC II restricted T cell Th1 immune response is of principal importance in establishing durable protective immunity. Immunoprotection experiments in mice using single recombinant vaccine proteins of *Coccidioides* have resulted in less than optimal survival and clearance of the fungus from the infected host compared to whole cell or multicomponent subcellular vaccines, indicating a lack of protective epitopes in the single protein vaccines. Therefore, it is likely that a protective recombinant protein subunit human vaccine against *Coccidioides* will contain genetically unrestricted (“promiscuous”) protective T cell epitopes, and will most likely be multivalent in nature. In this study we describe an immunoproteomic and bioinformatic approach for profiling a diverse immunogenic protein component of the coccidoidal parasitic cell wall. A phospholipase B (Plb), alpha-mannosidase (Amn1), and an aspartyl protease (Pep1) were selected as candidate vaccine proteins on the basis of their immunogenicity, cellular localization, predicted promiscuous T cell epitope content, and T cell reactivity. These antigens were evaluated individually, and in combination, for their protective efficacy in a pulmonary murine
model of infection. Each individual protein showed significant protection in infected mice as evaluated by survival after lethal challenge (53%-61% survival). A combinatorial vaccine composed of all three protective antigens enhanced survival in infected mice (86% survival) and significantly improved clearance of the pathogen from lungs of surviving mice by 90 d post-challenge. This strategy has been successful in producing the most comprehensive profile of immunogenic coccidioidal cell wall antigens to date, and lays the groundwork for the development of an epitope-driven, multivalent human vaccine against coccidioidomycosis.