Evaluation of Coccidioides posadasii antigens as recombinantly expressed monovalent, divalent, and chimeric vaccine candidates

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Evaluation of Two Homologous Coccidioides Posadasii Antigens as Recombinantly Expressed Monovalent, Divalent, and Chimeric Vaccine Candidates

Submitted by

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

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INTRODUCTION

Coccidioidomycosis (San Joaquin Valley fever) is caused by the dimorphic fungal respiratory pathogens, *Coccidioides immitis* and *Coccidioides posadasii* (Drutz and Catanzaro, 1978a; Fisher et al., 2002; Pappagianis, 1993a; Smith, 1940). *Coccidioides* spp. grow saprobically in the dry, alkaline soils found in the desert regions of North America, Mexico, and scattered areas of South America (Maddy, 1958; Pappagianis, 1988). Humans typically acquire coccidioidomycosis by inhalation of fungal arthroconidia that become aerosolized by the disruption of infested soil in endemic areas. Once inhaled, arthroconidia initiate a parasitic life cycle that is unique among the medically-important fungi.

*Coccidioides* spp. are formidable pathogens capable of causing serious pulmonary and sometimes fatal disseminated disease in immunocompetent people visiting or living in endemic regions. The clinical manifestations of coccidioidomycosis range from asymptomatic, primary respiratory disease (usually with uncomplicated resolution), persistent pulmonary or chronic progressive pulmonary disease, and in rare instances life threatening extrapulmonary dissemination (Pappagianis, 1988; Smith et al., 1946, 1961). Interestingly, individuals who develop self-limiting primary infection and recover are resistant to future encounters with *Coccidioides* (Pappagianis, 2001; Smith et al., 1946; Smith et al., 1961). Based on data from skin test surveys, it is believed that 25,000 to 100,000 infections are acquired annually in the United States alone (Drutz and Huppert, 1983; Pappagianis, 1980). The number of individuals who become infected with *Coccidioides* each year is influenced by the number of immunologically-naïve individuals relocating or visiting endemic areas, and climactic conditions such as the
length and timing of droughts and precipitation (Kolivras and Comrie, 2003; Kolivras et al., 2001). Recurrent epidemics combined with dramatic increases in tourism and migration of permanent residents to the Southwestern United States over the past 50 years has necessitated the classification of coccidioidomycosis as a re-emerging infectious disease (Kirkland and Fierer, 1996).

Due to the potential seriousness of the disease, combined with the length and expense of the treatment regimens, the cost of treatment for patients who acquire coccidioidomycosis can be very high. The annual per-patient cost, based on a retrospective study of 536 cases conducted between 1991 and 1993 at the Kern Medical Center in Bakersfield, California, was estimated at $8,096 (Caldwell et al., 1996). Non-disseminated cases averaged $5,400/yr, while patients who contracted the disseminated form of the disease faced an average cost of $48,000. Current costs incurred by patients with severe coccidioidal infection can climb to $300,000, and it is estimated that the annual cost of health care for management of patients with coccidioidomycosis in the United States is 60 million dollars (www.valleyfever.com). Based on the cost of patient treatment, geographic restriction of the disease, and evidence that natural coccidioidal infection provides lifelong resistance to re-infection, it has been suggested that the development of coccidioidal vaccine is justified (Cole et al., 2004; Cox and Magee, 2004).

Early *Coccidioides* vaccine development studies focused on the evaluation of viable (attenuated) and non-viable whole cell vaccines. Several of these studies demonstrated that immunization of mice with an attenuated strain of the pathogen provided protection against a lethal intranasal challenge with *Coccidioides* (Converse, 1965; Levine et al.,
1961). However, due to the persistence of viable organisms at the site of immunization and in some cases at other distal locations, a non-viable immunization strategy using formalin killed spherule (FKS) was adopted (Converse et al., 1963; Kong and Levine, 1967a). The efficacy of the FKS vaccine in both murine and non-human primate models of coccidioidomycosis prompted clinical trials in humans (Levine et al., 1962, 1965). Unfortunately, this study revealed no significant difference in the incidence or severity of coccidioidal cases between groups of individuals receiving the FKS vaccine or the placebo (Pappagianis, 1993b). Presumably this reflected, at least in part, the fact that only 1/1000\textsuperscript{th} of the dose per kilogram of body weight that was protective in mice was tolerated in humans (Ampel, 1994; Slagle et al., 1989). The failure of this trial, coupled with the undefined nature and compositional variability associated with viable and non-viable whole cell vaccines, has shifted the focus for the development of a vaccine against coccidioidomycosis to the identification of purified antigens which induce protective immune responses in immunized animals. The current belief is that a successful vaccine against coccidioidomycosis will be composed of multiple recombinantly-expressed \textit{Coccidioides} proteins that activate a Th1 pathway of immune response to different stages of the parasitic cycle, and contribute to host clearance of the pathogen from sites of infection (Cox and Magee, 2004; Deresinski, 1994; Magee and Cox, 1995, 1996). Progress towards this goal has been made by the discovery and subsequent evaluation of several recombinantly-expressed proteins of \textit{Coccidioides} as monovalent vaccines. These vaccine candidates confer varying degrees of protection against coccidioidal challenge, dependent on the strain of inbred mouse, route of challenge, and size of inoculum used (Abuodeh et al., 1999; Delgado et al., 2003; Herr et al., 2006; Jiang et al., 2005).
The most promising of these is a proline-rich, cell wall-associated antigen, Ag2/Pra (Dugger et al., 1996; Zhu et al., 1996b). Subcutaneous immunization of C57BL/6 or BALB/c mice with rAg2/Pra provided protection from an intraperitoneal challenge, as measured by reduction of coccidioidal burden in the lungs and spleen at 12-14 days post-challenge (Abuodeh et al., 1999; Jiang et al., 1999b; Kirkland et al., 1998a). In addition, subcutaneous immunization with rAg2/Pra significantly enhanced the survival of C57BL/6 and BALB/c mice following a intranasal challenge (natural route of infection) using inocula of ≤ 145 and ≤ 7 arthroconidia, respectively (Shubitz et al., 2002).

In an attempt to identify additional vaccine candidates, we have searched C. posadasii genome databases from The Institute for Genomic Research (TIGR) and the Whitehead Institute/MIT Center for Genome Research (www.tigr.org and www.genome.wi.mit.edu/annotation/fungi/fgi/) for homologs of AG2/PRA. We originally identified six homologs of AG2/PRA that all contain a conserved central proline/cysteine-rich domain (PCD), and are predicted to be surface expressed or secreted. We have named these homologs Pra2-Pra7. Using qualitative reverse transcription PCR assays we demonstrated that with the exception of PRA4, all the PRA mRNA transcripts are expressed during in vitro parasitic growth of C. posadasii and that transcripts from all members of the family are expressed in vivo within murine coccidioidal abscesses. The conserved amino acid sequences of the PCDs of Ag2/Pra and Pra2-Pra7 were aligned using the T-COFFEE algorithm (Notredame et al., 2000). Phylogenetic analysis of the aligned domains using the minimum evolution algorithm
revealed that the PCDs of Pra2 and Pra7 are the most closely related to Ag2/Pra (Rzhetsky and Net, 1993). Examination of recombinantly-expressed domains from all seven Pra family members by immunoblot analysis revealed that only rAg2/Pra, rPra2, and rPra7 PCDs react with sera from patients with coccidioidomycosis. These preliminary analyses facilitated the selection of PRA2 and PRA7 for further characterization and expression as full-length recombinant vaccine candidates against coccidioidomycosis.

The central hypothesis is that AG2/PRA and PRA2-PRA7 genes of C. posadasii are homologs whose translated products are antigenically related, and whose expression in vivo influences the nature of the host immune response to C. posadasii infection. Recombinant Ag2/Pra, rPra2 and rPra7 have been proposed as candidate vaccines against coccidioidomycosis. This project describes the results of testing this hypothesis by vaccination with recombinant full-length proteins, epitope mapping by use of overlapping peptide libraries, and selection of protective domains of Ag2/Pra and Pra2 to vaccinate and evaluate survival of two different strains of mice (BALB/c and C57BL/6). These mouse strains show distinct levels of susceptibility to Coccidioides infection and are representative of susceptible versus resistant human populations.

The specific aims of this project were to:

1. Complete the characterization and expression of AG2/PRA, PRA2, and PRA7,
2. Evaluate the seroreactivity of the selected full-length recombinant proline rich antigens (Pras) and conserved recombinant proline-cysteine rich domains of Ag2/Pra, Pra2-Pra7,
3. Assess the protective efficacy of rAg2/Pra, rPra2 and rPra7 (individually and in
combination) in a C57BL/6 murine model of coccidioidomycosis,

4. Assess the protective efficacy of rAg2/Pra and rPra2 (individually and in combination) in a BALB/c murine model of coccidioidomycosis,

5. Perform epitope-mapping experiments using the C57BL/6 and BALB/c models of coccidioidomycosis to localize T-cell and linear B-cell reactive epitopes in the selected Pra proteins, and

6. Develop a multivalent subunit vaccine composed of epitopes identified above for evaluation of its protective efficacy against coccidioidomycosis in C57BL/6 mice.
LITERATURE REVIEW

History of Coccidioidomycosis

Although the first documented cases of coccidioidomycosis were not reported until the late 1800’s, the discovery of an ancient Indian skeleton in Arizona with coccidioidal bone lesions provides evidence that it has been a disease endemic to the Southwest for thousands of years (Harrison et al., 1991). In 1892, Posadas isolated a protozoan-like organism from an Argentinian soldier with a 4 yr history of skin lesions resembling mycosis fungoides (Posadas, 1892; Wernicke, 1892). Several years later the first two cases in the United States were described by Rixford (Rixford and Gilchrist, 1896). Both cases had very different clinical presentations, the first patient exhibited a very slowly progressing disease that led to his death after nearly a decade; in contrast, the second patient succumbed to a rapidly progressing disease only 4 mo after the onset of symptoms. Rixford believed that the protozoan described by Posadas was closely related to the infectious agents in both the cases that he described and proposed the new genus classification (*Coccidioides*). However, since there were dramatic differences in clinical presentation and lesion development in both of the initial U.S. cases, the species designations of *immitis* and *pyogenes* were proposed for the first and second cases, respectively. The true fungal taxonomic affinity of *Coccidioides* was determined 4 yr later by Ophüls and Moffitt (1900). Several years later, Ophüls published a monologue that conclusively proved that the protozoan-like bodies observed in patient tissues belong to the life cycle of a pathogenic fungus which could be cultivated on artificial media (Ophüls, 1905). In this report he also fulfilled Koch’s postulates by initiating the development of coccidioidomycosis by inoculating animals with pure saprobic phase
cultures of the mold (obtained from lesions of previously-infected animals), and recovering the protozoan-like organism from lesions of the infected animal, which when grown on artificial media reverted to the saprobic phase of the pathogen. Until 1929, the only clinical form of coccidioidomycosis that had been recognized was a severe progressive disseminated disease known as coccidioidal granuloma, which was always fatal. During that year Harold Chope (a medical student) accidentally exposed himself to a pure culture of the mold form of *C. immitis* and instead of developing the disseminated fatal form of the disease, he presented with an acute self-limiting pneumonia and erythema nodosum (Dickson, 1937). Following the development of the coccidioidin skin test reagent, it was demonstrated that subclinical *C. immitis* infections were very common in endemic areas (Smith, 1940).

**Biology of Coccidioides**

**Phylogeny**

*Coccidioides* is a haploid ascomycete classified in the family Onygenaceae (order Onygenales), along with the human respiratory pathogens *Histoplasma*, *Blastomyces*, and *Paracoccidioides* (Currah, 1985; Pan et al., 1994; Sigler and Carmichael, 1976). Previously, *C. immitis* was believed to be the only etiological agent of coccidioidomycosis. However, molecular markers reveal differentiation among isolates from California (CA) versus isolates from Arizona and Texas (non-CA) providing evidence that North American populations of *Coccidioides* are genetically diverse (Burt et al., 1997; Fisher et al., 2000). More recently, Fisher and coworkers confirmed the molecular distinction between the CA and non-CA isolates by analyses of nucleotide sequence diversity between microsatellite loci of the isolates (Fisher et al., 2001). These
findings led to the proposal of a second species within the *Coccidioides* genera, *C. posadasii* which accommodates the non-CA clade and is widespread throughout most of the endemic regions of the U.S., Mexico and Central and South America (Fisher et al., 2002). The species designation of *C. immitis* was retained to include the isolates of the CA clade that are found primarily in the San Joaquin Valley of California.

**Geographic Distribution and Ecology**

The ecological niche of the saprobic phase of *Coccidioides* is the soil in the Lower Sonoran Life Zone of North America, Mexico, and scattered areas of South America (Maddy, 1958; Pappagianis, 1988). This bioclimatic zone is a rather harsh environment characterized by high temperatures (26°C-32°C), an annual rainfall of 5-20 in, and soil that is alkaline with a high salt content. Although it is difficult to recover *Coccidioides* from the soil (the majority of strains by far have been isolated from patients), the organism is usually found between 4-12 inches below the surface where it is cooler and more nutrients are available. It is believed that arthroconidia of *Coccidioides* are more resistant to drought than other organisms that compete for the same ecological niche and that prolonged periods of drought followed by heavy rainfalls can facilitate large “fungal blooms.” This supposition is supported by several recent outbreaks of coccidioidomycosis that have been associated with the climatic patterns described above (Kolivras and Comrie, 2003; Kolivras et al., 2001). The distribution of *Coccidioides* in the soil of the Lower Sonoran Life Zone is patchy and sparse, although ancient Indian burial sites have proved to be a rich source of the fungus (Lacy and Swatek, 1974). It is believed that burrowing rodents, armadillos, as well as wild and domesticated canines residing in the endemic areas of this fungus play important roles in the ecology of
*Coccidioides* by serving as natural reservoirs and facilitating the transfer of the organism to other areas suitable for colonization (Eulalio et al., 2001; Fowler et al., 1992; Harrison et al., 1991; Johnson et al., 2003; Pappagianis, 1988; Rubensohn and Stack, 2003). The death and decay of these coccidioidal animal reservoirs under natural conditions allows the fungus to revert to its saprobic form and return to the soil, while benefiting from the nutrients derived from the deceased host.

**Life cycle**

*Coccidioides* is a dimorphic fungus with a life cycle involving a saprobic phase characterized by mycelia that produce alternating barrel-shaped arthroconidia (enterothallic), and a parasitic phase that is unique amongst the medically-important fungi. Arthroconidia derived from the mycelial phase of *Coccidioides* can be aerosolized by the disruption of infested soil in endemic areas. The arthroconidia are approximately 3-6 x 2-4 µm in size, and when inhaled are small enough to reach the alveoli of a mammalian host (Cole and Samson, 1984). Once inhaled, arthroconidia initiate the parasitic life cycle by rounding up and displaying isotropic growth followed by synchronous nuclear divisions and segmentation (Kirkland and Cole, 2002). A large centralized vacuole can be found in young spherules that is surrounded by cytoplasm, which becomes progressively compartmentalized eventually giving rise to uninucleate compartments. These compartments further differentiate into endospores within the mature spherules that can reach 30-60 µm in diameter and contain up 300 endospores. The mature spherules rupture and release the endospores (2-4 µm in diameter) into the host tissues to begin the next generation of the parasitic cycle.
Clinical Manifestations

Most cases of primary pulmonary coccidioidomycosis are asymptomatic (~60%) or present as a mild self-limiting flu-like illness (~35%) (Smith et al., 1946, 1961). However, a number of patients with symptomatic primary pulmonary disease progress to persistent pulmonary or chronic progressive pulmonary coccidioidomycosis, and in rare instances life threatening extrapulmonary dissemination occurs (Pappagianis, 1988; Smith et al., 1946).

Primary Pulmonary Infection

Most patients who acquire primary pulmonary coccidioidomycosis are asymptomatic and the only indication that these individuals have been exposed to the pathogen is that they become reactive to intradermal injections with coccidioidin (convert to skin test positive). As stated above, approximately 35% of patients that acquire primary pulmonary coccidioidomycosis are symptomatic. Following exposure to the pathogen there is an incubation period that can vary dramatically (1-4 wk). It is believed that this variation in the observed incubation periods and the duration and severity of the disease are related to the magnitude of the exposure. Patients with symptomatic primary pulmonary disease have variable amounts of one or more of the following: fever, chest pain, fatigue, malaise, and anorexia. In addition, toxic erythema (a diffuse rash) is observed in 10% to 30% of patients. The appearance of this rash has no prognostic value but it often appears within a week of infection and disappears rapidly. Chest radiographs in patients with symptomatic pulmonary disease typically demonstrate pulmonary infiltrates, however, other roentgenographic findings vary greatly. An additional finding is that many patients with primary coccidioidomycosis and disseminated disease demonstrate peripheral blood
eosinophilia (Echols et al., 1982; Fiese, 1958; Schermoly and Hinthorn, 1988). Although the cause of the eosinophilia is unknown, it usually peaks 2-3 wk after the onset of clinical symptoms and does not appear to be associated with erythema nodosum or erythema multiforme (see below).

**Valley Fever**

Approximately 5% of all patients with primary pulmonary coccidioidomycosis develop Valley fever complex. These patients display specific cutaneous lesions known as erythema nodosum and erythema multiforme which are usually associated with the development of delayed-type hypersensitivity reactions to coccidioidin (Drutz and Catanzaro, 1978b; Fiese, 1958; Smith, 1940). These lesions may be found in association with arthralgias (desert rheumatism) and mild conjunctivitis. Fungal cells are not present in the lesions in patients with erythema multiforme and erythema nodosum, which are bright red to purple in color and are usually restricted to the upper body (multiforme) and the lower extremities (nodosum). The appearance of either of these cutaneous lesions usually signifies a favorable prognosis for the patient.

**Persistent Pulmonary Coccidioidomycosis**

The symptoms of primary pulmonary coccidioidomycosis usually disappear 2-3 wk after the initial onset of clinical symptoms. In patients that still demonstrate clinical symptoms and/or abnormal pulmonary radiographs beyond 6-8 wk (approximately 5%), either chronic or chronic progressive pulmonary disease will develop. Chronic pulmonary coccidioidomycosis is associated with one or more of the following symptoms: nodular or cavitary lesions, miliary disease, and chronic progressive pneumonia (Drutz and Catanzaro, 1978b; Winn, 1967, 1968; Winn et al., 1967). Pulmonary nodules are usually
benign but can become cavitary, however, eventually about 50% of these cavitary lesions close spontaneously with no surgical or chemotherapeutic intervention (Hyde, 1968). *Coccidioides* can remain viable in the nodular and cavitary lesions, which are very stable and persist for long periods of time. In one recorded case the organism was recovered in culture 15 yr after the initial infection (Cox and Smith, 1939). Although chronic pulmonary coccidioidomycosis usually remains stable, it may also become progressive immediately following primary disease or after long periods of stability. Malnutrition, old age, and immunosuppression can facilitate the reactivation and progression of chronic pulmonary coccidioidomycosis. Progression of chronic pulmonary coccidioidomycosis can involve increases in the size and/or numbers of nodules and cavities, progressive pneumonia, and lobular consolidation. These patients can have symptoms of cough, weight loss, fever, hemoptysis (coughing up blood), dyspnea (difficulty breathing), and chest pain. Progressive pulmonary coccidioidomycosis, particularly following previously stabilized disease usually leads to extrapulmonary dissemination or an aggressive pulmonary course with a fatal outcome.

**Disseminated Coccidioidomycosis**

Extrapulmonary dissemination of *Coccidioides* is dependent on the magnitude of exposure and the presence of one or more risk factors. The known risk factors for severe pulmonary and disseminated disease include being of African-American, Filipino or Asian race, 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). Dissemination may be limited to a single lesion in the skin, subcutaneous tissues, bone, meninges, reticuloendothelial
system, kidneys, or almost any other part of the body except the gastrointestinal system (Forbus and Bestebreurtje, 1946). Single lesion extrapulmonary dissemination usually has a favorable outcome unless the site is the meninges. In contrast, multifocal dissemination results in a very grim prognosis (mortality rate of greater than 50%).

**Host Defenses in Humans**

**Innate Immunity**

Polymorphonuclear leukocytes (PMNL) are among the first cells of the human immune system that respond to the *Coccidioides* arthroconidium (Forbus and Bestebreurtje, 1946). The early recruitment of PMNLs is in part due to chemotactic signaling via C5a, produced by the activation of the alternative pathway of the complement system by arthroconidia. However, histological studies of tissues from patients with coccidioidomycosis suggested that PMNLs may also be attracted by components released by the pathogen (Forbus and Bestebreurtje, 1946). This premise is supported by more recent data from chemotactic assays using human PMNLs stimulated with coccidioidin or spherulin (Galgiani et al., 1978). The *in vitro* interactions between human PMNLs and *Coccidioides* arthroconidia have been studied by multiple investigators (Drutz and Huppert, 1983; Frey and Drutz, 1986; Galgiani, 1985, 1986; Galgiani et al., 1978, 1982, 1984). These studies have revealed that the addition of *Coccidioides* immune sera to human PMNLs results in an increase in the phagocytic activity and respiratory burst of the immune cells. Nevertheless, more than 80% of the arthroconidia survive this encounter (Drutz and Huppert, 1983; Frey and Drutz, 1986; Galgiani, 1985). The maturation of arthroconidia into spherules (60 to 80 µm) makes them even less susceptible to PMNL killing presumably because the PMNLs (12 µm) are too small to ingest this parasitic form.
(Frey and Drutz, 1986; Galgiani, 1986). The further maturation and subsequent rupture of spherules releases endospores and also triggers the invasion of PMNLs into the surrounding area (Forbus and Bestebreurtje, 1946; Frey and Drutz, 1986). The endospores are small enough to be easily phagocytized by the PMNLs and have been shown to trigger a respiratory burst; however, the level of intracellular killing is no better than is observed in arthroconidium-PMNL interactions (Beaman and Holmberg, 1980b; Brummer et al., 1985; Frey and Drutz, 1986).

Macrophages not only express the machinery for MHC II antigen presentation but as phagocytic cells they are believed to be involved in the innate immunity to *Coccidioides*. Activation of the alternative pathway of the complement system by *Coccidioides* produces C5a, facilitating the recruitment of macrophages and other phagocytes to early sites of infection. However, multiple *in vitro* analyses of arthroconidium-endospore interactions with non-immune macrophage and peripheral blood monocytes (PBMCs) suggest that these cells are unable to kill the phagocytized pathogen efficiently (Beaman et al., 1981, 1983; Beaman and Holmberg, 1980a, b; Beaman and Pappagianis, 1985; Kashkin et al., 1977). Initially, Kashkin et al. reported that non-immune peritoneal macrophages from guinea pigs phagocytize, but do not effectively kill arthroconidia (Kashkin et al., 1977). These findings were supported by Beaman et al. using non-immune alveolar macrophages from monkeys, non-immune resident peritoneal macrophages from DBA/2 mice, and PBMCs from skin test-negative individuals (Beaman et al., 1981, 1983; Beaman and Holmberg, 1980a, b; Beaman and Pappagianis, 1985). These same studies also demonstrated that the interactions between endospores and these non-immune cells did not result in efficient killing of this alternative form of
the phagocytized pathogen. It is important that these studies used conventional assays for the determination of fungal viability. Several years later, using a newly-developed assay to measure the incorporation of radiolabeled $N$-acetylglucosamine (chitin precursor) by growing fungi, Galgiani et al. reported that PBMCs from both skin test positive and negative individuals killed or inhibited the growth of phagocytized arthroconidia (Ampel and Galgiani, 1991). These results have not been duplicated. One possible explanation for the inability of non-immune PBMCs or macrophages to kill phagocytized *Coccidioides* is the observed inhibition of phagosome-lysosome fusion (Beaman et al., 1981; Beaman and Pappagianis, 1985). However, the same investigators observed phagosome-lysosome fusion in macrophages infected in the presence of lymphocytes from immune mice (Beaman et al., 1981, 1983). Later it was demonstrated that co-incubation of monocytes/macrophages with IFN-$\gamma$ significantly enhances their anti-coccidioidal activity (Beaman, 1987, 1991).

Natural killer (NK) cells are believed to play a critical role in the innate immune response to *Coccidioides*. Usually NKs are confined to the peripheral blood, spleen, and bone marrow, but they are known to migrate to sites of inflammation in response to chemokines. Prior to the development of the adaptive immune response, NKs can be activated by IL-12 (derived from macrophages) at sites of inflammation. During the innate response, these activated NK cells are thought to be the main source of IFN-$\gamma$ in response to macrophage-derived IL-12, IL-18, and TNF-$\alpha$. In addition to producing IFN-$\gamma$, it has been suggested that NK cells have a direct cytotoxic effect on *Coccidioides* cells. Petkus and Baum reported that incubation of spherule/endospore phase *Coccidioides* cells with peripheral blood lymphocytes (PBL) depleted of macrophages and monocytes
resulted in a significant reduction in fungal viability (Petkus and Baum, 1987). These
authors found that this effect could be reversed by the removal of the NK cells by pre-
incubation of the macrophage/monocyte depleted PBLs with α-Leu11 (CD56) plus
complement. In addition, supernatants from macrophage / monocyte depleted PBLs co-
iculated with either Coccidioides or K562 cells also were found to be cytotoxic to
Coccidioides as demonstrated by a reduction in fungal viability. Although the above
results suggest that NK cells and a NK cell soluble factor are directly cytotoxic to
Coccidioides cells, additional studies need to be performed to confirm the findings. One
approach would be to assess the cytotoxicity of a NK cell line to Coccidioides cells.

One of the first events in the innate immune response at the site of infection is the
interaction between immature tissue dendritic cells and microbes and their products. The
immature dendritic cells initially bind the pathogen via pattern recognition receptors
(PRRs) that recognize structurally conserved pathogen-associated microbial products
(PAMPs). Initially, the PRR/PAMP interaction leads to the induction of
proinflammatory cytokines, including tumor necrosis factor alpha (TNF-α), IL-1, IL-6,
and IL-8. The polarization of dendritic cells upon maturation is also dependent on the
type of PRR/PAMP interaction that occurs (Kapsenberg, 2003). Recently, a Coccidioides
toluene spherule lysate (TSL) was shown to induce the maturation of peripheral blood
derived dendritic cells from healthy, non-immune (skin test negative) subjects (Richards
et al., 2001). Dendritic cell maturation was demonstrated by the increased cell surface
expression of HLA-DR, CD40, CD54, CD80, CD83, and CD86. The TSL pulsed
dendritic cells also were capable of stimulating non-adherent PBMCs to produce IFN-γ.
In a subsequent study, the same author showed that the anergy typically demonstrated by
PBLs from patients with disseminated coccidioidomycosis could be reversed by interaction with the coccidioidal-pulsed dendritic cells (Richards et al., 2002). Although these in vitro studies highlight the potential therapeutic value of antigen-pulsed dendritic cells, in vivo studies need to be done to establish if immunity to Coccidioides can be restored.

**Adaptive Immunity**

Early studies examining patients with different clinical manifestations of coccidioidomycosis revealed a correlation between the severity of disease and the observed profiles of adaptive immunity (Smith, 1948; Smith et al., 1950, 1956, 1961). Patients with primary, asymptomatic, or benign disease display strong skin test reactivity (to coccidioidin) and very low or non-existent levels of anti-Coccidioides complement fixation (CF) antibody. On the other hand, patients with chronic pulmonary, progressive pulmonary, or disseminated disease demonstrate a profile of adaptive immunity that is characterized by hyporesponsiveness or anergic reactions to coccidioidal skin testing and high titers of anti-Coccidioides IgG to the CF antigen. A reversal of this pattern is frequently observed in patients with severe coccidioidomycosis if they recover from active disease. Smith et al. also demonstrated a strong correlation between recovery from active disease and skin test reactivity (Smith, 1948, 1951). In these studies 75% of patients who were skin test positive to coccidioidin recovered from their disease, while only 17% of patients that were skin test negative recovered. In addition, it is important to note that skin test reactivity is retained in most patients who recover from primary infection, and these individuals are resistant to future encounters with Coccidioides (Pappagianis, 2001; Smith et al., 1946, 1961). The specificity of cutaneous anergy in
patients with coccidioidomycosis has been studied by multiple investigators (Catanzaro et al., 1975; Cox and Vivas, 1977; Gifford and Catanzaro, 1981; Smith, 1951). Interestingly, these studies reveal that about 50% of patients with severe disseminated multi-focal disease not only display anergy to coccidioidin but to a panel of recall antigens including candidin, mumps antigen, trichophyton, and streptodornase-streptokinase. Failure to respond to contact sensitization with dinitrochlorobenzene also has been reported in a number of patients with severe disseminated disease (Rea et al., 1976, 1977). These patterns of adaptive immune response in patients with coccidioidomycosis support the current belief that cellular immunity is critical for protection against coccidioidomycosis in humans.

**Murine Model of Coccidioidomycosis**

To date, almost all studies evaluating the protective efficacy of vaccines against *Coccidioides* have used a murine model of coccidioidomycosis. The availability of molecular and immunological reagents, low cost of breeding and housing mice compared to larger animals, and availability of inbred strains with differing susceptibilities to *Coccidioides* (BALB/c > C57BL/6 > DBA/2) are all important reasons for the observed preference for the murine model (Cox et al., 1988; Kirkland and Fierer, 1983b). However, several differences in the course of coccidioidal disease have been observed between humans and mice. One of these differences is the predisposition of DBA/2 (relatively resistant) and BALB/c (highly susceptible) mice to allow dissemination of the disease to the liver and spleen after pulmonary challenge with only 10 arthroconidia (Cox et al., 1988). Conversely, this pattern of disease is only frequently observed in human patients following exposure to a large infectious dose of *Coccidioides*. Another
difference is that infected (non-immunized) mice do not have detectable titers of CF antibody, which in human patients has been used as a prognostic tool (Beaman et al., 1979; Kong et al., 1965). Although these differences are important to consider when evaluating coccidioidal vaccine candidates, an analogous relationship between the outcome of disease and pattern of acquired immune response in humans and mice has been observed (see description below).

Host Defenses in Experimentally Infected Mice

There is considerable evidence from murine models of coccidioidomycosis that cellular immunity is essential for resistance. Initially it was demonstrated that in comparison to their T-cell sufficient counterparts, neonatal or adult thymectomized and congenitally athymic BALB/c mice were more susceptible to a coccidioidal challenge (Beaman et al., 1977; Clemons et al., 1985; Hicks and Northey, 1967). Subsequently, other investigators demonstrated that splenic T cells but not B cells or sera from FKS-immune DBA/2 mice could adoptively transfer protection against *Coccidioides* (Beaman et al., 1977, 1979). More recently, Cox et al. established that primarily CD4$^+$ T cells are responsible for the adoptive transfer of protective immunity from FKS-immune to naïve DBA/2 mice, but optimal transfer of immunity requires both CD4$^+$ and CD8$^+$ spleen cells (Cox and Magee, 1998). It has been demonstrated that one way immune T cells provide protection against *Coccidioides* is by activating murine macrophages to a fungicidal state. *In vitro* analysis of murine alveolar and peritoneal macrophages have revealed that these cells effectively phagocytize *Coccidioides* arthroconidia and endospores but fail to kill them (Beaman et al., 1981, 1983). However, co-incubation of these macrophages with immune lymphocytes results in a significant increase in the killing of phagocytized arthroconidia.
and endospores (37% and 25% respectively). The same authors demonstrated that this enhanced killing of the phagocytized arthroconidia is positively correlated with an increase in phagosome-lysosome fusion, from 13% using macrophages alone or in combination with non-immune lymphocytes to 61% when macrophages were co-incubated with immune lymphocytes. Additionally, depletion of T cells from the immune lymphocyte population prior to co-incubation with macrophages resulted in a reversion of phagosome-lysosome fusion to 26%. A subsequent study by the same author demonstrated that antibody to murine IFN-γ could reduce the ability of immune lymphocytes to activate murine macrophages to a fungicidal state, and in the reciprocal experiment showed that the addition of IFN-γ enhanced macrophage activation (Beaman, 1987). Further support for the protective role of cellular immunity in host defense against Coccidioides is illustrated in a study by Cox et al. (1988). These investigators measured footpad hypersensitivity to coccidioidin at multiple time points post-infection in both BALB/c and DBA/2 mice. Initially both strains of mice demonstrated significant footpad hypersensitivity by day 9 post-infection that increased in strength by day 12. At later time points (days 15 and 18), the susceptible BALB/c mice became anergic to coccidioidin while resistant DBA/2 mice maintained a robust delayed-type hypersensitivity reaction through the end of the experiment.

Multiple studies examining the role of cytokines in murine models of coccidioidomycosis have been conducted. Evaluation of the amount of proinflammatory cytokines (TNF-α, IL-1α, and IL-6) in lung homogenates from both BALB/c and DBA/2 mice at various times after a pulmonary challenge revealed that the levels of these cytokines were essentially the same in both strains of mice (Cox and Magee, 1995). A
subsequent study was conducted to evaluate the levels of IFN-γ and IL-4 in lung homogenates of BALB/c and DBA/2 mice at 3 d intervals following an intranasal challenge with *Coccidioides* (Magee and Cox, 1995). These investigators observed that in comparison to susceptible BALB/c mice, pulmonary IFN-γ levels in DBA/2 mice were not only increased earlier in the course of disease (6 versus 9 d), but also were maintained at significantly higher levels in these resistant animals for the remainder of the experiment. The reciprocal experiment revealed that in comparison to DBA/2 mice, IL-4 levels in the lung homogenates of BALB/c mice were significantly greater at 9 d post-challenge but thereafter (days 12 and 15) both strains exhibited similar levels. In the same study, susceptible BALB/c mice were treated with murine rIFN-γ before and during the course of coccidioidal infection. These mice demonstrated significant reductions in lung, liver and spleen fungal burden 13 d after an intraperitoneal challenge. However, administration of rIFN-γ to BALB/c mice does not significantly protect these mice against a pulmonary challenge with *Coccidioides* in comparison to non-treated controls. Conversely, neutralization of endogenous IFN-γ in resistant DBA/2 mice significantly reduced their resistance to both intraperitoneal and pulmonary challenge with *Coccidioides* compared to non-treated controls. In a subsequent study, the same authors examined the effects of administering rIL-12 to BALB/c mice 1 d prior to and during the course of coccidioidal infection initiated by an intraperitoneal challenge (Magee and Cox, 1996). Upon sacrifice 12 d after intraperitoneal challenge with *Coccidioides*, mice that received treatment with rIL-12 demonstrated significantly lower fungal burdens in their lungs, livers, and spleens. Measurement of the Th1- and Th2-associated cytokines in the lung homogenates of rIL-12 treated mice and untreated BALB/c mice revealed an
enhanced expression of IFN-γ and IL-4, respectively. The reciprocal experiment neutralizing endogenous IL-12 in DBA/2 mice, by treatment with a monoclonal antibody prior to intraperitoneal challenge with *Coccidioides* and during the course of the subsequent infection, significantly reduced the natural resistance of these mice to disease. Other investigators have provided additional evidence that increased levels of Th2 associated cytokines are expressed by susceptible mice in experimentally induced coccidioidomycosis. Fierer et al. demonstrated that BALB/c, C57BL/6, and CAST/Ei mice express higher levels of IL-10 and IL-4 than resistant DBA/2 mice during the course of a coccidioidal infection that was initiated by intraperitoneal challenge (Fierer et al., 1998). Importantly, these same authors observed that IL-10 knockout mice on a C57BL/6 background were as resistant to an intraperitoneal challenge with *Coccidioides* as DBA/2 mice, as assessed by measurement of fungal burden in lungs and spleen 14 d post-challenge.

Currently, there is little evidence that antibody-mediated immunity is important for resistance to coccidioidomycosis. Initially, Kong et al. reported that passive transfer of serum from mice vaccinated with FKS did not provide protection (Kong et al., 1965). These findings were later supported by additional murine studies, which confirmed that passive transfer of immune serum does not provide protection against a coccidioidal challenge, and demonstrated that passively transferred immune B cells are non-protective as well (Beaman et al., 1977, 1979). Only recently has there been any evidence that B cells and/or antibodies may also contribute significantly to vaccine-induced protection against *Coccidioides*. By using gene expression microarray analysis and B cell deficient MuMT mice, Magee et al. revealed that B cells and/or antibody play a role in the
acquired protective immunity against *Coccidioides* induced by immunization of mice with a FKS vaccine (Magee et al., 2005).

**Vaccine Candidates**

Individuals who develop coccidioidomycosis and recover are resistant to future encounters with *Coccidioides* (Pappagianis, 2001; Smith et al., 1946, 1961). On this basis it is argued that a human vaccine against coccidioidal infections can be produced (Cole et al., 2004; Cox and Magee, 2004). The geographic limitation of the disease, cost of patient treatment, and evidence that natural coccidioidal infection provides lifelong immunity has encouraged investigators to pursue the development of a vaccine against coccidioidomycosis.

**Viable and Nonviable Cell Vaccines**

In 1896, Rixford and Gilchrist were the first to provide evidence that immunization with viable cells could protect against a subsequent infection with *Coccidioides* (Rixford and Gilchrist, 1896). These findings were confirmed and extended by multiple investigators (Converse et al., 1963, 1964; Pappagianis et al., 1960, 1961). Converse et al. reported that subcutaneous immunization of cynomolgous monkeys with $10^1$ to $10^8$ viable arthroconidia provided protection against a subsequent pulmonary challenge (7,000 arthroconidia) with *Coccidioides* (Converse et al., 1964). In this study monkeys appeared to be healthy during the four-month observation period following pulmonary challenge, however, examination of pulmonary lung cultures upon conclusion of the experiment revealed that 20% of the animals failed to clear *Coccidioides* from this organ. Additional studies revealed a potentially serious concern with viable *Coccidioides* vaccines. Following vaccination *Coccidioides* was found to persist not only at the sites of
immunization, but also was frequently observed at distal sites (Converse et al., 1963; Kong and Levine, 1967a). In an attempt to ameliorate the risk of disease from immunization, studies evaluating irradiated mutants and serially passaged attenuated strains of *Coccidioides* were conducted (Kong and Levine, 1967b; Pappagianis et al., 1961). Unfortunately, both mutant strains and the serially passaged attenuated strain of *Coccidioides* reverted to their virulent phenotypes *in vivo*.

An experimental, FKS vaccine also has been shown to protect mice against disseminated coccidioidal infection, indicating that a non-viable immunization strategy is feasible (Cox et al., 1988; Levine et al., 1965). Between 1980 and 1985, a double-blinded human trial was conducted using the FKS vaccine versus a placebo (Pappagianis, 1993b). The study involved almost 3000 volunteers, but only a minority of the vaccinated individuals became skin test-positive to *Coccidioides*. 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. Possibly this was due to the fact that only $1/1000^{th}$ of the dose per kilogram of body weight that was protective in mice was tolerated in humans. *In vitro* studies with FKS were performed on human PBMCs obtained from skin test-positive and -negative volunteers. The PBMCs from both donor groups produced elevated levels of inflammatory cytokines upon exposure to FKS, which could account for the toxicity of the vaccine in humans (Ampel, 1994; Slagle et al., 1989).

**Cell-Derived Antigenic Fraction Vaccines**

The unacceptable risks associated with a viable cell vaccination strategy, combined with the failure of the FKS vaccine in the early 1980s during clinical trials, initiated a shift in the focus of coccidioidal vaccine development. All subsequent research has focused on
the evaluation of complex native antigenic fractions, recombinantly-expressed antigens, or genes as candidate vaccines.

To date, two complex native antigenic fractions isolated from spherules have been evaluated in murine models of coccidioidomycosis. The first of these fractions is a PBS extract of spherule cell walls. This fraction was initially isolated by Pappagianis et al. via the mechanical disruption of spherules followed by incubation in PBS supplemented with 2% chloroform for 5d with constant agitation (Pappagianis et al., 1979). This PBS extract combined with either complete Freund’s adjuvant (CFA) or alum provided NAMRU mice with protection against a pulmonary challenge with \(10^3\) *Coccidioides* arthroconidia. These studies have never been reproduced, and it has never been determined whether the protective component/s of this fraction are soluble, colloid or both. The second sub-cellular fraction that has been evaluated for its protective efficacy in murine models of coccidioidomycosis is the 27K fraction. This fraction was initially isolated by Zimmer et al. via the mechanical disruption of FKS and centrifugation of the homogenate at 27,000xg to yield a slightly opalescent supernatant that was designated 27K (Zimmer et al., 1990). Subsequently, Zimmermann et al. immunized outbred Swiss-Webster mice with 1 mg of 27K admixed with alum three times at 1wk intervals. The mice that received the 27K with alum demonstrated significantly enhanced survival over a 13wk period following intranasal challenge with 5,000 arthroconidia \((P = 0.003)\) and 15,000 arthroconidia \((P = 0.04)\) (Zimmermann et al., 1998). An additional study that was initially reported in a review by Cox et al. as unpublished data, highlights the ability of the 27K fraction to protect BALB/c mice from an intranasal challenge with 30 arthroconidia of *Coccidioides* (significantly reduced fungal burden in the lungs and
spleen) (Cox and Magee, 2004). The 27K fraction has been shown to provide substantial protection against *Coccidioides* to susceptible BALB/c mice and an outbred population of Swiss-Webster mice. However, the prospects for use of the 27K fraction as a human vaccine are unlikely because of its undefined nature and the potential for compositional variation in different preparations of the fraction that could induce significant variations in host response to this multicomponent immunogen. Current efforts to develop a vaccine against coccidioidomycosis, therefore, have focused on the identification of purified antigens which elicit a protective immune response.

**Recombinant Protein Vaccines**

The current belief is that a successful vaccine against coccidioidomycosis will be composed of multiple recombinantly-expressed *Coccidioides* proteins, which can provide the necessary antigenic diversity to induce protective immunity against *Coccidioides* in a heterogeneous population. Progress towards this goal has been made by the discovery and subsequent evaluation of several recombinantly-expressed proteins of *Coccidioides* as monovalent vaccines. These studies have identified recombinant vaccine candidates that confer varying degrees of protection against coccidioidal challenge dependent on the strain of inbred mouse, route of challenge, and size of inoculum used (Abuodeh et al., 1999; Ampel, 1994; Delgado et al., 2003; Jiang et al., 1999b; Kirkland and Cole, 2002; Kirkland et al., 1998a, b; Li et al., 2001; Shubitz et al., 2002; Slagle et al., 1989).

**T cell Reactive Protein**

A series of experiments by Cole et al. and Kirkland et al. designed to identify immunoreactive components within a water soluble fraction isolated from *Coccidioides* arthroconidia led to the identification and cloning of a T cell reactive protein (TCRP) by
Wyckoff et al. (Cole et al., 1987; Kirkland et al., 1991; Wyckoff et al., 1995). The tcrP gene contains a 1,197-bp ORF, which encodes a putative 45.2-kDa protein that is not predicted to be surface expressed or secreted and demonstrates a high level of sequence relatedness to two mammalian proteins (Wyckoff et al., 1995). Subsequently, tcrP was ligated into the pET28b plasmid, and the 48-kDa recombinant protein was expressed and purified from Escherichia coli (Kirkland et al., 1998b). In this same study, the authors demonstrated that rTCRP was able to induce proliferation and secretion of IFN-γ from FKS-immune T cells isolated from BALB/c mice, however it only provided modest protection against an intraperitoneal challenge with Coccidioides to the same strain of inbred mice. This finding combined with the high level of homology between tcrP and two mammalian genes suggest that rTCRP would not be a suitable component for a vaccine against Coccidioides.

**SOWgp**

Cole et al. initially described the growth and subsequent shedding of a lipid-rich membranous outer wall layer (SOW) by spherules of Coccidioides in a defined salts medium (Cole et al., 1988b). These authors described a detergent extraction method for purification of the SOW fraction and demonstrated that patient sera recognized both intact spherules and purified SOW (Cole et al., 1988a, b). In addition, the crude SOW and the isolated detergent extract of SOW demonstrated the ability to induce proliferation of Coccidioides-immune lymphocytes from (BALB/c X DBA/2)F₁ mice (Cole et al., 1988a). The crude SOW fraction was later shown to provide protection to BALB/c and C3H mice against an intraperitoneal challenge with 50 arthroconidia (Kirkland et al., 1998b). Subsequently, the gene that encodes SOWgp, the major glycoprotein component
of SOW was cloned and sequenced from *C. posadasii* (strain C735) by Hung et al. (Hung et al., 2000; Kirkland and Cole, 2002). The *sow* gene is composed of a 1,266-bp ORF whose translated product is a 422 amino acid protein with a predicted molecular weight (mature protein) of 39.5 kDa. A number of experiments have demonstrated that SOWgp is surface expressed and parasitic phase specific (Hung et al., 2000, 2002; Kirkland and Cole, 2002). A rSOWgp vaccine study was conducted on BALB/c mice to determine if this antigen could provide protection against an intraperitoneal challenge with 50 arthroconidia (Kirkland and Cole, 2002). In this study, BALB/c mice were immunized subcutaneously with 15 µg of rSOWgp mixed with incomplete Freund’s adjuvant (IFA), followed by two booster immunizations at 2wk and 4 wk of the same antigen in CFA. Quantitative analysis of fungal burdens in the lungs and spleens of the mice 14d after the challenge revealed that the group of BALB/c mice that had been immunized with rSOWgp had significantly lower pulmonary and splenic CFU’s than mice receiving adjuvant alone. In addition, splenocytes isolated from rSOWgp-immune mice displayed Th1 polarized qualitative recall responses to stimulation with rSOWgp *in vitro* (Kirkland and Cole, 2002). Recently, Hung et al. reported that SOWgp functions as an adhesin to extracellular matrix proteins (laminin > fibronectin > collagen) and as a potential virulence factor of *Coccidioides* (Hung et al., 2002).

**Urease**

In 1997, Yu et al. cloned the *Coccidioides* gene that encodes urease (Yu et al., 1997). The *URE* gene is composed of a 2,517-bp ORF that is expressed maximally during the endosporulation of mature *Coccidioides* spherules (Kirkland and Cole, 2002; Yu et al., 1997). The translated product of this gene is 839 amino acids in length with a predicted
molecular weight of 91.5 kDa. The URE protein sequence displays a high level of sequence identity with URE proteins from *Cryptococcus neoformans*, *Schizosaccharomyces pombe*, *Canavalia ensiformis*, *Helicobacter pylori*, *Klebsiella aerogenes*, *Bacillus pasteurii*, and *Proteus mirabilis* (Cox and Magee, 2004). A subsequent study by Li et al. showed that immune splenic and lymphatic T cells isolated from BALB/c mice immunized with rURE and CpG/IFA (strong Th1 biasing adjuvant) responded to *in vitro* re-stimulation with homologous antigen by proliferation and increased expression of IFN-γ and IL-2 but not IL-10, IL-4, or IL-5 (Li et al., 2001). Animal protection experiments were conducted using BALB/c mice that were immunized and boosted with either 5, 15, 30 or 60 µg of rURE plus CpG/IFA as adjuvant and were subsequently challenged by the intraperitoneal route with 100 *Coccidioides* arthroconidia. The assessment of survival in the control and rURE-immune groups over a 40d period following challenge revealed that rURE provides dose dependent protection against *Coccidioides*. A significant enhancement of survival was only demonstrated in the groups of mice that received 30 or 60 µg of rURE (44% and 47% at day 40 post-challenge, respectively) (Li et al., 2001). In addition, these authors tested the protective efficacy the *URE* gene expressed by the pSecTag2A.URE plasmid construct in immunized BALB/c mice. Survival was assessed over the 40d period following an intraperitoneal challenge with 100 arthroconidia. Mice that were immunized with the URE construct demonstrated significantly enhanced survival (83%) compared to mice immunized with vector only (17%). While neither of the two surviving BALB/c mice from the control group cleared the fungus from their lungs or spleen, immunization with
the pSecTag2A.URE construct provided sterilizing immunity to of the majority of mice (8 of 10) that survived in this group (Li et al., 2001).

**HSP60**

Using degenerate primers designed from known sequences of other fungal heat shock proteins, the gene that encodes the *Coccidioides* HSP60 protein was cloned and subsequently expressed by Thomas et al. (1997). The *hsp60* gene is composed of a 1,782-bp ORF whose translated product is a 594 amino acid protein with a predicted molecular weight of 62.4 kDa. These authors provided evidence that HSP60 was expressed during parasitic phase growth of *Coccidioides*, and T cells isolated from rHSP60-immune BALB/c mice vigorously proliferated in response to *in vitro* stimulation with homologous antigen. In a subsequent study, BALB/c mice that were immunized and boosted with rHSP60 plus CpG/IFA as adjuvant were then challenged by the intraperitoneal route with 100 *Coccidioides* arthroconidia (Li et al., 2001). The assessment of survival in the control and rHSP60-immune groups over a 40d period following challenge revealed that rHSP60 does not provide a significant level of protection against *Coccidioides*. These results combined with the fact that the *Coccidioides* HSP60 protein demonstrates 72% identity to the HSP60 proteins of *Homo sapiens* have resulted in the abandonment of this vaccine candidate (Cox and Magee, 2004).

**Coccidioides-specific Antigen (CSA)**

This antigen was initially recognized as a heat stable exoantigen present in all culture filtrates of *Coccidioides* strains, but absent in the culture filtrates of other closely related pathogenic fungi (Kaufman and Standard, 1978). In 1995, Pan et al. cloned the
*Coccidioides* gene that encodes CSA (Pan and Cole, 1995). The *CSA* gene is composed of a 543-bp ORF that encodes a 181-amino-acid serine protease with a predicted molecular mass of 19.8 kDa. A preliminary report has examined the protective efficacy of rCSA in C57BL/6 mice by both the intraperitoneal and intranasal route of challenge (Yu et al., Abstract ASM 103 Gen Meeting 2003; poster F-111). This study demonstrated that C57BL/6 mice immunized with rCSA in CpG/monophosphoryl lipid oil (MPL) adjuvant were significantly protected from an intraperitoneal challenge with 49 arthroconidia as assessed by significant reductions in pulmonary and splenic fungal burden. More importantly, rCSA-immune C57BL/6 mice also were protected from an intranasal challenge as measured by an increased rate of survival at 60d post-challenge compared to controls (30% versus 0%, respectively). In addition, in the same study these authors reported that co-immunizing C57BL/6 mice with rCSA and rAg2/Pra (1-106) in CpG/MPL adjuvant increased the survival of these mice to 90%.

**GEL-1**

Using a bioinformatics strategy to search the recently sequenced *C. posadasii* genome (www.tigr.org) for proteins that are predicted to be surface expressed, Delgado et al. identified a β-1,3 glucanosyltransferase that was named *GEL-1* (Delgado et al., 2003). The translated product of the *GEL-1* gene is a 447-amino acid protein with a predicted signal peptide and GPI anchor that was shown to be maximally expressed during the endosporulating spherule stage of parasitic growth. In the same report, rGEL-1 was expressed and purified from *E. coli* and was used to subcutaneously immunize BALB/c and C57BL/6 mice. The protective efficacy of rGEL-1 combined with CpG/IFA was evaluated by challenging BALB/c mice and C57BL/6 mice via intraperitoneal and
intranasal routes, respectively (Delgado et al., 2003). These experiments revealed that as little as 1 μg of rGEL-1 could protect immunized BALB/c mice from an intraperitoneal challenge with 100 arthroconidia as measured by significant reductions of fungal burden in the lungs and spleen 12d post-challenge. The protection experiments involving C57BL/6 mice that were immunized with 1 μg of rGEL-1 revealed that this group demonstrates a significant increase in survival 60d post intranasal challenge with 80 arthroconidia.

**ELI-Ag1**

Recently, Ivey et al. utilized an expression library immunization strategy (ELI) to identify a novel coccidioidal vaccine candidate (ELI-Ag1) (Ivey et al., 2003). The original ELI process was initially developed by Barry et al. and was adapted for use in the identification of potential vaccine candidates from *Coccidioides* (Barry et al., 1995). Expression libraries were generated from cDNA isolated from *in vitro* grown parasitic phase cultures of *Coccidioides*. These libraries were split into pools that were evaluated for their ability to protect BALB/c mice from an intraperitoneal challenge with *Coccidioides*. Protective pools were subdivided and tested for protective efficacy repeatedly until a single protective clone containing the *ELI-Ag1* gene was identified. The translated product of the *ELI-Ag1* gene is a 224 amino acid protein with a predicted signal peptide, GPI anchor, and chitin binding domain. Genetic vaccination with the *ELI-Ag1* pBKCMV construct provides BALB/c mice with a significantly increased rate of survival for a period of 40d following an intraperitoneal challenge with 2,500 arthroconidia (Ivey et al., 2003). To date *ELI-Ag1* has not been tested as a recombinant antigen for its protective capacity alone or in combination with other antigens.
AG2/PRA

Early studies have revealed that immunoprotective components of the spherule wall could be extracted by alkali treatment (Lecara et al., 1983), or toluene extraction followed by deglycosylation with hydrogen fluoride (Dugger et al., 1991). The Cox laboratory cloned a protein that was present in the alkali-soluble, water-soluble preparation of the spherule wall, and referred to it as antigen2 (Ag2) (Zhu et al., 1996a, b). This nomenclature was based on a reference system which relies on two-dimensional immunoelectrophoretic separation of crude preparations of saprobiic and parasitic phase antigens, designated coccidioidin and spherulin (Huppert et al., 1978). The AG2 gene was reported to encode a proline-rich protein with a predicted molecular mass of 19.4 kDa. The deduced protein sequence contains 10 repeats (TXX'P), predicted signal peptide and C-terminal GPI anchor, a conserved cysteine fungal extracellular membrane (CFEM) domain, and 24 potential O-glycosylation sites (Kulkarni et al., 2003).

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 difference in molecular size of the predicted and electrophoretically-separated, deglycosylated native protein is accounted for by its high proline content. Northern blot analysis of AG2/PRA gene expression revealed that levels of specific mRNA increased during stages of spherule maturation (Peng et al., 1999), which correlated with the immunolocalization of the antigen in the parasitic cell wall (Galgiani et al., 1992). In addition, little allelic diversity was detected in the gene isolated from multiple strains of C. posadasii, suggesting that
the rAg2/Pra vaccine should provide comparable protection against a wide range of isolates (Johannesson et al., 2004).

In 1996, the Cox laboratory expressed the cloned *AG2/PRA* gene in *E. coli* as a glutathione S-transferase (GST) fusion protein and demonstrated that it could elicit footpad hypersensitivity responses (DTH) in FKS-immune mice (Zhu et al., 1996a). The protective capacity of the recombinant GST-Ag2/Pra fusion protein was tested by Jiang et al. (1999b). BALB/c mice were immunized three times at weekly intervals with 100 µg of the rAg2/Pra GST fusion protein. The first of these immunizations was given intramuscularly with RIBI 730 adjuvant, and the two subsequent boosts were given subcutaneously with RIBI 700. One month following the final boost, these mice were challenged via the intraperitoneal route with 250 arthroconidia of *Coccidioides*. The fungal burden was measured in the lungs, liver, and spleen of control and rAg2/Pra-immune BALB/c mice 12d after challenge. Compared to GST immune controls, mice that were immunized with rAg2/Pra demonstrated significant reductions in their splenic and hepatic fungal burden. However, a significant decrease in pulmonary fungal burden was not observed. As part of the same experiment, equivalently treated groups of mice were evaluated for 30d following an intraperitoneal challenge with 250 arthroconidia. The rates of survival were almost identical for the control GST/adjuvant-treated mice and the rAg2/Pra GST fusion-immune mice (< 20%). The evaluation of genetic immunization with *AG2/PRA* cDNA ligated into the pVR1012 plasmid was also completed by Jiang et al. (1999b). BALB/c mice were given immunizations intramuscularly three times at weekly intervals with 50 µg of either *AG2/PRA*-pVR1012 or empty pVR1012 and challenged 1 mo after the final boost with 2,500 arthroconidia.
Evaluation of pulmonary, hepatic, and splenic fungal burden in mice vaccinated with AG2/PRA-pVR1012 12d post-challenge revealed a 2-log or greater reduction of CFU’s in all three organs compared to mice immunized with vector alone. As part of the same experiment, equivalently treated groups of mice were evaluated for 40d following an intraperitoneal challenge with 2,500 arthroconidia. Survival of the mice receiving the AG2/PRA-pVR1012 vaccine was 100% while only 9% of the vector control mice survived to 40d. The fungal burden in the surviving animals from either group was not reported, however, the observed protective efficacy of the AG2/PRA-pVR1012 vaccine was associated with the induction of footpad hypersensitivity and the production of IFN-γ in immune BALB/c mice. To complete this study, Jiang et al. also evaluated the ability of AG2/PRA-pVR1012 vaccine to enhance survival and reduce fungal burden in BALB/c mice challenged by the intranasal route with 50 arthroconidia (Jiang et al., 1999b). Although mice that received AG2/PRA-pVR1012 prior to challenge demonstrated reductions in hepatic and splenic fungal burden, genetic vaccination with this construct did not enhance clearance of Coccidioides from their lungs or reduce mortality. In a subsequent study Jiang et al. reported that co-administration of a pVR1012 plasmid containing IL-12 cDNA with the AG2/PRA-pVR1012 construct provides BALB/c mice with significantly enhanced protection against an intraperitoneal challenge with 2,500 arthroconidia compared to AG2/PRA-pVR1012 alone (Jiang et al., 1999a). The enhanced protection was associated with an increase in the production of IFN-γ to levels higher than observed in BALB/c mice that received only the AG2/PRA-pVR1012 vaccine. Analysis of the anti-Coccidioides IgG isotype response in the same group of mice unexpectedly revealed the predominance of the IgG1 isotype both before and after
challenge. Unfortunately, the same study revealed that the co-administration of a pVR1012 plasmid containing IL-12 cDNA with the \textit{AG2/PRA}-pVR1012 construct did not enhance the clearance of \textit{Coccidioides} from the lungs of BALB/c mice following an intranasal challenge with 50 arthroconidia. Recently, Jiang et al. reported that genetic vaccination of BALB/c mice with cDNA encoding the first 18 amino acids (signal peptide) of Ag2/Pra provides BALB/c mice with significant protection against an intraperitoneal challenge with 2,500 arthroconidia of \textit{Coccidioides} as assessed by reductions in fungal burden in their lungs and spleen (Jiang et al., 2002). Although the level of protection afforded by genetic immunization with the \textit{AG2/PRA}(1-18)-pVR1012 construct was less than that of the previously described full length genetic vaccine (\textit{AG2/PRA}(1-194)-pVR1012), the difference in protective efficacy was not found to be significant. As part of the same experiment, the protective efficacy of the \textit{AG2/PRA}(19-194)-pVR1012 construct also was evaluated. This construct also was shown to provide enhanced protection, but at a significantly lower level than the \textit{AG2/PRA}(1-18)-pVR1012 and \textit{AG2/PRA}(1-194)-pVR1012 constructs. The authors also demonstrate that the protection provided by genetic immunization with \textit{AG2/PRA}(1-18)-pVR1012 is associated with increased IFN-\textgamma response and a lack of anti-\textit{Coccidioides} antibody. A peptide corresponding to the \textit{AG2/PRA}(1-18)-pVR1012 coding sequence was synthesized and tested for protective efficacy by the authors in this same study. BALB/c mice were immunized subcutaneously three times at weekly intervals with 10 \(\mu\)g of synthetic peptide combined with CFA. One month after the last boost the mice were challenged by the intraperitoneal route with 2,500 arthroconidia. Determination of the fungal burden in the lungs and spleen at 12d post-challenge revealed that the peptide immune mice had
decreased fungal loads in these organs, however, the reduction was less than the corresponding genetic vaccine.

In 1998, the Galgiani laboratory expressed the cloned \textit{AG2/PRA} gene in \textit{E. coli} as a histidine-tagged recombinant protein and demonstrated that could induce proliferative responses in T cells isolated from rAg2/Pra- and FKS-immune mice (Kirkland et al., 1998a). The protective capacity of rAg2/Pra fusion protein was tested in the same study by immunizing BALB/c with rAg2/Pra. The first immunization was given subcutaneously using 5 $\mu$g of rAg2/Pra in IFA adjuvant, and a second immunization was given 4wk later by the same route with CFA as the adjuvant. One month following the final boost, these mice were challenged via the intraperitoneal route with 50 arthroconidia of \textit{Coccidioides}. Measurement of fungal burden in these mice 12d after challenge revealed significant reductions in the number of viable organisms present in the lungs and spleen of rAg2/Pra-immune mice compared to adjuvant controls. In a subsequent study Abuodeh et al. compared the protective efficacy of the recombinantly expressed Ag2/Pra and a \textit{AG2/PRA} genetic vaccine in BALB/c and C57BL/6 mice (Abuodeh et al., 1999). Both inbred strains of mice were immunized subcutaneously with 5 $\mu$g of rAg2/Pra in MPL adjuvant followed 4wk later by an identical boost. One month later vaccinated mice were challenged by the intraperitoneal route with either 50 (BALB/c) or 500 (C57BL/6) arthroconidia. Fourteen days after challenge the pulmonary and splenic fungal burdens in rAg2/Pra-immune BALB/c and C57BL/6 mice were significantly reduced in comparison to PBS-immune mice. Unfortunately an appropriate control (adjuvant) was not included for this experiment. The same authors also evaluated the protective efficacy of genetic immunization of BALB/c and C57BL/6 mice with
AG2/PRA cDNA ligated into the pVR1020 plasmid. Both strains of inbred mice were given two immunizations intramuscularly 4wk apart with 100 µg of either AG2/PRA-pVR1020 or empty pVR1020 and challenged via the intraperitoneal route 1mo after the boost with either 100 (BALB/c) or 500 (C57BL/6) arthroconidia. The evaluation of fungal burden in challenged mice 2wk later revealed that genetic vaccination with AG2/PRA cDNA significantly reduced pulmonary and splenic coccidioidal loads in C57BL/6 but not BALB/c mice. It was noted by the authors that splenocytes from both strains of inbred mice immunized with either the recombinant or AG2/PRA gene vaccine demonstrated significant proliferative responses and secreted IFN-γ but not IL-4 when stimulated with homologous antigen in vitro. Although there seems to be a Th1 polarized immune response to Ag2/Pra in the context of the MPL adjuvant or as a DNA vaccine, the predominant anti-Ag2/Pra IgG isotype was shown to be IgG1. This paradoxical finding supports the observations of Jiang et al. (1999a).

Although the intraperitoneal challenge model is valuable for the screening of recombinant proteins as potential vaccine candidates against coccidioidomycosis (Delgado et al., 2003; Kirkland et al., 1998a, b) it has an obvious pitfall. The primary concern is that the intraperitoneal challenge does not mimic the natural route of coccidioidal infection (inhalation of arthroconidia) (Polesky et al., 1999). Previous investigators have shown that challenging mice via the intraperitoneal route not only requires a much larger inoculum of arthroconidia to achieve equivalent severity of disease, it also facilitates an artificial progression of disease from the visceral organs (i.e. spleen and liver) into the lungs (Fierer et al., 1990; Kirkland and Fierer, 1983a, b; Scalarone and Huntington, 1983). The influence of the route of challenge on the
development and outcome of disease in murine models of other respiratory fungal pathogens such as *Blastomyces dermatitidis* (Calich and Kashino, 1998; Morozumi et al., 1981) also has been documented. The only evaluation of the protective efficacy of recombinantly expressed Ag2/Pra following an intranasal challenge with *Coccidioides* was conducted by Shubitz et al. (2002). In this study BALB/c and C57BL/6 mice were immunized with either 1 or 5 µg of rAg2/Pra in MPL and boosted in an identical fashion 4wks later. Vaccinated BALB/c mice challenged by the intranasal route with seven or less arthroconidia demonstrated significantly increased survival compared to controls. However, none of the surviving BALB/c mice from the control group or the rAg2/Pra-immune group showed evidence of infection at necropsy. This raises the question whether they had actually been infected or that few or none of the seven arthroconidia in the inoculum actually made it beyond the upper airways of these mice. A more substantial level of protection was afforded to rAg2/Pra-immune C57BL/6 mice challenged by the intranasal route. These mice could withstand an intranasal challenge with 145 or less arthroconidia and still demonstrated significantly increased survival compared to controls. Unfortunately, quantitative assessment of pulmonary and splenic fungal burden was not performed on BALB/c or C57BL/6 mice that survived until the end of the 56d post-challenge evaluation period.

In an attempt to localize the protective domains within Ag2/Pra, Peng et al. generated genetic vaccine constructs containing cDNA’s that encode overlapping pieces of Ag2/Pra that were tested for their ability to protect BALB/c mice from an intraperitoneal challenge (Peng et al., 2002). Comparisons of BALB/c mice immunized with *AG2/PRA*(1-106)-pVR1020, *AG2/PRA*(27-106)-pVR1020, *AG2/PRA*(90-151)-pVR1020,
and AG2/PRA(90-194)-pVR1020 revealed that only mice vaccinated with either AG2/PRA(1-106) or AG2/PRA(27-106) had significantly reduced fungal burden following an intraperitoneal challenge with 50 arthroconidia. These results were confirmed by immunizing BALB/c mice with recombinantly expressed Ag2/Pra domains that corresponded to the previously tested genetic vaccines.

A recent paper by Kirkland et al. has shed some light on the requirements for the generation of protective immunity in C57BL/6 mice immunized with rAg2/Pra plus CpG (Kirkland et al., 2006). Using rAg2/Pra plus CpG-immunized C57BL/6 mice with targeted mutations in IL-12α (p35), IFN-γ, CD4, CD8, and MHC II these authors demonstrated that protection against an intraperitoneal challenge with Coccidioides requires IL-12, IFN-γ, and MHC II restricted T cells but not cytotoxic T cells. These authors did not address or test the possibility of antibody or B cells playing a role in the protection that rAg2/Pra affords C57BL/6 mice. This oversight may be an important issue since there is recent evidence that B cells and/or antibodies also may contribute significantly to vaccine-induced protection against Coccidioides (Magee et al., 2005). Several investigations have demonstrated that rAg2/Pra is recognized by sera from patients with coccidioidomycosis (Galgiani et al., 1996; Wieden et al., 1996; Zhu et al., 1997). The most recent of these investigations demonstrated that a centrally localized domain within Ag2/Pra (CFEM aa 19-96) harbors both continuous and conformational B cell epitopes which are recognized by patient, murine, and hyper-immune goat sera (Zhu et al., 1997).
Evaluation of Two *Coccidioides posadasii* Antigens (Ag2/Pra and Pra2) as Candidate Vaccines Suggests Immunological Complementation in Protection Against Murine Coccidioidomycosis

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ABSTRACT

Evaluation of recombinant proteins of *C. posadasii* for their protective efficacy in a murine model of coccidioidomycosis has revealed several potential vaccine candidates. One of the most promising is a proline rich-antigen (Ag2/Pra). However, contradictory results on the protection afforded by this antigen have been obtained depending on the strain of mice which are immunized, route of challenge (intranasal [i.n.] vs. intraperitoneal [i.p.]), size of inoculum, and the methods used to evaluate protection and immune response of the vaccinated animals. Earlier studies of C57BL/6 and BALB/c mice have demonstrated that immunization with recombinant antigen (rAg2/Pra) followed by i.p. challenge results in significant reduction of coccidioidal burden in the lungs and spleen of the vaccinated animals at 12-14 days post-challenge compared to controls. Although it is possible to induce disseminated coccidioidomycosis by i.p. inoculation, natural infection of the mammalian host with *C. posadasii* occurs via the i.n. route. In our current studies of this latter murine model, we have shown that rAg2/Pra vaccination results in a chronic pulmonary infection over a 90 day period after i.n. challenge. In addition, this report includes the introduction of Pra2, an Ag2/Pra homolog that has recently been isolated from *C. posadasii*. We present the results of the evaluation of the protective efficacy of rAg2/Pra and rPra2 singly and in combination in C57BL/6 mice following a lethal intranasal challenge, and preliminary evidence suggesting that rAg2/Pra and rPra2 may immunologically complement each other as vaccine candidates. The current belief is that a successful vaccine against coccidioidomycosis will be composed of multiple immunodominant proteins, activate a Th1 pathway of immune response, stimulate cellular immunity to different stages of the
parasitic cycle, and contribute to host clearance of the pathogen from sites of infection. These data support the need for further evaluation of protective efficacy of novel vaccine candidates against coccidioidomycosis singly, and more importantly in combination with established protective vaccine candidates.

**INTRODUCTION**

*Coccidioides posadasii*, formerly known as the non-California (non-CA) *Coccidioides species*, is an etiological agent of coccidioidomycosis (San Joaquin Valley fever) (24, 63). *Coccidioides spp.* are dimorphic fungal respiratory pathogens which grow saprobically in the dry, alkaline soils found in the desert regions of North America, Mexico, and scattered areas of South America (43, 52). Disruption of the soil in these areas causes aerosolization of the arthroconidia of *Coccidioides* which when inhaled initiate a parasitic life cycle that is unique amongst the medically important fungi. *Coccidioides spp.* are primary pathogens that are capable of causing serious pulmonary and sometimes fatal disseminated disease in immunocompetent people visiting or living in endemic regions. Based on data from skin test surveys, it is believed that 25,000 to 100,000 infections are contracted annually in the United States alone (19, 53). The number of individuals that become infected with *Coccidioides* each year is influenced by the number of immunologically naïve individuals relocating or visiting endemic areas and climatic conditions such as the length and timing of droughts and precipitation (38, 39). Dramatic increases in population of endemic areas in the United States over the last 50 years and recurrent epidemics of coccidioidal infections in the Southwestern United
States has prompted the classification of coccidioidomycosis as a reemerging infectious disease (32).
Most cases of pulmonary coccidioidomycosis are asymptomatic or present as a mild self-limiting flu-like illness (62, 64). However, a number of patients develop persistent pulmonary or chronic progressive pulmonary coccidioidomycosis, and in rare instances life threatening extrapulmonary dissemination occurs (52, 62). Currently it is estimated that the annual cost of health care for management of patients with coccidioidomycosis in the United States is 60 million dollars (www.valleyfever.com).
It is important to note that individuals who develop coccidioidomycosis and recover are resistant to future encounters with Coccidioides (54, 62, 64). On this basis it is argued that a human vaccine against coccidioidal infection can be produced (12, 13). The geographic limitation of the disease, cost of patient treatment, and evidence that natural coccidioidal infection provides lifelong immunity has encouraged investigators to pursue the development of a vaccine against coccidioidomycosis.
The current belief is that a successful vaccine against coccidioidomycosis will be composed of multiple immunodominant proteins, activate a Th1 pathway of immune response, stimulate cellular immunity to different stages of the parasitic cycle, and contribute to host clearance of the pathogen from sites of infection (13, 16, 44, 45). Therefore, recent efforts to develop a vaccine against coccidioidomycosis have focused on the identification of purified recombinant antigens that elicit a protective immune response in murine models of coccidioidomycosis. The majority of protective antigens that have been characterized to date are products of the parasitic phase of the fungus (11, 15, 29, 37, 42) (Yu, J. J.et al. Abstract ASM 103 Gen Meeting 2003; poster F-111).
most promising of these is a proline-rich, cell wall-associated antigen (Ag2/Pra) (20, 69). The level of protection provided by immunization with rAg2/Pra is dependent on the strain of mouse that has been immunized, route of challenge (intranasal [i.n.] vs. intraperitoneal [i.p.]), size of inoculum, and the methods used to evaluate protection and immune response of the vaccinated animals. Earlier studies of C57BL/6 and BALB/c mice have demonstrated that immunization with the recombinant antigen (rAg2/Pra) followed by i.p. challenge results in significant reduction of coccidioidal burden in the lungs and spleen of the vaccinated animals at 12-14 days post-challenge compared to controls (1, 31, 35). While rAg2/Pra-vaccinated C57BL/6 mice challenged by the i.n. route showed prolonged survival, BALB/c mice were not protected against pulmonary infection. Although it is possible to induce disseminated coccidioidomycosis by i.p. inoculation, natural infection of the mammalian host with C. posadasii occurs via the i.n. route. To date, a single study has been published which evaluated survival of mice immunized with rAg2/Pra followed by i.n. challenge. The fungal burden of the survivors was not determined at the conclusion of the 56-day experiment (61).

In an attempt to identify additional vaccine candidates, we have utilized the genome database derived from the C. posadasii sequencing project underway at The Institute for Genomic Research (TIGR). The database searches revealed a homolog of Ag2/Pra that showed high sequence identity to the previously described, immunoprotective antigen. The studies reported here include the introduction of Pra2 as a novel vaccine candidate against coccidioidomycosis, evaluation of the protective efficacy of rAg2/Pra and rPra2 singly and in combination in C57BL/6 mice following a lethal intranasal challenge, and preliminary evidence suggesting that rAg2/Pra and rPra2 may immunologically
complement each other as vaccine candidates. Our current hypothesis is that vaccination with the combination of rAg2/Pra plus rPra2 is more effective in the stimulation of a robust and durable protective immune response against coccidioidomycosis than immunization with either of the single antigens.

MATERIALS AND METHODS

Culture conditions and parasitic cell development.

*Coccidioides posadasii* isolate C735 was used throughout this study, and was originally designated as *C. immitis* (24). The isolate was obtained from a patient with disseminated coccidioidomycosis. The saprobic and parasitic phases of the fungus were grown *in vitro* under conditions previously described (27). Parasitic phase cultures were harvested at various times after inoculation with arthroconidia (36-132 h) as reported (26).

Mice.
Eight- to 10-wk-old female C57BL/6 mice from the National Cancer Institute (Bethesda, MD) were used. Animals were maintained in an American Association for the Accreditation of Laboratory Animal Care-approved animal facility.

Genome database analysis and gene discovery.

The *C. posadasii* genome sequencing project was initiated in 2001 at the Institute for Genomic Research (Rockville, MD) and was supported by the National Institutes of Health (Bethesda, MD). The project involved a whole-genome shotgun strategy for determination of the 29-Mb genome sequence (51). The *C. posadasii* genome database has been completed to 8X coverage. The genomic sequences have been assembled into unique contigs and incorporated into a public database (www.tigr.org). In addition, 53,664 EST’s have been sequenced and assembled into 9,312 unique cDNA sequences.
The translated sequence of AG2/PRA gene was used to query the C. posadasii genome database using the basic local alignment search tool (BLAST) (2). Sequence alignments were conducted using the translated nucleotide sequences of the contigs. A 1000-bp fragment of a single contig was selected on the basis of its high translated sequence homology (3.0e-30) to Ag2/Pra (GenBank accession numbers AF013256 and U32518) (20, 69). Sense and antisense primer sequences were selected and synthesized on the basis of regions of the translated fragment that aligned with the amino acid sequence of the Ag2/Pra protein of C. posadasii. These primers were employed in a PCR reaction with genomic template DNA of C. posadasii isolate C735, and a 933-bp product was amplified. The nucleotide sequences of the sense and antisense primers were 5’-TGGCCGTTGACAATTCTTTG-3’ and 5’-TTTCTGCGCGAGTCTCCTAAG-3’, respectively. The amplification conditions included an initial denaturation step at 95°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min. The 933-bp PCR amplicon was ligated into the pCR2.1 TOPO cloning vector (Invitrogen, Carlsbad, CA), and the nucleotide sequence of the insert was determined as reported previously (28). This homolog of AG2/PRA of C. posadasii was referred to as PRA2.

Characterization of the translated product of PRA2.

The T-COFFEE algorithm (49) was used to align the translated Ag2/Pra and Pra2 sequences. Gaps were treated as non-informative when calculating identity and similarity percentages. The PROSITE database was used to identify conserved motifs in Pra2 with homology to reported proteins (21), and the PSORT database was used for prediction of cellular localization of Pra2 (47).
Quantitative real-time PCR analyses of AG2/PRA and PRA2 expression in vitro.

To assess the levels of expression of AG2/PRA and PRA2 during different stages of the parasitic cycle of C. posadasii, we employed a quantitative real-time PCR (QRT-PCR) assay. Total RNA was separately isolated from near-synchronous, parasitic-phase cultures after 36, 96, and 132 h of incubation in defined glucose salts medium, as previously reported (28). RNA was isolated using the RNeasy protocol, and on-column DNase digestion was conducted as described by the manufacturer (Qiagen, Chatsworth, CA.). Reverse transcription was performed using the following protocol: a reaction mixture which contained 500 ng of oligo (dT)$_{17}$, 0.5 mM deoxynucleoside triphosphates, 500 ng of template RNA, and RNase/DNase free water up to 12 µl was incubated at 65°C for 5 minutes to remove secondary structure in the RNA then immediately transferred to ice. First-strand buffer, 10 mM dithiothreitol, and 40 U of RNaseOUT™ RNase inhibitor (Invitrogen) were added and the samples were incubated at 42°C for 2 minutes. Finally, 200 U of Superscript II reverse transcriptase (Invitrogen) was added to the samples, which were incubated for 50 min at 42°C followed by an enzyme inactivation step at 70°C for 15 min. Oligonucleotide primers used for the real-time PCR assays were designed to amplify cDNA products that can be discriminated from genomic DNA products based on size. The sequences of the AG2/PRA-specific sense and antisense primers were 5’-ATGCAGTTCTCTCACGCTCT-3’ and 5’-TGGTGGGATGTCAATTGGGAC-3’, respectively. This primer pair amplified a 315-bp product using single-stranded template cDNA generated from the parasitic cell-derived RNA preparations. The sequences of the PRA2-specific sense and antisense primers were 5’-ATCCACCTTGCGCCCTATG-3’ and 5’-TCAACTCCTATCTCCTCCTCGT-
3’, respectively. This primer pair amplified a 331-bp product using single-stranded template cDNA generated from the parasitic cell-derived RNA preparations. A 191-bp amplicon used for normalization of the assay was derived from the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene of C. posadasii (GenBank accession no. AF288134) (28). Previously, GAPDH has been shown to be constitutively expressed in Coccidioides (J.-J. Yu, C.-Y. Hung, P. W. Thomas, and G. T. Cole, Abstr. 99th Gen. Meet. Am. Soc. Microbiol. 1999, abstr.F-52, p. 305, 1999). The sequences of the sense and antisense primers used for amplification of this constitutive gene were 5’-TATGAAGAAGGCCTCTGCCAA-3’ and 5’-ACCTGCGGTAGCATCGAAGAT-3’, respectively. Approximately 500 ng of single stranded cDNA from each developmental stage was used as template for the QRT-PCR analyses of gene expression. Control PCR and nucleotide sequence analyses confirmed that only single amplicons were generated by each of the above primer pairs, and the products were the appropriate fragments of the AG2/PRA, PRA2, and GAPDH genes. The QRT-PCR assays were performed using a SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) in the second step of the two-step RT-PCR protocol described by the manufacturer. PCR conditions were as follows: 50°C for 2 min and then 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. The assays were performed using a LightCycler system (Roche Applied Science, Indianapolis, IN). The data from the QRT-PCR assays were analyzed using the Light Cycler Relative Quantification Software (version 1.0) supplied by the manufacturer of the instrument. The comparative (CT) method was used for quantification of gene expression.
RT-PCR analysis of *AG2/PRA* and *PRA2* expression *in vivo*.

To determine if the *AG2/PRA* and *PRA2* genes were expressed by *C. posadasii in vivo* reverse transcription polymerase chain reaction assays (RT-PCR) were conducted using total RNA as template, which was obtained from uninfected lung and pulmonary abscesses of *C. posadasii* infected mice. Three C57BL/6 mice (females, 8 weeks old) were challenged by the intranasal (i.n.) route with approximately 100 arthroconidia of *C. posadasii* as previously described (42). Infected lung tissue obtained from each mouse sacrificed at 14 days post-challenge was used as a source of total RNA for separate RT-PCR assays of *AG2/PRA* and *PRA2* expression. Total RNA was isolated using TRIZOL reagent (Invitrogen), followed by DNase digestion (Promega, Madison, WI) and a final purification was done using the RNeasy protocol conducted as described by the manufacturer (Qiagen). Reverse transcription was performed using a standard protocol. The reaction mixture which contained 500 ng of oligo (dT)$_{17}$, 0.5 mM deoxynucleoside triphosphates, 500 ng of template RNA, and RNase/DNase free water up to 12 µl was incubated at 65°C for 5 minutes to remove secondary structure in the RNA then immediately transferred to ice. First-strand buffer, 10 mM dithiothreitol, and 40 U of RNaseOUT™ RNase inhibitor (Invitrogen) were added and the samples, which were incubated at 42°C for 2 minutes. Finally, 200 U of Superscript II reverse transcriptase (Invitrogen) was added to the samples, which were incubated for 50 min at 42°C followed by an enzyme inactivation step at 70°C for 15 min. The same oligonucleotide primers and PCR conditions for amplification of the 315-bp *AG2/PRA* and the 331-bp *PRA2* fragments as described above were used for the RT-PCR.
Expression and purification of recombinant Ag2/Pra and Pra2.

Total RNA from *C. posadasii* strain C735 was extracted from 132-h spherules and reverse transcribed with Superscript II and oligo(dT)$_{17}$ (Invitrogen) as described above. The resulting cDNA was used as a template for PCR amplification of Ag2/Pra and Pra2 coding sequences, catalyzed by Advantage 2 polymerase as described by the manufacturer (Clontech, Mountain View, CA). Amplification conditions were as follows: 95°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min. The sequences of the Ag2/Pra-specific sense and antisense primers were 5’-ATAGGCAGCCATATGCAGTTCTCTCAGCCTCT-3’ and 5’-GAATTCAAGCTTCTGAGTCTGATAGTCTAAATTTAC-3’, respectively. This primer pair amplified a 625-bp product using single-stranded template cDNA. The sequences of the *PRA2*-specific sense and antisense primers were 5’-ATAGGCAGCCATATGAAGTTCTCTCACACCCTC-3’ and 5’-GAATTCAAGCTTCTCGAGTCAACTCCTATCTTCCCTCGT-3’, respectively. This primer pair amplified a 412-bp product using single-stranded template cDNA. Each of sense and antisense primers for Ag2/Pra and Pra2 include a restriction site (shown in bold) to facilitate subcloning. The resulting 625-bp and 412-bp products were gel purified and were ligated into the cloning vector (Invitrogen). The orientation, frame, and sequence of the cDNA inserts were confirmed by automated DNA sequencing on an ABI 310 sequencer (Applied Biosystems). The inserts were excised from pCR2.1 TOPO with *Nde*I and *Xho*I and ligated into pET32b (Ag2/Pra) and pET28b (Pra2) to yield the pET32-*AG2/PRA* and pET28-*PRA2* plasmid constructs (Novagen, Madison, WI). The
pET plasmid constructs were transformed into *E. coli* BL21(DE3)slyD\textsuperscript{−} (59) (kindly provided to us by Ry Young, Texas A&M University). This host strain was chosen because it lacks the FK-506 binding protein, which was previously found to co-purify with recombinant Ag2/Pra (35). Isolated bacterial colonies were grown at 37°C with shaking at 225 rpm, until the absorbance at 600 nM was 0.6 (about 3 to 4 h), at which time the expression of rAg2/Pra and rPra2 was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and the cells were incubated for another 4 h at 37°C. Histidine-tagged recombinant Ag2/Pra and Pra2 were purified from the supernatant by batch metal affinity chromatography under denaturing conditions on Ni-nitrilotriacetic acid (Ni-NTA) agarose per manufacturer’s instructions (Novagen). Eluted histidine-tagged proteins were dialyzed against renaturation buffer (150 mM NaCl, 4 mM reduced glutathione, 40 nM oxidized glutathione, 20 mM Tris, pH 7.5) to remove urea, imidazole, and to renature the proteins. The thioredoxin fusion peptide and histidine tags were removed from the purified His-tagged proteins by proteolysis with biotinylated thrombin (Novagen), and the thrombin was removed with a small aliquot of streptavidin-agarose as suggested by the supplier (Novagen). The cleaved proteins were purified by collecting the flow through (non-bound fraction) after batch metal affinity chromatography under native conditions on Ni-NTA agarose (Novagen). Purified recombinant proteins were concentrated and passed over an E-TOX endotoxin removal column following the manufacturer’s protocol (Sterogene Bioseparations, Carlsbad, CA). The total protein content was determined by bicinchoninic acid with bovine serum albumin as a standard (Pierce, Rockford, IL). The level of endotoxin contamination was determined with a
Limulus amebocyte lysate QCL-1000 kit (Biowhittaker, Walkersville, MD). The recombinant antigens used in this study had ≤0.85 IU of endotoxin per µg of protein.

**Surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS)**

SELDI analysis was performed using an aliphatic reverse phase chip (H4 Protein Chip™, Ciphergen, Palo Alto, CA). The bait surfaces on the chip were pretreated with 2 µl of acetonitrile (Sigma, St. Louis, MO). Shortly before the acetonitrile completely evaporated, 1 µl of purified recombinant antigen in 0.1 M phosphate-buffered saline (PBS; pH 7.4) was applied to the bait surface. The analyte was allowed to concentrate by air-drying. The unbound material was removed by washing three times with 5 µl of high-pressure liquid chromatography-grade water (Fisher Scientific, Pittsburgh, Pa.). Five hundred nanoliters of a saturated solution of 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid, Sigma), the energy-absorbing molecule of choice, was applied to the washed surface of the chip and allowed to crystallize. The sample was then subjected to mass analysis in a PBSII laser desorption/ionization mass spectrometer (Ciphergen) using the Ciphergen ProteinChip® software (Ciphergen). Data were collected by averaging 30-50 laser shots with an intensity of 280 and a detector sensitivity of 10.

**Tandem mass spectrometry (LC-MS/MS) analysis.**

Recombinant Ag2/Pra and rPra2 were separated by SDS-PAGE as described above. The Coomassie blue-stained protein bands (31.5 kDa, 14.5 kDa) were excised from the gel and subjected to in-gel trypsin digestion at 37°C overnight using sequencing grade, modified trypsin (Promega) in 100 mM ammonium bicarbonate. Peptides were then extracted from the gel with 60% acetonitrile: 0.1% TFA and concentrated in a speed-vac.
The concentrated samples were separated on a reverse phase column (75 µm id x 5 cm x 15 µm Aquasil C18 PicoFrit column, New Objectives), eluted from the column using a binary gradient of 1% acetic acid/acetonitrile (5-95% acetonitrile in 35 min) and directly introduced into an ion-trap tandem mass spectrometer (LCQ Deca XP Plus, ThermoFinnigan) equipped with a nanospray source. 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 collision induced dissociation (CID) spectrum of the most abundant ion from the full MS scan was obtained. The CID spectra were either manually interpreted or searched against the 6 frame translation of the 8X C. posadasii genome database (www.tigr.org) using the TurboSEQUEST software package version 3.0 (Finnigan). The amino acid sequences of peptides matching corresponding CID spectra were then compared with the amino acid sequences of rAg2/Pra and rPra2.

**Immunization and animal challenge.**

Immunoprotection experiments were conducted with C57BL/6 mice (females, 8 weeks old) supplied by the National Cancer Institute (Bethesda, MD). Mice were immunized subcutaneously (s.c.) with either rAg2/Pra, rPra2, or rAg2/Pra in combination with rPra2 plus CpG/IFA adjuvant by using essentially the same protocol as previously described (42). The adjuvant used was unmethylated CpG dinucleotides present in a synthetic oligodeoxynucleotide (ODN) preparation (CpG ODN; Integrated DNA Technologies, Inc., Coralville, Iowa). This same adjuvant has been described in evaluations of other candidate vaccines (42, 50). The CpG ODN sequence used to immunize mice was TCCATGACGTTCCTGACGTT (CpG motifs are underlined). The oligonucleotides were dissolved in PBS (1 mg/ml) and used as stock solution for the vaccination
experiments. Mice were first immunized s.c. with either adjuvant alone in PBS (10 µg of CpG prepared in 50 µl of PBS plus 50 µl of incomplete Freund’s adjuvant [Sigma]) (42) or with the same CpG adjuvant plus either rAg2/Pra, rPra2, or rAg2/Pra in combination with rPra2. The mice were then boosted by s.c. immunization 14 days later with the same amount of immunogens plus adjuvant. The animals were subsequently challenged with either 63, 72, or 76 viable arthroconidia by the i.n. route 4 weeks after the last immunization. Mice were scored for survival over a 90-day period post-challenge. Survival differences between groups of i.n.-infected mice were analyzed for statistical significance by the Kaplan-Meier method as previously reported (42). In addition, fungal burden in the lungs and spleen of challenged mice were evaluated in surviving animals at either 15, 20, 30, 40, 60, and 90 days post-challenge or at the termination of the experiment. The CFU per organ were expressed on a log scale, and the Mann-Whitney U test was used to compare the medians of the experimental groups as described previously (35). The detection limit of the CFU assay is 10 colonies per organ homogenate (log_{10} CFU = 1).

**ELISPOT analysis.**

Ninety-six well PVDF plates (Millipore, Bedford, MA) were coated overnight at 4°C with 50 µl/well of 15 µg/ml anti-murine IFN-γ mAb (AN18, Mabtech, Nacka Strand, Sweden). Wells were washed and unoccupied sites blocked with RPMI 1640 supplemented with 2%FCS (ATCC, Manassas, VA) for 2 h at 37°C under 5% CO₂. Each well was seeded with 2x10^5 murine CD4⁺ splenocytes from C57BL/6 mice which were immunized and boosted with either rAg2/Pra, rPra2, or PBS as described above, suspended in 50 µl of RPMI 1640 supplemented with 10% FCS and 1%
penicillin/streptomycin (ATCC). CD90− splenocytes were used as APCs and isolated from naïve C57BL/6 mice and used at a concentration of 2.5x10^5 cells/well in 50 µl of RPMI 1640 supplemented with 10% FCS and 1% penicillin/streptomycin. Finally, a range of concentrations of recombinant proteins were added in 50 µl of RPMI 1640 supplemented with 10% FCS and 1% penicillin/streptomycin. ELISPOT plates were incubated at 37°C for 24 hours under 5% CO₂. Wells were washed five times with 200 µl of PBS, and then 100 µl/well of 1 ug/ml anti-murine IFN-γ biotinylated mAb (R4-6A2-biotin, Mabtech, Nacka Strand, Sweden) was added and allowed to incubate for 2 hours at room temperature. Wells were washed as above and 100 µl/well of strepavidin-alkaline phosphatase [1:1000] was added for 1 hour (Mabtech). Wells were washed again as above and 100 µl of BCIP/NBT substrate solution (Mabtech) was added to each well until development was complete. The ELISPOT plates were allowed to dry overnight and were shipped to an ELISPOT plate evaluation service to be analyzed (Zellnet Consulting, Fort Lee, NJ). To control for non-specific background spots, all wells were corrected by subtracting the number of spots detected in an identically treated well that contained PBS-immune CD4⁺ T cells.

Immune and control CD4⁺ T cells were isolated by depletion of non-CD4⁺ T cells using a CD4⁺ T cell isolation kit (Miltenyi Biotec, Gladbach, Germany). CD90⁻ splenocytes were isolated by depletion of CD90⁺ cells using anti-CD90 (Thy 1.2) microbeads (Miltenyi Biotec). The purity of the isolated CD4⁺ and CD90⁻ cell preparations were verified by staining with FITC conjugated anti-CD4 and anti-CD90 mAbs (Miltenyi Biotec) followed by analysis of the labeled cells using an Epics Elite Flow Cytometer.

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RESULTS

Identification of \textit{PRA2} gene in the \textit{C. posadasii} genome database.

BLAST searches (tblastn) of the \textit{C. posadasii} genome database (www.tigr.org) with the translated sequence of the \textit{AG2/PRA} gene (either AF013256 or U32518) revealed a 372-bp sequence encoding a homologous gene (3.0e-30) that we have designated as proline-rich antigen 2 (\textit{PRA2}). Sense and antisense primer sequences were selected to amplify a region of genomic DNA from \textit{C. posadasii}, which contains the predicted open reading frame of \textit{PRA2}. Nucleotide sequence analysis of the PCR product confirmed that it is identical to the contig sequence originally identified in the \textit{C. posadasii} genome database. This genomic sequence has been deposited in the GenBank database under accession # AY102922. Nine additional ORFs from \textit{C. posadasii} have been identified in separate contigs of the genome database, which have significant sequence identity to \textit{AG2/PRA} and \textit{PRA2}, contain a conserved proline-cysteine-rich domain, and are predicted to be secreted or surface expressed. Attempts are under way to clone and express these full-length genes, which we suggest encode members of a family of related proline-rich antigens of \textit{C. posadasii}. The complete coding sequence of \textit{PRA2} was amplified using gene specific primers and cDNA template derived from the total RNA of 132-hour parasitic-phase cultures of \textit{C. posadasii} to yield a 0.4 kb product. Sequencing of this amplicon revealed that the coding sequence of the \textit{PRA2} gene is 372 bp, and confirmed the positions of two predicted introns of 62 and 64 bp in length respectively. The
complete coding sequence of \textit{PRA2} has been deposited in the GenBank database under accession \# AY102921.

\textbf{Comparison of Ag2/Pra and Pra2 protein sequences.}

The cDNA sequences of \textit{AG2/PRA} and \textit{PRA2} were translated into amino acid sequences of 194 aa and 124 aa in length, respectively. When aligned using the T-COFFEE algorithm (49), these protein sequences share 69\% identity and 91\% similarity (Figure 1A). The aligned proteins show highest homology within the first 97 amino acids of their N-termini. This finding is of particular interest since the N-terminal domain of Ag2/Pra (amino acids 1-106) has been suggested to be responsible for the protective efficacy of this antigen in C57BL/6 and BALB/c mice (30, 56). In addition, both Ag2/Pra and Pra2 amino acid sequences are proline- and alanine-rich (12.9 vs. 13.7\% Pro, and 14.4 vs. 12.9\% Ala respectively). SignalP analyses of Ag2/Pra and Pra2, revealed signal peptides at the N-termini of both proteins (Figure 1B), which are 18 aa in length and have predicted cleavage sites at A\textsuperscript{18}/Q\textsuperscript{19} \url{http://www.cbs.dtu.dk/services/SignalP} (48). The PROSITE database was used to identify conserved motifs in Pra2 with homology to reported proteins (21), and the PSORT database was used for prediction of cellular localization of Pra2 (47). These analyses identified a conserved cysteine fungal extracellular membrane (CFEM) domain (Figure 1B) located within amino acids 19-96 of Ag2/Pra and Pra2. The consensus motif for the CFEM domain can be summarized as follows: PXC[A/G]X\textsubscript{2}CX\textsubscript{8-12} CX\textsubscript{1-3}[X/T]DX\textsubscript{2-3}CX\textsubscript{9-14}CX\textsubscript{3-4}CX\textsubscript{15-16}C where ‘X’ is any residue, with a range indicated (40). The CFEM domains found in Ag2/Pra and Pra2 are also proline-rich 13.16\% and 12.99\% respectively. Although no functional data is currently available, several fungal proteins with CFEM domains including Ag2/Pra and
Pra2 are expressed in morphological stages that are involved in pathogenesis of Magnaporthe grisea, Candida albicans, and Coccidioides sp. (9, 17, 41, 55). Previous investigations of this formerly nameless domain within Ag2/Pra demonstrated that it contains both linear and conformational B-cell reactive epitopes, which we suggest are primarily responsible for patient seroreactivity to this antigen (67). Downstream of the CFEM domain of Ag2/Pra and Pra2, the translated sequences of these proteins diverge significantly. A noteworthy feature of Ag2/Pra is the presence of a TXX’P (X=A,H,E and X’=A,V,E) tandem repeat domain located proximal to its C-terminus which is not found in Pra2. The TXX’P domain of Ag2/Pra is similar to the P-region of the P1 adhesion molecule of Streptococcus mutans, which is known to be an important structural component necessary for surface expression of this bacterial protein (6, 58). As mentioned previously, both Ag2/Pra and Pra2 are predicted to have N-terminal signal peptides. However the C-terminus of Pra2 does not contain a predicted GPI anchor motif and the mature, native Pra2 is believed to be secreted. This premise is supported by immunoblot analyses, which demonstrate that Pra2 but not Ag2/Pra is present in concentrated media from in vitro parasitic phase cultures of C. posadasii (data not shown).

Analysis of expression of AG2/PRA and PRA2.

Quantitative real time polymerase chain reaction assays (QRT-PCR) were used to evaluate expression of the C. posadasii AG2/PRA and PRA2 transcripts during in vitro growth of the parasitic phase of the fungus. Specifically, transcript levels of AG2/PRA and PRA2 produced by round cells (36 h), segmenting spherules (96 h), and the endosporulation stage of the parasitic cycle (132 h) were compared. QRT-PCR data
analysis was conducted by application of the comparative ($C_T$) method, and data are presented as the ratio of the amount of either $AG2/PRA$ or $PRA2$ to $GAPDH$ transcript in each sample. The expression ratios at various time points during in vitro parasitic stages of growth reveal distinct expression patterns between these two homologs. Transcript levels of $AG2/PRA$ progressively increase during in vitro growth at 36 h, 96 h, and 132 h time points (Figure 2A). In contrast, $PRA2$ transcript levels are virtually undetectable at 96 h, and are expressed maximally at 132 h during in vitro growth (Figure 2B). To determine if $AG2/PRA$ and $PRA2$ are expressed in vivo, RT-PCR was performed using cDNA templates derived from total RNA of uninfected and $C. posadasii$-infected murine lung tissue. The detection of specific 315-bp $AG2/PRA$ and 331-bp $PRA2$ amplicons (Figure 2C) was demonstrated using cDNA template from $C. posadasii$ infected murine lung tissue, indicating that both $AG2/PRA$ and $PRA2$ genes of $C. posadasii$ are expressed during infection.

**Production and analysis of recombinant Ag2/Pra and Pra2.**

The expression of rAg2/Pra and rPra2 began by amplifying the cDNA sequences of $AG2/PRA$ and $PRA2$ genes of $C. posadasii$ by PCR. The amplified cDNA sequences of 625-bp ($AG2/PRA$) and 427-bp ($PRA2$) were initially ligated into pGEM T-easy plasmid for identification of clones with correct open reading frames and engineered restriction sites. The verified $AG2/PRA$ and $PRA2$ cDNA sequences were subcloned into either pET32b to yield the pET32b-$AG2/PRA$ expression plasmid, or into pET28b to yield the pET28b-$PRA2$ expression plasmid. Both the $AG2/PRA$ and $PRA2$ cDNAs include the nucleotide sequences which encode their respective signal peptides. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to examine the
thrombin digested, purified recombinant proline-rich antigens obtained from *E. coli* strain BL21(DE3) SlyD-, which had been transformed with either the pET32b-*AG2/PRA* or pET28b-*PRA2* plasmid constructs and then induced with IPTG (isopropyl-β-D-thiogalactopyranoside) (Figure 3A). This analysis revealed single protein bands with estimated molecular masses of 31.5 kDa for rAg2/Pra and 14.5 kDa for rPra2. The migration patterns of rAg2/Pra and rPra2 were higher than their predicted molecular weights (22.1 kDa and 13.2 kDa). This finding was consistent with previous reports, which demonstrated that rAg2/Pra and spherule-derived deglycosylated Ag2/Pra migrate aberrantly on SDS-PAGE gels (25, 35, 68). In order to more accurately determine the molecular masses of rAg2/Pra and rPra2, the recombinant proteins were subjected to surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) analysis. Small aliquots (approximately 0.2µg of each) of purified rAg2/Pra and rPra2 previously analyzed by SDS-PAGE above were bound to pre-treated bait surfaces of a H4 aliphatic reverse phase chip for SELDI-TOF MS analysis. Examination of the rAg2/Pra and rPra2 spectra revealed that the molecular weights of both recombinant antigens are nearly identical to their calculated predicted molecular masses when evaluated by this technique (Figure 3B). Recombinant Ag2/Pra and rPra2 were also subjected to tandem mass spectrometry analysis. Coomassie blue-stained protein bands (31.5 kDa, 14.5 kDa) were excised from the gel (Figure 3A) digested with trypsin, separated on a reverse phase column, and then directly introduced into an ion-trap tandem mass spectrometer. Analysis of the collision induced dissociation (CID) spectra from the excised bands yielded two peptides from the rAg2/Pra 31.5 kDa band (ISVSNIVVDQCSK and PTASTPAEPFGAGSNVR), and a single peptide from the
rPra2 14.5 kDa band (AGVPISIPPADTR). The peptides isolated from the 31.5 kDa band were both identical to predicted sequences found in the translated AG2/PRA gene (amino acids 73-85 and 161-177), and the peptide isolated from the 14.5 kDa band was identical to a predicted sequence found in the translated PRA2 gene (amino acids 87-99).

**Evaluation of survival and fungal burden in rAg2/Pra- and PBS-immunized mice post-intranasal challenge with C. posadasii.**

In parallel experiments, C57BL/6 mice were immunized s.c. with either rAg2/Pra or PBS in adjuvant (CpG in IFA) and then challenged by the i.n. route with a lethal inoculum of C. posadasii (72 viable arthroconidia). The first experiment evaluated the survival of rAg2/Pra- and PBS-immunized mice (n=15 per group) over the course of 90 days post i.n.-challenge (Figure 4A). Control PBS-immunized mice (open circles) began to die about 14 days post i.n. challenge and in this experiment none survived beyond 36 days. In contrast, mice immunized with rAg2/Pra (shaded circles) showed significantly greater survival when compared to the control mice ($P \leq 0.0001$). As the experiment progressed, the percentage of surviving mice in the rAg2/Pra-immunized group was reduced from 80% to 73.3% at day 60, and finally to 66% at 88 days post-challenge. The second experiment assessed the fungal burden in lungs of rAg2/Pra-immunized mice equivalently challenged, and sacrificed at 15, 20, 30, 40, and 60 days post-challenge (Figure 4B). Data for the assessment of the residual fungal burden in the lungs of rAg2/Pra-immunized mice at 90 days post-challenge was determined by sacrificing the surviving rAg2/Pra animals from the first experiment. Due to the rapid progression of disease, fungal burdens were not determined in PBS immunized mice. As part of the same experiment, fungal burden was also evaluated in the spleens of rAg2/Pra-
immunized mice. With the exception of one animal at day 15, the spleens of all mice were below the detection limit of the CFU assay (data not shown). In contrast, 55-88% (64% at day 90) of surviving rAg2/Pra-immunized mice had ~ 4-5 $\log_{10}$ colony forming units (CFU) in their lungs during the course of the entire experiment.

**Evaluation of survival and fungal burden in C57BL/6 mice immunized with rAg2/Pra or rPra2 alone or in combination post-intranasal challenge with C. posadasii.**

Two independent experiments were conducted using C57BL/6 mice that were immunized with either PBS, rAg2/Pra alone (0.2µg or 1µg), rPra2 alone (0.2µg or 1µg), or the combination of rAg2/Pra plus rPra2 (1µg of each) in adjuvant, followed by i.n. challenge with a lethal inoculum of *C. posadasii* (63 or 76 viable arthroconidia). Survival was scored over the course of 90 days post-challenge, and fungal burden in the lungs and spleen was assessed in all surviving animals upon termination of the experiment (day 90). The individual experiments revealed very similar results, therefore the survival curves and fungal burden data for the two experiments were combined and are presented graphically in figure 5A and 5B. The groups of mice immunized with either 0.2µg (open circles n=16) or 1µg of rAg2/Pra (shaded triangles n=30) demonstrate significantly improved survival when compared to PBS-immunized mice (shaded circles n=31) (both $P \leq 0.0001$). The final percentages of surviving rAg2/Pra-immune mice (0.2µg or 1µg) at day 90 post-challenge are 68.75% and 80% respectively. Although the improvement in survival is not as dramatic as rAg2/Pra-immune mice, animals immunized with 0.2µg of rPra2 (open triangles n=16) or 1µg of rPra2 (shaded squares n=31) also demonstrate significantly enhanced survival compared to PBS-immune controls ($P \leq 0.004$ and $P \leq$
0.001). Upon termination of the experiment the percentage of surviving mice in the rPra2-immune groups was 31.25% (0.2µg) and 22.58% (1µg). Mice immunized with the combination of 1µg rAg2/Pra plus 1µg rPra2 (open diamonds n=30) also have a highly significant improvement in survival (%) when compared to the PBS-immune control group (P≤ 0.0001). The final percentage of surviving rAg2/Pra plus rPra2-immune (1µg of each) mice at day 90 post-challenge was 73.33%. The differences in the rates of survival for the rAg2/Pra-immune (0.2µg and 1µg) and rAg2/Pra plus rPra2-immune (1µg of each) mice were not found to be statistically significant (P≥ 0.765).

Upon conclusion of the survival experiment fungal burden was assessed in the lungs and spleen of all surviving animals (Figure 5B). The fungal burden in spleens of all surviving mice sacrificed at 90 days post-challenge were below the detection limit of the CFU assay (data not shown). As demonstrated previously in figure 4B at 90 days post-challenge, the majority of surviving mice immunized with 1µg of rAg2/Pra had detectable fungal burden in their lungs (67%). Statistical analysis of the survival curves and fungal burden in the lungs of rAg2/Pra (1µg)-immunized mice at day 90 post-challenge from the previous experiment (Figure 4) and equivalently treated mice in this experiment demonstrated no significant differences (P≤ 0.612 and P≤ 0.69). Although the percentage of surviving 0.2µg rAg2/Pra-immunized mice with detectable fungal burden in the lungs was slightly lower (63.6%) than that of the 1µg group, the difference in fungal burden between these groups was not found to be statistically significant (P≤ 0.637). In addition to the low rates of survival, 50% or greater of the mice in the rPra2 (0.2µg or 1µg)-immune groups that did survive had detectable fungal burden in their lungs at 90 days post-challenge (data not shown). In contrast to the single antigens, the
majority of surviving mice immunized with the combination of rAg2/Pra plus rPra2 were found to have no detectable fungal burden in their lungs. The differences in pulmonary fungal burden between rAg2/Pra alone (0.2µg or 1µg) and the combination of rAg2/Pra plus rPra2 were found to be statistically significant ($P \leq 0.033$ and $P \leq 0.008$).

**IFN-γ ELISPOT analyses.**

The finding that the protection afforded by rAg2/Pra can be complemented by rPra2 suggests that these two highly homologous proteins may be antigenically diverse. To explore this possibility, C57BL/6 mice were immunized as described above and CD4$^+$ T cells were isolated from rAg2/Pra- and rPra2-immune animals for ELISPOT analysis. The number of CD4$^+$ T cells secreting IFN-γ in response to *ex vivo* stimulation with varying concentrations of homologous or heterologous full-length recombinant antigen was quantified (Figure 6). These data reveal that when compared to the media control, significant numbers of CD4$^+$ T cells from rAg2/Pra-immune C57BL/6 mice (black bars) secrete IFN-γ in response to *ex vivo* stimulation with various amounts of full length homologous antigen ($P \leq 0.001$). When a range of concentrations of heterologous antigen were used to stimulate identical cells *ex vivo*, the response was no different or significantly lower than the media control. Similar results were demonstrated in the corresponding experiment utilizing CD4$^+$ T cells from rPra2-immune C57BL/6 mice (white bars) stimulated *ex vivo* with the same range of concentrations of homologous and heterologous antigen (Figure 6). Significant numbers of CD4$^+$ T cells from rPra2-immune C57BL/6 mice were shown to secrete IFN-γ in response to several different concentrations of homologous antigen ($P \leq 0.0002$), but not to the heterologous antigen.
DISCUSSION

Almost all reports that have evaluated the protective efficacy of rAg2/Pra as a vaccine candidate in murine models of coccidioidomycosis have opted for challenging via the intraperitoneal route. These investigations have demonstrated that s.c. immunization of C57BL/6 and BALB/c mice with rAg2/Pra significantly reduced fungal burden in the lungs and spleen of these mice at the time of sacrifice (14 days post infection) when compared to controls (1, 31, 35). Although the i.p. challenge model is valuable for the screening of recombinant proteins as potential vaccine candidates against coccidioidomycosis (15, 35, 37) it has an obvious pitfall. The primary concern is that i.p. challenge does not mimic the natural route of coccidioidal infection (inhalation of arthroconidia) (57). Previous investigators have shown that challenging mice via the i.p. route not only requires a much larger inoculum of arthroconidia to achieve equivalent severity of disease, it also facilitates an artificial progression of disease from the visceral organs (i.e. spleen and liver) into the lungs (22, 33, 34, 60). The influence of the route of challenge on the development and outcome of disease in murine models of other respiratory fungal pathogens such as Blastomyces dermatitidis (8, 46) has also been documented. An additional concern is that the all but one of the studies evaluating the protective efficacy of rAg2/Pra following an i.p. challenge have elected to determine fungal burden 12-14 days post-challenge rather than evaluate survival and fungal burden over a longer period of time. Interestingly, when survival was assessed in BALB/c mice thirty days after an i. p. challenge with Coccidioides (250 arthroconidia) rAg2/Pra-immune animals did not demonstrate improved survival when compared to controls (31).
To date, only one study has been published which evaluated the survival of mice immunized with rAg2/Pra following an intranasal challenge (61). Fungal burden was not reported upon conclusion of this 56 day survival experiment. In this report, subcutaneous immunization with rAg2/Pra was shown to significantly enhance the survival of C57BL/6 and BALB/c mice using inocula of ≤ 145 and ≤ 7 arthroconidia, respectively. Although the results of this study support the need for further investigation of rAg2/Pra as a vaccine candidate against coccidioidomycosis, it is essential that future studies assess pulmonary fungal burden in surviving mice upon termination of the experiment. The issue of clearance in surviving animals should be of significant interest in coccidioidal vaccine development, since it has been documented that patients with pulmonary coccidioidomycosis who do not completely clear the pathogen are at risk of disease reactivation (7, 18, 65, 66). To address the need for more complete published data concerning the protective efficacy of rAg2/Pra, we decided to measure survival and fungal burden in the lungs and spleen of rAg2/Pra-immune C57BL/6 mice at 15, 20, 30, 40, 60, and 90 days post-intranasal challenge. The C57BL/6 mouse was selected for these studies since it is intermediately susceptible to pulmonary challenge in contrast to the extreme susceptibility of BALB/c mice (lethal inoculum by i.n. route is ≥ 10 arthroconidia) (61). These initial animal experiments demonstrate that compared to PBS-immune controls, rAg2/Pra provides significantly enhanced survival after a lethal i.n.-challenge with C. posadasii, but over half (64% at day 90) of surviving mice failed to clear the organism from their lungs. These data suggest that rAg2/Pra does provide a significant level of initial protection to i.n. Coccidioides challenged C57BL/6 mice (increased survival %), but the response does not necessarily facilitate the clearance of
the organism, rather in some mice it may induce a chronic state of disease. Even though rAg2/Pra does not appear to facilitate pulmonary clearance in the majority of surviving C57BL/6 mice following i.n. challenge, it has been proposed to be the most promising known recombinant vaccine candidate against coccidioidomycosis (61). In the current study we have described a homolog of AG2/PRA (PRA2) whose translated sequence when aligned with Ag2/Pra displays substantial homology with the N-terminal fragment of this protective antigen. This observation combined with evidence that Pra2 is expressed during in vitro parasitic growth and pulmonary coccidioidal abscesses (in vivo), encouraged us to examine whether immunization with rPra2 alone or in combination with rAg2/Pra would protect C57BL/6 mice from a lethal i.n.-challenge with C. posadasii. As observed in our initial animal experiment, the survival (%) of mice immunized with 1µg of rAg2/Pra was significantly improved over that of PBS-immune controls. When we reduced the amount of rAg2/Pra used to vaccinate mice five-fold (0.2µg), the level of survival (%) was not significantly reduced from the higher vaccine dosage. This finding suggests that additional studies using the same model of coccidioidomycosis to examine varying reductions in dose of rAg2/Pra may be productive. Examination of rPra2-immune mice revealed that both groups (0.2µg and 1µg) displayed significantly enhanced survival when compared to the PBS-immune controls. Unfortunately, the increase in survival although significant was not as striking as that observed between rAg2/Pra-immune and control groups of mice. This result was somewhat surprising since prior investigations have established that the N-terminal portion of Ag2/Pra (amino acids 1-106) contains the immunoprotective domain(s) that are responsible for the protective efficacy of this antigen in C57BL/6 and BALB/c mice.
Despite the high level of homology shared by their N-termini, rPra2 was found to be considerably less protective than rAg2/Pra based on survival following i.n. challenge. As part of the same experiment, mice immunized with the combination of rAg2/Pra (1µg) plus rPra2 (1µg) were scored for survival over 90 days post intranasal challenge. Survival (%) in this group of mice was significantly enhanced when compared to PBS-immune controls, but there was no significant improvement in survival compared to mice immunized with either dose of rAg2/Pra alone. At the conclusion of the survival experiment the fungal burdens in the lungs and spleen of the remaining mice were determined. Interestingly, the surviving mice that were immunized with rAg2/Pra plus rPra2 (1µg of each) had significantly lower fungal burden in their lungs compared to animals that were immunized with rAg2/Pra singly at 0.2 or 1µg. The latter indicates that although no significant difference in survival between all three groups was observed, the protective efficacy (reduction of pulmonary fungal burden) of rAg2/Pra alone can be improved by the addition of rPra2. Although Ag2/Pra and Pra2 are highly homologous, their additive protective effect does not appear to be caused simply by an increased dosage of antigen. Preliminary studies using C57BL/6 mice immunized with 0.2µg, 1µg, and 2µg of rAg2/Pra revealed no significant difference in the level of protection (survival and pulmonary fungal burden) following a lethal i.n. challenge (data not shown). The results indicate that the protection afforded by rAg2/Pra can be complemented by the addition of rPra2, suggesting that these two highly homologous proteins may be antigenically diverse. To explore this possibility, we isolated rAg2/Pra- and rPra2-immune splenic CD4⁺ T cells from C57BL/6 mice for IFN-γ ELISPOT analysis. We chose to initially focus on immune CD4⁺ T cells since there is considerable evidence...
from murine models of coccidioidomycosis that cellular immunity is essential for resistance. Previously, other investigators demonstrated that splenic T cells but not B cells or sera from FKS-immune DBA/2 mice could adoptively transfer protection against *Coccidioides* (4, 5). More recently, Cox et al. established that primarily CD4+ T cells are responsible for the adoptive transfer of protective immunity from FKS-immune to naïve DBA/2 mice, but optimal transfer of immunity requires both CD4+ and CD8+ spleen cells (14). In addition, Kirkland et al. demonstrated that IL-12, IFN-γ, and MHC II restricted T cells, but not cytotoxic T cells are responsible for the protective efficacy observed in C57BL/6 mice immunized with rAg2/Pra plus CpG following i.p. challenge with *Coccidioides* (36). Clinical observations that AIDS patients and immunosuppressed transplant patients are both prone to develop disseminated disease also highlights the importance of cell-mediated immunity in resistance to coccidioidomycosis (3, 10, 23).

Our IFN-γ ELISPOT analyses of rAg2/Pra- and rPra2-immune CD4+ T cells stimulated *ex vivo* with homologous and heterologous antigen revealed that although both of these proteins are capable of generating antigen specific Th1 effector populations, they do not appear to share any common epitopes which can be presented in the context of murine H-2b (*I-A^b^* and *I-E^b^*). These data support the hypothesis that the divergent C-terminus of Pra2 or minor differences in the amino acid sequences of the conserved N-terminal portions of these antigens are responsible for the complementation of protective efficacy observed in rAg2/Pra-, rPra2-immune C57BL/6 mice following a lethal challenge with *C. posadasii*. The current opinion is that a successful vaccine against coccidioidomycosis should be composed of multiple recombinant antigens. The findings in this report indicate that the level of protection afforded by rAg2/Pra alone can be improved upon by
the addition rPra2. In future experiments we will map the $H-2^b$ restricted CD4$^+$ epitopes of rAg2/Pra and rPra2 in C57BL/6 mice using overlapping synthetic peptides.
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**Figure 1.** Sequence alignment and primary structure of *C. posadasii* Ag2/Pra and Pra2. (A) Comparison of the translated, full length ORFs of the AG2/PRA and PRA2 genes of *C. posadasii* after alignment using the T-COFFEE algorithm (49). An asterisk indicates amino acid identity; a colon indicates a conservative substitution; a single dot indicates a semi-conserved substitution. (B) Graphical representation of Ag2/Pra and Pra2 of *C. posadasii*, showing the presence of predicted signal peptides and conserved fungal extracellular membrane (CFEM) domains in each. Tandem TXX’P repeats and a predicted GPI-anchor unique to the C-terminal of Ag2/Pra are also depicted above.
Figure 2. Evaluation of AG2/PRA and PRA2 transcript levels in vitro and in vivo. (A)(B) Quantitative real time polymerase chain reaction assays (QRT-PCR) were used to evaluate relative expression of the C. posadasii AG2/PRA and PRA2 transcripts compared to GAPDH during in vitro growth of parasitic phases of the fungus (36h, 96h, and 132h). (C) Qualitative reverse transcription polymerase chain reaction assays (RT-PCR) were performed to determine if AG2/PRA and PRA2 transcripts are present in cDNA templates derived from total RNA of uninfected and C. posadasii infected murine lung tissues. Results shown are representative of three separate preparations of total RNA isolated from three sets of uninfected and infected mice.
Figure 3. Assessment of purified *C. posadasii* rAg2/Pra and rPra2 quality.
(A) Purified rAg2/Pra and rPra2 were electrophoresed on a 10-20% Tricine gel and the coomassie blue-stained protein bands (31.5 kDa, 14.5 kDa) were excised and subjected to LC-MS/MS analysis. (B) SELDI analysis was performed on purified rAg2/Pra and rPra2. The low intensity peaks observed at 11,023 and 6,704 daltons represent double charged molecules of rAg2/Pra and rPra2 respectively.
Figure 4. Evaluation of survival and fungal burden in lungs of rAg2/Pra-, and PBS-immune C57BL/6 mice following i.n. challenge with C. posadasii.

(A) C57BL/6 mice were immunized by the s.c. route with rAg2/Pra (1µg) plus adjuvant or PBS plus adjuvant alone then challenged by the i.n. route with C. posadasii (72 viable arthroconidia). Mortality was determined at days 1 through 90 post-challenge for each of 15 mice per group. (B) Fungal burdens were assessed in the lungs of equivalently challenged rAg2/Pra-immune mice at 15, 20, 30, 40, 60, and 90 days post-challenge.
Figure 5. Evaluation of protective efficacy of rAg2/Pra and rPra2 alone and in combination in C57BL/6 mice challenged by the i.n. route with C. posadasii. (A) C57BL/6 mice were immunized by the s.c. route with either PBS, rAg2/Pra (0.2 or 1µg), rPra2 (0.2 or 1µg), or a combination of rAg2/Pra plus rPra2 (1µg each) in adjuvant. Immunized mice were challenged by the i.n. route with C. posadasii (63-72 viable arthroconidia), and mortality was determined at days 1 through 90 post-challenge. (B) Fungal burdens were assessed in the lungs of rAg2/Pra-, rPra2-, and rAg2/Pra plus rPra2-immune mice at 90 days post-challenge.
Figure 6. ELISPOT assays of IFN-γ production by immune CD4⁺ T cells in response to full-length rAg2/Pra or rPra2. CD4⁺ T cells obtained from C57BL/6 mice immunized with rAg2/Pra or rPra2 were assayed for number of IFN-γ producing immune T cells in response to ex vivo stimulation with the homologous or heterologous antigen. Each well of the ELISpot microtiter plate contained 2.5 x 10⁵ antigen presenting cells isolated from isogenic, naïve mice, and 2 x 10⁵ immune splenic CD4⁺ T cells.
Epitope Mapping of Ag2/Pra and Pra2 Provides Evidence of Diverse Immune Responses Induced by These Two Coccidioidal Vaccine Candidates in Both C57BL/6 and BALB/c Mice

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ABSTRACT

Vaccination of C57BL/6 and BALB/c mice with the combination of rAg2/Pra plus rPra2 enhanced protection against a lethal intranasal challenge with *Coccidioides*, compared to vaccination with either of the single antigens. Enhanced protection was based both on higher percentage of surviving mice (as demonstrated by the BALB/c strain) or significantly better clearance of the fungal burden (C57BL/6). Despite the related primary structures of Ag2/Pra and Pra2, we have identified differences in their helper T cell (Th) and continuous B cell epitope profiles using immune CD4⁺ T cells and sera from both C57BL/6 and BALB/c mice. Enzyme-linked immunospot (ELISPOT) assays were used to screen overlapping synthetic peptides (20-mers with 8 residue offsets), derived from the sequences of Ag2/Pra and Pra2, for their ability to stimulate IFN-γ recall responses from immune CD4⁺ splenocytes. The same overlapping peptides were also immobilized on ELISA plates and used to screen homologous and heterologous immune sera obtained from BALB/c and C57BL/6 mice for continuous B cell epitopes. Our mapping results provide evidence of diverse immune responses induced by Ag2/Pra and Pra2 in both C57BL/6 and BALB/c mice, which provide these animals with a larger *Coccidioides* specific immune repertoire than immunization with either of the single antigens.
INTRODUCTION

Coccidioides spp. are dimorphic fungal respiratory pathogens which grow saprobically in the dry alkaline soils of the desert regions of North America, Mexico, and scattered areas of South America (29, 30). Disruption of the soil in these areas causes aerosolization of arthroconidia, which when inhaled initiate the parasitic life cycle of this fungus. Coccidioides spp. are primary pathogens capable of causing serious pulmonary and sometimes fatal disseminated disease in immunocompetent people visiting or living in endemic regions. Based on data from skin test surveys, it is believed that 25,000 to 100,000 infections are acquired annually in the United States alone (14, 31). Most cases of pulmonary coccidioidomycosis are asymptomatic or present as a mild self-limiting flu-like illness (38, 39). However, a number of patients develop persistent pulmonary or chronic progressive pulmonary coccidioidomycosis, and in rare instances life-threatening extrapulmonary dissemination occurs (30, 38). Currently it is estimated that the annual cost of health care for management of patients with coccidioidomycosis in the United States is 60 million dollars (www.valleyfever.com). Individuals who develop coccidioidomycosis and recover are resistant to future encounters with Coccidioides (33, 38, 39). On this basis it is argued that a human vaccine against coccidoidal infections can be produced (9, 11). The geographic limitation of the disease, cost of patient treatment, and evidence that natural coccidioidal infection provides lifelong immunity has encouraged investigators to pursue the development of a vaccine against coccidioidomycosis. The current belief is that a successful vaccine against coccidioidomycosis will be composed of multiple recombinantly expressed Coccidioides proteins, which can provide the necessary antigenic diversity needed to induce protective
immunity against *Coccidioides* in a heterogeneous population. Progress towards this goal has been made by the discovery and subsequent evaluation of several recombinantly expressed proteins of *Coccidioides* as monovalent vaccines. These studies have identified recombinant vaccine candidates that confer varying degrees of protection against coccidioidal challenge dependant on the strain of inbred mouse, route of challenge, and size of inoculum used (1, 13, 21, 22, 24, 26, 28, 36). The evaluation of combinations of the monovalent candidates is a compulsory next step in the development of a multivalent vaccine against coccidioidomycosis. We have begun this work by demonstrating that compared to vaccination with either of the single antigens, a combined rAg2/Pra plus rPra2 vaccine provides enhanced protection to C57BL/6 mice against a lethal intranasal (i.n.) challenge with *Coccidioides* (17). In this report we have extended these findings by evaluating the protective efficacy of the rAg2/Pra plus rPra2 combination in BALB/c mice and have mapped the Th and continuous B cell epitopes of Ag2/Pra and Pra2 induced by immunization of either C57BL/6 or BALB/c mice with these recombinant antigens.

**MATERIALS AND METHODS**

**Mice.**

Eight- to 10-wk-old female C57BL/6 (*H-2^b^*) and BALB/c (*H-2^d^*) mice from the National Cancer Institute (Bethesda, MD) were used for all of the following experiments. Animals were maintained in an American Association for the Accreditation of Laboratory Animal Care-approved animal facility.
Strains, media, and growth conditions.

*Coccidioides posadasii* isolate C735 was used throughout this study, and was originally designated as *C. immitis* (16). The strain was isolated from a patient with disseminated coccidioidomycosis. The saprobic phase of the fungus was grown in vitro on GYE agar (1% glucose, 0.5% yeast extract, 1.5% agar) at 30°C for 3 to 4 weeks prior to harvesting arthroconidia used in animal challenge experiments.

Peptides and Antigens.

Based on the sequences of Ag2/Pra and Pra2, two complete synthetic peptide libraries were synthesized (Mimotopes, Raleigh, NC). These libraries are composed of 23 (Ag2/Pra) and 14 (Pra2) synthetic icosameric peptides designed to overlap adjacent peptides by 12 amino acids. Recombinant Ag2/Pra and rPra2 were expressed and purified as previously described (17). Briefly, oligonucleotide primers were designed to amplify cDNA fragments from the *AG2/PRA* and *PRA2* genes that encode the Ag2/Pra and Pra2 proteins. The *AG2/PRA* and *PRA2* cDNA’s were purified, digested with *Nde*I and *Xho*I and ligated into pET32b (Ag2/Pra) and pET28b (Pra2) to yield the pET32-*AG2/PRA* and pET28-*PRA2* plasmid constructs (Novagen, Madison, WI). The pET plasmid constructs were transformed into *E. coli* BL21(DE3)slyD− (35) (kindly provided to us by Ry Young, Texas A&M University). Transformants were grown at 37°C with shaking at 225 rpm, until the absorbance at 600 nM was 0.6 (about 3 to 4 h), at which time the expression of rAg2/Pra and rPra2 was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and the cells were incubated for another 4 h at 37°C. Histidine-tagged recombinant Ag2/Pra and Pra2 were purified from the supernatant by batch metal affinity chromatography under denaturing conditions on Ni-nitrilotriacetic acid (Ni-NTA)
agarose per manufactures instructions (Novagen). The purified recombinant protein preparations were concentrated and subjected to endotoxin removal on a E-TOX column (Sterogene Bioseparations, Carlsbad, CA). Protein content of the preparations was determined by bicinchoninic acid using bovine serum albumin as a standard (Pierce, Rockford, IL), and the level of endotoxin contamination was determined with a Limulus amebocyte lysate QCL-1000 kit (Biowhittaker, Walkersville, MD). The recombinant antigens used in this study had ≤0.85 IU of endotoxin per µg of protein. In order to assess the purity of the recombinant antigens and confirm their identity, surface enhanced laser desorption/ionization time-of-flight mass spectrometry SELDI-TOF MS and tandem mass spectrometry (LC-MS/MS) analysis were performed as previously described (17).

**Immunization and animal challenge.**

Immunoprotection experiments were conducted with BALB/c mice (females, 8 weeks old) supplied by the National Cancer Institute (Bethesda, MD). Mice were immunized subcutaneously (s.c.) with either rAg2/Pra (1 µg), rPra2 (1 µg), or rAg2/Pra in combination with rPra2 (1 µg of each) plus CpG/IFA adjuvant by using the same protocol as previously described (28). The unmethylated CpG dinucleotides present in a synthetic oligodeoxynucleotide (ODN) preparation was used as an adjuvant (Integrated DNA Technologies, Inc., Coralville, Iowa). The CpG ODN sequence used to immunize mice was TCCATGACGGTCTCGACGT (CpG motifs are underlined). Mice were first immunized s.c. with either adjuvant alone in PBS (10 µg of CpG prepared in 50 µl of PBS plus 50 µl of incomplete Freund’s adjuvant [Sigma, St.Louis MO]) or with CpG adjuvant plus rAg2/Pra, rPra2, or rAg2/Pra in combination with rPra2. The mice were then boosted by s.c. immunization 14 days later with the same amount of immunogens
plus adjuvant. The animals were subsequently challenged with 84 viable arthroconidia from *C. posadasii* strain C735 by the i.n. route 4 weeks after the last immunization. Mice were scored for survival over a 50-day period post challenge. Survival differences between groups of i.n.-challenged mice were analyzed for statistical significance by the Kaplan-Meier method as previously reported (28). Fungal burden in the lungs and spleen of challenged mice were evaluated at the termination of the experiment on surviving animals. The CFU per organ were expressed on a log scale, and the independent-samples *t*-test was used to compare the means of the experimental groups. The lower limit of detection for the CFU assay is 10 colonies per organ homogenate (log$_{10}$ CFU = 1).

**Production of antiserum against rAg2/Pra and rPra2.**

Chromatographically isolated recombinant Ag2/Pra and Pra2 were used to immunize C57BL/6 and BALB/c mice (8 weeks old) for production of specific antiserum as reported previously (18). Briefly, groups of six mice (C57BL/6 and BALB/c) were immunized s.c. with either 2 µg of the purified rAg2/Pra or rPra2 in 50 µl of PBS, to which 50 µl of complete Freund’s adjuvant (Sigma) was added. The mice were boosted twice at 2-week intervals with the same amount of immunogen plus incomplete Freund’s adjuvant. Mice were sacrificed and exsanguinated by cardiac puncture at 2 weeks after the third boost. Antisera were combined from 3 mice per group to yield 2 pools of sera from each group for use in ELISA.

**IFN-γ enzyme-linked immunospot assay (ELISPOT).**

C57BL/6 and BALB/c mice were primed and boosted s.c. with PBS, 1 µg of rAg2/Pra, or rPra2 plus adjuvant (CpG in IFA) as described above. Murine splenocytes from 5 mice per group were pooled and CD90$^{-}$ and CD4$^{+}$ T cells were isolated from immune animals
for IFN-γ ELISPOT analysis. Immune and control CD4⁺ T cells were isolated by depletion of non-CD4⁺ T cells using a CD4⁺ T cell isolation kit (Miltenyi Biotec, Gladbach, Germany). CD90⁻ splenocytes were used as APCs and purified by depletion of CD90⁺ cells using anti-CD90 (Thy 1.2) microbeads (Miltenyi Biotec). The purity of the isolated CD4⁺ and CD90⁻ cell preparations were verified by staining with FITC conjugated anti-CD4 and anti-CD90 mAbs (Miltenyi Biotec) followed by analysis of the labeled cells using an Epics Elite Flow Cytometer (Beckman Coulter, Fullerton, CA).

Ninty-six well PVDF plates (Millipore, Bedford, MA) were coated overnight at 4°C with 50 µl/well of 15 µg/ml anti-murine IFN-γ mAb (AN18, Mabtech, Nacka Strand, Sweden). Wells were washed and unoccupied sites blocked with RPMI 1640 supplemented with 2% FCS (ATCC, Manassas, VA) for 2 h at 37°C under 5% CO₂. Each well was seeded with 2x10⁵ murine CD4⁺ splenocytes from either C57BL/6 or BALB/c mice which were immunized and boosted with rAg2/Pra, rPra2, or PBS as described above, suspended in 50 µl of RPMI 1640 supplemented with 10% FCS and 1% penicillin/streptomycin (ATCC). CD90⁻ splenocytes were isolated from either naïve C57BL/6 or BALB/c mice and used at a concentration of 2.5x10⁵ cells/well in 50 µl of RPMI 1640 supplemented with 10% FCS and 1% penicillin/streptomycin. Finally, ex vivo stimuli (recombinant proteins or peptides) were added in 50 µl of RPMI 1640 supplemented with 10% FCS and 1% penicillin/streptomycin. ELISPOT plates were incubated at 37°C for 24 hours under 5% CO₂. Wells were washed five times with 200 µl of PBS, before adding 100 µl of 1 µg/ml anti murine IFN-γ biotinylated mAb (R4-6A2-biotin, Mabtech) per well and allowed to incubate for 2 hours at room temperature. Wells were washed as above and 100 µl of strepavidin-alkaline phosphatase [1:1000] was
added to wells for 1 hour (Mabtech). Wells were washed as above and 100 µl of BCIP/NBT substrate solution (Mabtech) was added to each well until development was complete. Developed ELISPOT plates were allowed to dry overnight and were shipped to an ELISPOT plate evaluation service to be analyzed (Zellnet Consulting, Fort Lee, NJ). To control for non-specific background spots, all wells were corrected by subtracting the number of spots detected in an identically treated well that contained PBS-immune CD4+ T cells. The independent-samples t-test was used for statistical comparison of the mean number of IFN-γ spots per group.

**Enzyme linked immunosorbent assay (ELISA).**

Ninty-six well flat bottomed high binding polystyrene plates (Corning Incorporated Life Sciences, Acton, MA) were coated overnight at 4°C with 100 µl/well of 0.1 M carbonate buffer (pH 9.5) containing either 1 µg/ml recombinant protein or 3.5 µg/ml of peptide. Coated plates were washed 4x with 300 µl of phosphate buffered saline containing 0.05% Tween-20 (pH 7.4). Unoccupied sites were blocked with 200 µl of phosphate buffered saline supplemented with 10% FCS (ATCC, Manassas, VA) for 2 h at 25°C. Pre-immune, rAg2/Pra-immune, and rPra2-immune sera harvested from C57BL/6 and BALB/c mice (described above) were diluted in blocking buffer [1:250]. Diluted sera (100 µl) was added to each well and incubated for 2 hours at 25°C. Unbound antibody was removed by washing the wells 4x as described above. Detection of antigen specific murine IgG was achieved by incubating 100 µl/well of horseradish peroxidase conjugated goat α-mouse IgG heavy and light chain antibody [1:2000] for 1 hour at 25°C (Southern Biotech, Birmingham, AL). Unbound secondary antibody was removed by washing the wells 4x as described above. After washing, 100 µl of TMB substrate was added to each
well and allowed to react in the dark for thirty minutes at 25°C (BD Biosciences, Franklin Lakes, NJ). The enzymatic conversion of the TMB substrate was stopped by the addition of 50 µl of 2N H₂SO₄. Endpoint colorimetric analysis was performed by reading the absorbance of each sample at 450nm on a microplate reader (Molecular Devices, Sunnyvale, CA). The independent-samples t-test was used for statistical comparison of the mean absorbance readings between groups.

RESULTS

Epitope mapping of Ag2/Pra- and Pra2-specific CD4⁺ T cell responses induced by the immunization of C57BL/6 mice with full-length recombinant proteins

We previously established that the protective efficacy afforded by rAg2/Pra could be complemented by rPra2 in C57BL/6 mice (17). In this same report, ELISPOT analyses revealed that rAg2/Pra- and rPra2-immune CD4⁺ T cells from C57BL/6 mice respond to homologous but not heterologous full-length proteins by secreting IFN-γ. To further clarify this finding, C57BL/6 mice were primed and boosted s.c. with either 1µg of rAg2/Pra or rPra2 plus adjuvant (CpG in IFA) and CD4⁺ T cells were isolated from immune animals for epitope mapping using IFN-γ ELISPOT analysis. To facilitate the mapping of MHC II H-2b restricted epitopes, two synthetic peptide libraries were generated based on the amino acid sequences of Ag2/Pra and Pra2. The libraries consist of a series of icosameric peptides (20-mers) designed to overlap adjacent peptides by 12 amino acids (with the exception of 1P23 which overlaps 1P22 by 14 aa). The locations of the individual peptides are depicted to scale above graphical representations of Ag2/Pra and Pra2, which highlight the key features of each protein (Figure 1).
Abbreviated nomenclature, position on respective full-length antigen, and amino acid sequence for each individual 20-mer in the Ag2/Pra and Pra2 peptide libraries is listed in Table 1. The Ag2/Pra library and the Pra2 library are composed of 23 overlapping peptides (1P1-1P23) and 14 overlapping peptides (2P1-2P14), respectively. The number of immune CD4+ T cells secreting IFN-γ in response to ex vivo stimulation with full-length recombinant antigens and panels of individual icosamers was quantified (Figure 2). Stimulation of rAg2/Pra- and rPra2-immune CD4+ T cells (black bars and white bars, respectively) with full-length rAg2/Pra and rPra2 or their related peptides (1P1-1P23 and 1P1-2P14) reveals distinct patterns of recall response (Figure 2A and 2B). Consistent with our previous report, significant levels of IFN-γ producing cells are observed when rAg2/Pra- and rPra2-immune CD4+ T cells are stimulated with homologous but not heterologous full-length proteins. These striking differences in response to the full-length antigens are reflected by the recall profiles of rAg2/Pra- and rPra2-immune cells to their homologous and heterologous peptide panels. In comparison to the media control, rAg2/Pra-immune cells (black bars) demonstrate significant recall responses to rAg2/Pra, 1P7, 1P12, 1P13, 1P15, and 1P16 ($P \leq 0.0005$). Based on the pattern and intensity of these five reactive peptides we predict that they translate into three unique MHC II $H-2^b$ restricted epitopes (Table 2). The first of these three epitopes is characterized by a single reactive peptide (1P7), while the two remaining epitopes are each spanned by two reactive peptides (1P12 / 1P13 and 1P15 / 1P16). The prediction that 1P12 / 1P13 and 1P15 / 1P16 correspond to two epitopes is based on examination of the intensity of recall responses to these peptides coupled with the knowledge that naturally processed Th epitopes are usually 13-25 amino acids in length (34). Additional IFN-γ ELISPOT
analysis using rPra2-immune cells (white bars) revealed significant recall responses of to
rPra2, 1P6, 2P6, 2P13, and 2P14 when compared to the media control ($P \leq 0.003$). The
intensity and pattern of the recall responses to 2P6, 2P13, and 2P14 indicate that these
reactive peptides translate into two unique MHC II $H-2^b$ restricted epitopes of Pra2
(Table 2). Interestingly, rPra2-immune CD4$^+$ cells respond to a peptide derived from
Ag2/Pra (1P6) while rAg2/Pra-immune CD4$^+$ cells do not respond to 1P6 or 2P6. One
possible explanation for this finding could be the near identity of amino acids 43-57 of
Ag2/Pra and Pra2, which when compared have only one highly conserved amino acid
substitution. The similarity of these peptides may allow rPra2-immune CD4$^+$ T cells
specific for an $H-2^b$ restricted epitope (2P6) that was induced by immunization with full-
length rPra2 to also respond to 1P6 ex vivo.

Identification of continuous B cell epitopes of Ag2/Pra and Pra2 induced by the
immunization of C57BL/6 mice with full-length recombinant proteins

The same libraries of synthetic overlapping icosameric peptides for Ag2/Pra and Pra2
(Table 1) used for the T cell epitope mapping studies were immobilized on ELISA plates
and used to screen homologous and heterologous immune sera from C57BL/6 mice for
continuous B cell epitopes. Sera from C57BL/6 mice primed and boosted with either
rAg2/Pra or rPra2 was pooled and used to map the continuous B cell epitopes of Ag2/Pra
and Pra2. When tested by an immunoblot assay, $\alpha$-rAg2/Pra serum recognizes rAg2/Pra,
and to a lesser degree rPra2. In contrast, immunoblot analysis of $\alpha$-rPra2 serum indicates
that only homologous recombinant antigen is recognized (data not shown). To permit the
localization of continuous B cell epitopes, these antisera were tested against the
immobilized icosameric peptides spanning the entire amino acid sequences of Ag2/Pra
and Pra2. As shown in figure 3A and B, murine \( \alpha \)-rAg2/Pra (black bars) and \( \alpha \)-rPra2 (white bars) are capable of recognizing multiple peptides, indicating complex polyclonal responses to both proteins. The majority of the antibody-binding activity of the \( \alpha \)-rAg2/Pra serum (black bars) is restricted to a central immunogenic domain comprised of the reactive icosamers: 1P7, 1P9, 1P11, 1P13, 1P14, and 1P15 (Figure 3A). These peptides correspond to four discrete epitopes of Ag2/Pra (Table 2). A fifth continuous B cell epitope was identified by antibody recognition of 1P20 and is located near the C-terminus of Ag2/Pra. All of these peptides demonstrate significantly increased reactivity to the \( \alpha \)-rAg2/Pra serum compared to the control \((P \leq 0.03)\). As anticipated from the results of the immunoblot analysis, the antibody-binding activity of the \( \alpha \)-rAg2/Pra serum against the immobilized Pra2 icosamers revealed a continuous B cell epitope localizing to icosamer 2P7. Conversely, the antibody-binding activity of the \( \alpha \)-rPra2 serum (white bars) is restricted to two icosamers that are both derived from Pra2 (Figure 3B). These two reactive peptides, 2P7 and 2P11 demonstrate significantly increased reactivity to \( \alpha \)-rPra2 serum compared to the control \((P \leq 0.0001)\). These reactive icosamers represent two discrete continuous B cell epitopes of Pra2 (Table 2). Evidently, the immunization of C57BL/6 mice with rPra2 does not induce the production of antibody capable of recognizing heterologous peptides as was observed in assays utilizing \( \alpha \)-rAg2/Pra serum. However, it does appear that \( \alpha \)-rPra2 serum is capable of recognizing one or more discontinuous (conformational) B cell epitope/s in rAg2/Pra as evidenced by antibody binding to this full-length antigen (Figure 3A). Pre-immune control sera from C57BL/6 mice did not recognize full-length antigens or any of Ag2/Pra or Pra2 icosameric peptides (data not shown).
Evaluation of protective efficacy in BALB/c mice immunized with rAg2/Pra and rPra2 alone or in combination following intranasal challenge with *C. posadasii*.

Dramatic differences in susceptibility to coccidioidal disease following intranasal challenge with arthroconidia have been observed between several inbred strains of mice (BALB/c > C57BL/6 > DBA/2) (23). We decided to evaluate the protective efficacy of the combined of rAg2/Pra plus rPra2 vaccine using a murine model that is highly susceptible to intranasal challenge with *Coccidioides*. BALB/c mice were immunized with PBS, rAg2/Pra alone (1 µg), rPra2 alone (1 µg), or the combination of rAg2/Pra plus rPra2 (1 µg of each) in adjuvant, followed by i.n. challenge with a lethal inoculum of *C. posadasii* (84 viable arthroconidia, strain C735). Survival was scored over the course of 50 days post-challenge, and fungal burden in the lungs and spleen was assessed in all surviving animals upon termination of the experiment (day 50). The survival curve data for the experiment is presented graphically in figure 4. Mice immunized with 1 µg of rAg2/Pra (open triangles) demonstrate improved survival compared to control mice immunized with adjuvant alone (shaded circles). However, this moderately increased rate of survival was not found to be significantly different than the survival rate of control mice (*P*≤ 0.122). The final percentage of rAg2/Pra-immune mice surviving at day 50 post-challenge was 30%. The percentage of surviving mice immunized with 1 µg of rPra2 (shaded squares) was found to be identical to that of the control group (10%). Mice immunized with the combination of 1 µg rAg2/Pra plus 1 µg rPra2 (open diamonds) demonstrate a significant improvement in their rate of survival compared to the adjuvant control group (*P*≤ 0.049). The final percentage of surviving rAg2/Pra plus rPra2-immune (1 µg of each) mice at day 50 post-challenge was 60%. Upon conclusion of the survival
experiment fungal burden was assessed in the lungs and spleen of all surviving animals. There was no detectable fungal burden in the spleens of any surviving mice sacrificed at 50 days post-challenge. However, substantial pulmonary fungal burden (4-5 log<sub>10</sub>) remained in four of the six surviving mice immunized with rAg2/Pra plus rPra2 (data not shown).

**IFN-γ ELISPOT analysis of rAg2/Pra-and rPra2-immune CD4<sup>+</sup> T cells from BALB/c mice**

BALB/c mice were immunized as described above and CD4<sup>+</sup> T cells were isolated from rAg2/Pra- and rPra2-immune animals for ELISPOT analysis. The number of CD4<sup>+</sup> T cells secreting IFN-γ in response to *ex vivo* stimulation with varying concentrations of homologous or heterologous full-length recombinant antigen was quantified (Figure 5). These data show that compared to the media control, significant numbers rAg2/Pra-immune CD4<sup>+</sup> T cells from BALB/c mice (black bars) secrete IFN-γ in response to *ex vivo* stimulation with various amounts of full length homologous antigen (*P* ≤ 0.012). Stimulation of rAg2/Pra-immune CD4<sup>+</sup> cells *ex vivo* with heterologous antigen concentrations of 1 µg-10 µg/mL resulted responses which were less intense, but still significantly higher than the media controls (*P* ≤ 0.0018). Similar results were demonstrated in the corresponding experiment utilizing CD4<sup>+</sup> T cells from rPra2-immune C57BL/6 mice (white bars) stimulated *ex vivo* with varying concentrations of homologous and heterologous antigen. In comparison to media controls, significant numbers of CD4<sup>+</sup> T cells from rPra2-immune BALB/c mice were shown to secrete IFN-γ in response to stimulation with all concentrations of homologous antigen tested (*P* ≤ 0.0013) and concentrations ranging from 1 µg-10 µg/mL of heterologous antigen (*P* ≤ 0.0013).
With the exception of 0.1 µg/mL rPra2, the strength of responses to ex vivo stimulation of immune CD4+ T cells with several concentrations of heterologous recombinant antigen was found to be significantly less than observed with homologous antigen ($P \leq 0.04$).

**Epitope mapping of Ag2/Pra- and Pra2-specific CD4+ T cell responses induced by the immunization of BALB/c mice with full-length recombinant proteins**

Panels of overlapping icosamers corresponding to Ag2/Pra and Pra2 (Table 1) were used to stimulate immune CD4+ T cells to facilitate identification of MHC II $H-2^d$ restricted T cell epitopes induced by immunization of BALB/c mice with either rAg2/Pra or rPra2 (Figure 6). In contrast to the previous C57BL/6 epitope mapping results, significant numbers of IFN-γ producing cells are observed when rAg2/Pra- and rPra2-immune CD4+ T cells are stimulated with homologous and heterologous full-length antigens. As expected, the recall profiles of rAg2/Pra- and rPra2-immune cells to their homologous and heterologous peptide panels are congruent with the responses to the full-length antigens. In comparison to the media control, rAg2/Pra-immune cells (black bars) demonstrate significant recall responses to rAg2/Pra, rPra2, 1P1, 1P8, 1P9, 1P10, and 2P10 ($P \leq 0.022$). These five peptides translate into two MHC II $H-2^d$ restricted epitopes that are exclusive to Ag2/Pra, and a common MHC II $H-2^d$ restricted epitope induced by immunization with either rAg2/Pra or rPra2 (Table 3). The corresponding experiment using rPra2-immune CD4+ T cells (white bars) revealed significant recall responses to rPra2, rAg2/Pra, 2P1, 2P9, 1P9, 2P10, and 1P10 compared to the media control ($P \leq 0.0016$). Our prediction is that these results are indicative of a Pra2 specific MHC II $H-2^d$
restricted epitope, and a common MHC II $H-2^d$ restricted epitope induced by immunization with either rAg2/Pra or rPra2 (Table 3).

**Identification of continuous B cell epitopes of Ag2/Pra and Pra2 induced by the immunization of BALB/c mice with full-length recombinant proteins**

Panels of Ag2/Pra and Pra2 derived icosameric peptides (Table 1) identical to those employed in previous mapping experiments, were used to identify continuous B cell epitopes of Ag2/Pra and Pra2 induced by immunization of BALB/c mice with full-length recombinant proteins. When tested by an immunoblot assays, both $\alpha$-rAg2/Pra and $\alpha$-rPra2 sera (raised in BALB/c mice) recognize homologous and to a lesser degree heterologous full-length antigen (data not shown). These antisera were tested against the immobilized icosameric peptides spanning the entire amino acid sequences of Ag2/Pra and Pra2. As shown in figure 7, murine $\alpha$-rAg2/Pra (black bars) and $\alpha$-rPra2 (white bars) are capable of recognizing multiple icosameric peptides, indicating complex polyclonal responses to both proteins. Analysis of antibody-binding activity of $\alpha$-rAg2/Pra serum (black bars) to Ag2/Pra- and Pra2-derived icosamers revealed that 1P5-1P9, 1P11-1P17, 1P19, 1P20, and 2P7 demonstrate significantly increased reactivity compared controls ($P \leq 0.016$). The reactive Ag2/Pra peptides correspond to seven discrete continuous B cell epitopes of Ag2/Pra recognized by homologous antiserum (Table 3). As anticipated from the results of the immunoblot analysis, the antibody-binding activity of the $\alpha$-rAg2/Pra serum against the immobilized Pra2 peptides revealed a continuous B cell epitope localizing to icosamer 2P7 (Figure 7B). Analysis of antibody-binding activity of $\alpha$-rPra2 serum (white bars) to Pra2 and Ag2/Pra derived icosamers indicated that 2P1, 2P6, 2P7, 2P11, 2P14, and 1P7 demonstrate significantly
increased reactivity compared to controls ($P \leq 0.032$). The reactive Pra2 icosamers represent four discrete continuous B cell epitopes of Pra2 recognized by homologous serum (Table 3). Giving further support to the results of the immunoblot analyses, the antibody-binding activity of the $\alpha$-Pra2 serum against the immobilized Ag2/Pra peptides revealed a continuous B cell epitope localizing to icosamer 1P7 (Figure 7A). Pre-immune control sera from BALB/c mice did not recognize full-length antigens or any Ag2/Pra or Pra2 icosameric peptides (data not shown).

**DISCUSSION**

Previously, we demonstrated that, compared to vaccination with either of the single antigens, a combined rAg2/Pra plus rPra2 vaccine provides enhanced protection to C57BL/6 mice against a lethal intranasal challenge with *Coccidioides* (17). We believe that despite their closely related primary structures (69% identity, 91% similarity), there are significant differences in the fine specificities of immune responses to rAg2/Pra and rPra2, that contribute to their ability to complement each other in a combined recombinant vaccine. To explore this possibility, we chose to initially focus on the characterization of CD4$^+$ T cell populations induced by immunization of C57BL/6 mice ($H-2^b$) with either rAg2/Pra or rPra2. This decision was based on considerable evidence that cell mediated immunity is essential for resistance to coccidioidomycosis (3-5, 7, 12, 15, 25). Our initial findings revealed that rAg2/Pra- and rPra2-immune CD4$^+$ T cells from C57BL/6 mice respond to *ex vivo* stimulation with homologous but not heterologous full-length protein (17). In order to extend these findings we decided to map the exact locations of Ag2/Pra and Pra2 MHC II $H-2^b$ restricted epitopes. IFN-$\gamma$
ELISPOT assays were utilized to detect recall responses of rAg2/Pra- and rPra2-immune CD4+ T cells stimulated with panels of individual peptides derived from the amino acid sequences of Ag2/Pra and Pra2. Analyses of rAg2/Pra-immune CD4+ T cells, revealed significant recall responses to five icosameric peptides corresponding to three distinct MHC II H-2b restricted epitopes of Ag2/Pra (Figure 2A, Table 2). The reciprocal experiment assessing recall responses of rPra2-immune CD4+ T cells in response to ex vivo stimulation with panels of individual peptides identified two distinct MHC II H-2b restricted epitopes of Pra2 (Figure 2B, Table 2). These results indicate that while the amino acid sequences of Ag2/Pra and Pra2 are very similar, immunization of C57BL/6 mice with either recombinant antigen results in the induction of a population of CD4+ T cells that exhibits specificity for MHC II H-2b restricted epitopes derived only from the recombinant protein used for vaccination.

To further investigate the level of antigenic diversity between Ag2/Pra and Pra2, we also mapped the continuous B cell epitopes recognized by sera from C57BL/6 mice immunized with either rAg2/Pra or rPra2. Evaluation of the reactivity of immobilized Ag2/Pra and Pra2 derived icosameric peptides with α-rAg2/Pra and α-rPra2 sera by ELISA revealed the presence of seven continuous B cell epitopes. Five of these continuous B cell epitopes were identified within Ag2/Pra and were only recognized by homologous antisera. Conversely, one of the two continuous B cell epitopes found in Pra2 was recognized by both homologous and heterologous sera (Figure 3B, Table 2). These results correlate with our previous immunoblot analyses which demonstrate that rAg2/Pra- but not rPra2-immune sera are capable of recognizing heterologous antigen. The combined results of the continuous B cell and MHC II H-2b restricted epitope
mapping of Ag2/Pra and Pra2 provide clear support for our previous supposition that C57BL/6 mice mount diverse antigenic responses to both antigens.

Further analysis of the combined data from these mapping studies has revealed the presence of a potentially important 28 amino acid reactive domain (aa 41-68) within Ag2/Pra and Pra2 which is highly conserved between these two antigens (82 % identity, 96 % similarity). This domain contains both MHC II \( H-2^b \) restricted (1P7, 2P6) and continuous B cell epitopes (1P7, 2P7) of both antigens. The work of other investigators has revealed that peptides containing both B cell and T cell epitopes are valuable in the development of peptide based vaccines because they are able to autonomously trigger T- and B cell cooperation \textit{in vivo} (6). In the future, this constellation of Ag2/Pra and Pra2 epitopes may prove to be valuable in the development of a multivalent, peptide-based vaccine against coccidioidomycosis.

In contrast to the protective efficacy observed in C57BL/6 mice, subcutaneous immunization with rAg2/Pra alone does not provide significant protection to BALB/c mice (as assessed by enhanced survival or reduction of pulmonary fungal burden) against an intranasal challenge of eight or more arthroconidia (36). This single study on the protective efficacy of immunization of BALB/c mice with rAg2/Pra followed by an intranasal challenge reported that vaccinated mice challenged with seven arthroconidia demonstrated significantly increased survival compared to controls. However, none of the surviving mice from the control group or the rAg2/Pra-immune group showed evidence of infection at necropsy. This raises the question whether they had actually been infected or that few or none of the seven arthroconidia in the inoculum actually made it beyond the upper airways of these mice. The lack of protective efficacy afforded
BALB/c mice by subcutaneous immunization with rAg2/Pra may be due to the differences in susceptibility of inbred strains of mice to coccidioidal disease following intranasal challenge (BALB/c > C57BL/6 > DBA/2) (23). Even though the level of protective efficacy provided by subcutaneous vaccination of BALB/c mice with rAg2/Pra alone is questionable, we were encouraged by the results of our previous protection experiments using C57BL/6 mice (17). Therefore we decided it would be valuable to evaluate the protective efficacy of rAg2/Pra and rPra2 singly and in combination following intranasal challenge in a highly susceptible murine model of coccidioidomycosis. To increase the stringency of this study, we elected to challenge BALB/c mice by the intranasal route with more than ten times the minimal number of arthroconidia previously shown to abrogate the protective effects of immunization with rAg2/Pra (84 arthroconidia). Our studies confirmed the findings of Shubitz et al. (36) that BALB/c mice immunized with rAg2/Pra alone (1 µg) do not demonstrate significantly increased survival compared to adjuvant-immunized control mice following an intranasal challenge with greater than seven arthroconidia of Coccidioides. Similarly, we did not observe a significant improvement in the survival of rPra2-immune mice (1 µg) compared to adjuvant controls. In contrast, BALB/c mice immunized with the combination of rAg2/Pra (1 µg) plus rPra2 (1 µg) did demonstrate significantly enhanced survival in comparison to the adjuvant control group. This is the first time that immunization with any recombinant protein(s) alone or in combination has significantly enhanced the survival of highly susceptible BALB/c mice following an intranasal challenge with more than seven arthroconidia of Coccidioides. More importantly, BALB/c mice immunized with either rAg2/Pra plus rPra2 or formalin-killed spherules
(FKS) exhibit comparable levels of survival following intranasal challenge with similar numbers of arthroconidia (84 versus 100) (10). At the conclusion of the survival experiment fungal burdens in the lungs and spleen of the remaining mice were determined. None of the surviving animals had detectable fungal burdens in their spleens, but 4 of the 6 surviving rAg2/Pra plus rPra2 immunized mice demonstrated significant fungal burden in their lungs. This result was not surprising since even immunization with FKS does not provide sterilizing immunity to BALB/c mice challenged by the intranasal route (40).

As previously observed in C57BL/6 mice, the immunization of BALB/c mice with rAg2/Pra plus rPra2 provides superior protection against intranasal challenge with *Coccidioides* than immunization with either of the single antigens. The additive effect of rAg2/Pra plus rPra2 may be due to the induction of heterogeneous immune responses to Ag2/Pra and Pra2 by BALB/c mice. To explore this possibility, CD4$^+$ T cells were isolated from rAg2/Pra- and rPra2-immune BALB/c mice for IFN-γ ELISPOT analysis. These assays revealed that rAg2/Pra-immune and rPra2-immune CD4$^+$ T cells display significant recall responses to *ex vivo* stimulation with full-length homologous and heterologous antigens (Figure 5). These results suggest that in contrast to what was observed with C57BL/6 mice (*H-2^b*), there are antigen specific as well as cross-reactive MHC II *H-2^d* restricted epitope(s) within Ag2/Pra and Pra2 that are induced by the immunization of BALB/c mice by these antigens. It is impossible to determine from these results the number of antigen specific and cross-reactive MHC II *H-2^d* restricted epitopes Ag2/Pra and Pra2 contain, but it is likely that both antigens harbor at least one unique epitope as evidenced by the higher IFN-γ recall responses to homologous antigens.
seen in figure 5. In order to determine the quantity and locations of the MHC II $H-2^d$ restricted epitopes within Ag2/Pra and Pra2, we decided to examine the recall responses of rAg2/Pra- and rPra2-immune CD4$^+$ T cells from BALB/c mice to *ex vivo* stimulation with panels of overlapping peptides derived from both antigens by IFN-γ ELISPOT. Analyses of rAg2/Pra-immune CD4$^+$ T cells revealed significant recall responses to five icosameric peptides corresponding to two unique MHC II $H-2^d$ restricted epitopes of Ag2/Pra and a single cross-reactive epitope shared by Ag2/Pra and Pra2 (Figure 6A, Table 3). The reciprocal experiment assessing recall responses of to *ex vivo* stimulation with panels of individual peptides confirmed the presence of the previously identified cross-reactive epitope shared by Ag2/Pra and Pra2. These same assays using rPra2-immune CD4$^+$ T cells also revealed a unique MHC II $H-2^d$ restricted epitope of Pra2 (Figure 6B, Table 3). Two of the three unique MHC II $H-2^d$ restricted epitopes identified in Ag2/Pra and Pra2 are associated with the predicted signal peptides of each of these antigens (1P1 and 2P1). Interestingly, the signal peptide of Ag2/Pra, administered to BALB/c mice as a gene vaccine or synthetic peptide, has been shown to induce protective immunity against an intraperitoneal *Coccidioides* challenge (20). These results demonstrate that immunization of BALB/c mice with either rAg2/Pra or rPra2 leads to the induction CD4$^+$ T cell populations displaying specificity for MHC II $H-2^d$ restricted epitopes derived from homologous and heterologous antigens.

To further characterize the immune responses to rAg2/Pra and rPra2 by BALB/c mice, we decided to map the continuous B cell epitopes recognized by immune sera from BALB/c mice. Evaluation of the reactivity of immobilized icosamers with α-rAg2/Pra and α-rPra2 sera by ELISA revealed the presence of 11 continuous B cell epitopes
Six of the seven continuous B cell epitopes identified within Ag2/Pra were only recognized by homologous sera. The seventh continuous B cell epitope located within amino acids 41-68 of Ag2/Pra and Pra2 (peptides 1P6, 1P7, 2P6, and 2P7) is recognized by both homologous and heterologous sera. This cross-reactive continuous B cell epitope of Ag2/Pra and Pra2 was one of four epitopes recognized by rPra2-immune sera raised in BALB/c mice (Figure 7B, Table 3). The other three continuous B cell epitopes identified in Pra2 were only recognized by homologous sera. These findings are supported by immunoblot results which demonstrate that rAg2/Pra- and rPra2-immune sera recognize both homologous and heterologous full-length antigen. The combined continuous B cell and MHC II H-2d restricted epitope mapping of Ag2/Pra and Pra2 provides evidence of unique and conserved antigenic responses to these proteins by BALB/c mice.

There are numerous reports in the literature on the protective efficacy of recombinant Coccidioides antigens (8, 13, 19, 26, 28) (Yu, J. J. et al. Abstract ASM 103 Gen Meeting 2003; poster F-111). None of these reports has provided clear evidence of the ability of any single recombinant vaccine candidate to adequately protect both C57BL/6 and BALB/c mice from intranasal challenge with Coccidioides. In direct contrast to these findings, multiple investigators have demonstrated that immunization of all strains of inbred mice tested can be protected from intranasal challenge by immunization with either a whole killed cell (FKS) or a complex cell extract from parasitic Coccidioides cultures (27K fraction) (10, 11, 21, 27, 40). There are two fundamental reasons why whole killed cells and the 27K fraction have been unilaterally more effective than monovalent recombinant protein vaccines. The first is that both the FKS and 27K
vaccines display adjuvant-like properties due to microbial products they contain (2, 10, 21, 27, 37, 40). The second and more important factor is the complex array of *Coccidioides* antigens that the immune system is exposed to by immunization with these vaccines. The complex nature of these vaccines provides the antigenic diversity necessary for the induction of comprehensive immune responses against *Coccidioides* by all individuals in a heterogeneous population. Unfortunately, evaluation of the FKS vaccine in a human population revealed that it was ineffective (32). Presumably this was due to the fact that only 1/1000th of the dose per kilogram of body weight that was protective in mice was tolerated in humans (2, 37). The failure of this vaccine trial coupled with the undefined nature and compositional variability of FKS and the 27K fraction has necessitated a shift in the coccidioidomycosis vaccine development strategy. The current belief is that a successful vaccine against coccidioidomycosis will be composed of multiple recombinantly expressed *Coccidioides* proteins, which can provide the necessary antigenic diversity needed to induce protective immunity against *Coccidioides* in a heterogeneous population. Progress towards this goal has been made by the discovery and subsequent evaluation several recombinantly expressed proteins of *Coccidioides* as monovalent vaccines. These studies have identified recombinant vaccine candidates that confer varying degrees of protection against coccidioidal challenge dependant on the strain of inbred mouse, route of challenge, and size of inoculum used (1, 2, 13, 21, 22, 24, 26, 28, 36, 37). The evaluation of combinations of the monovalent candidates is a compulsory next step in the development of a multivalent vaccine against coccidioidomycosis.
We have begun this work by demonstrating that compared to vaccination with either of the single antigens a combined rAg2/Pra plus rPra2 vaccine provides enhanced protection to C57BL/6 and BALB/c mice against a lethal intranasal challenge with *Coccidioides* (17). The results of this paper provide evidence of diverse immune responses induced by these two vaccine candidates in both C57BL/6 and BALB/c mice, which provide these animals with a larger *Coccidioides* specific immune repertoire than immunization with either of the single antigens. Although these results are very promising, we realize that the epitopes we have identified within Ag2/Pra and Pra2 may be genetically restricted. Therefore it is probable that mice with different haplotypes (other than *H-2b* and *H-2d*) will differ in their ability to present these or other epitopes of these two antigens. At this point it is impossible to ascertain the optimal number of recombinant proteins needed to provide a heterogeneous population with the antigenic diversity necessary for the induction of a protective immune response against *Coccidioides*. It is very likely that future investigations will have to focus on the identification of non-genetically restricted epitopes as well as rational design of recombinant chimeric antigens to make the production of an effective multivalent vaccine against *Coccidioides* economically feasible.
REFERENCES


**Table 1:** Ag2/Pra and Pra2 peptide libraries used for Th and continuous B cell epitope mapping studies.

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Table 2: Summary of $H-2^b$ restricted Th epitopes and continuous B cell epitopes.

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122
Table 3: Summary of $H-2^d$ restricted Th epitopes and continuous B cell epitopes.

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FIGURES:

Figure 1: Locations of overlapping icosamers derived from the amino acid sequences of Ag2/Pra and Pra2. The positions of all 37 synthetic peptides are indicated above scale representations of Ag2/Pra and Pra2. These diagrams illustrate the presence of predicted signal peptides and conserved fungal extracellular membrane (CFEM) domains Ag2/Pra and Pra2. Tandem TXX’P repeats and a predicted GPI-anchor unique to the C-terminal of Ag2/Pra are also depicted above.
Figure 2: Ex vivo identification of Ag2/Pra and Pra2 H-2b restricted Th epitopes. CD4+ T cells harvested from C57BL/6 mice immunized with either rAg2/Pra or rPra2 (black and white bars, respectively) were assayed for the presence of IFN-γ recall responses to ex vivo stimulation with overlapping synthetic peptides. Results are shown as number of spots per 2 x 10^5 immune splenic CD4+ T-cells and are representative of two independent experiments. (A) ELISPOT assays of IFN-γ production by rAg2/Pra- and rPra2-immune CD4+ T cells harvested from C57BL/6 mice, in response to ex vivo stimulation with overlapping synthetic peptides derived from Ag2/Pra. (B) ELISPOT assays of IFN-γ production by rAg2/Pra- and rPra2-immune CD4+ T cells harvested from C57BL/6 mice, in response to ex vivo stimulation with overlapping synthetic peptides derived from Pra2.
Figure 2: *Ex vivo* identification of Ag2/Pra and Pra2 H-2b restricted Th epitopes. CD4*+* T cells harvested from C57BL/6 mice immunized with either rAg2/Pra or rPra2 (black and white bars, respectively) were assayed for the presence of IFN-γ recall responses to *ex vivo* stimulation with overlapping synthetic peptides. Results are shown as number of spots per 2 x 10^5 immune splenic CD4*+* T-cells and are representative of two independent experiments. (A) ELISPOT assays of IFN-γ production by rAg2/Pra- and rPra2-immune CD4*+* T cells harvested from C57BL/6 mice, in response to *ex vivo* stimulation with overlapping synthetic peptides derived from Ag2/Pra. (B) ELISPOT assays of IFN-γ production by rAg2/Pra- and rPra2-immune CD4*+* T cells harvested from C57BL/6 mice, in response to *ex vivo* stimulation with overlapping synthetic peptides derived from Pra2.
Figure 3: Identification of continuous B cell epitopes of Ag2/Pra and Pra2 induced by the immunization of C57BL/6 mice with full-length recombinant proteins. Full-length antigen and peptide specific IgG responses induced by vaccination with either rAg2/Pra or rPra2 were determined by ELISA. Antisera from rAg2/Pra- or rPra2-immune C57BL/6 mice [1:250] (black and white bars, respectively) were screened for IgG antibody-binding activity to panels of icosameric peptides using horseradish peroxidase conjugated α-murine IgG (H+L) [1:2000] secondary antibody. IgG responses of pre-immune control sera to full-length proteins and peptides were all less than 0.05 O.D. units. Results are representative of three independent experiments. (A) ELISA assays of IgG antibody-binding activity of α-rAg2/Pra and α-rPra2 sera raised in C57BL/6 mice to overlapping synthetic peptides derived from Ag2/Pra. (B) ELISA assays of IgG antibody-binding activity of α-rAg2/Pra and α-rPra2 sera raised in C57BL/6 mice to overlapping synthetic peptides derived from Pra2.
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Figure 4: Evaluation of protective efficacy of rAg2/Pra and rPra2 alone and in combination in BALB/c mice challenged by the i.n. route with C. posadasii.

BALB/c mice were immunized by the s.c. route with either PBS, rAg2/Pra (1 µg), rPra2 (1 µg), or a combination of rAg2/Pra plus rPra2 (1 µg of each) in adjuvant (n=10 mice per group). Immunized mice were challenged by the i.n. route with C. posadasii (84 viable arthroconidia), and mortality was determined at days 1 through 50 post-challenge. Survival differences between groups of i.n.-challenged mice were analyzed for statistical significance by the Kaplan-Meier method.
**Figure 5:** *Ex vivo* identification of Ag2/Pra and Pra2 *H-2d* restricted Th epitopes. CD4⁺ T cells harvested from BALB/c mice immunized with either rAg2/Pra or rPra2 (black and white bars, respectively) were assayed for the presence of IFN-γ recall responses to *ex vivo* stimulation with overlapping synthetic peptides. Results are shown as number of spots per 2 x 10⁵ immune splenic CD4⁺ T-cells and are representative of two independent experiments. (A) ELISPOT assays of IFN-γ production by rAg2/Pra- and rPra2-immune CD4⁺ T cells harvested from BALB/c mice, in response to *ex vivo* stimulation with overlapping synthetic peptides derived from Ag2/Pra. (B) ELISPOT assays of IFN-γ production by rAg2/Pra- and rPra2-immune CD4⁺ T cells harvested from BALB/c mice, in response to *ex vivo* stimulation with overlapping synthetic peptides derived from Pra2.
Figure 6: Ex vivo identification of Ag2/Pra and Pra2 H-2d restricted Th epitopes. CD4+ T cells harvested from BALB/c mice immunized with either rAg2/Pra or rPra2 (black and white bars, respectively) were assayed for the presence of IFN-γ recall responses to ex vivo stimulation with overlapping synthetic peptides. Results are shown as number of spots per 2 x 10^5 immune splenic CD4+ T-cells and are representative of two independent experiments. (A) ELISPOT assays of IFN-γ production by rAg2/Pra- and rPra2-immune CD4+ T cells from BALB/c mice in response to ex vivo stimulation with overlapping synthetic peptides derived from Ag2/Pra. (B) ELISPOT assays of IFN-γ production by rAg2/Pra- and rPra2-immune CD4+ T cells from BALB/c mice in response to ex vivo stimulation with overlapping synthetic peptides derived from Pra2.
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Figure 7: Identification of continuous B cell epitopes of Ag2/Pra and Pra2 induced by the immunization of BALB/c mice with full-length recombinant proteins. Full-length antigen and peptide specific IgG responses induced by vaccination with either rAg2/Pra or rPra2 were determined by ELISA. Antisera from rAg2/Pra- or rPra2-immune BALB/c mice [1:250] (black and white bars, respectively) were screened for IgG antibody-binding activity to panels of icosameric peptides using horseradish peroxidase conjugated α-murine IgG (H+L) [1:2000] secondary antibody. IgG responses of pre-immune control sera to full-length proteins and peptides were all less than 0.05 O.D. units. Results are representative of three independent experiments. (A) ELISA assays of IgG antibody-binding activity of α-rAg2/Pra and α-rPra2 sera raised in BALB/c mice to overlapping synthetic peptides derived from Ag2/Pra. (B) ELISA assays of IgG antibody-binding activity of α-rAg2/Pra and α-rPra2 sera raised in BALB/c mice to overlapping synthetic peptides derived from Pra2.
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Generation of Three Divalent Chimeric Vaccines Derived From Ag2/Pra and Pra2 and Evaluation of Their Protective Efficacy Against Coccidioidal Challenge

R. A. Herr¹, C. Y. Hung¹, M. J. Gardner², G. T. Cole¹

¹Medical University of Ohio, Toledo, OH and ²The Institute for Genomic Research, Rockville, MD.
ABSTRACT

Previously we have demonstrated that administration of a combined rAg2/Pra plus rPra2 vaccine provides superior protection to C57BL/6 mice against a lethal intranasal challenge with *Coccidioides* than vaccination with either of the single antigens (14, 15). The subsequent mapping of the Th and continuous B cell epitopes of Ag2/Pra and Pra2 induced by the immunization of C57BL/6 (*H-2*b) mice with either rAg2/Pra or rPra2 facilitated the rational design of three divalent chimeric antigens composed of immunoreactive domains selected from both antigens. The selected immunoreactive domains from Ag2/Pra and Pra2 were combined using an overlap PCR strategy to yield the divalent chimeric expression constructs encoding C1, C2, and C3. The most inclusive divalent chimeric vaccine construct that we generated is C1, whose translated product contains all of the Th and continuous B cell epitopes induced by immunization of C57BL/6 mice with rAg2/Pra and rPra2. The translated products of the divalent chimeric vaccines C2 and C3 were each respectively less inclusive containing fewer Ag2/Pra and Pra2 Th and continuous B cell epitopes. The three recombinantly expressed and purified divalent chimeric antigens were evaluated for their ability to protect C57BL/6 mice from both sub-lethal and lethal intranasal challenges with *Coccidioides* (47 and 90 arthroconidia, respectively). The results of these protection experiments provide evidence that the three divalent recombinant chimeric antigens we designed afford a level of protective efficacy that in some cases is statistically no different than the full-length rAg2/Pra plus rPra2 divalent vaccine. However, comparisons of pulmonary fungal burdens (sub-lethal challenge) and survival rates (lethal challenge) indicate that the chimeric antigens in all cases are noticeably (and in some cases statistically) less
protective than the combination of rAg2/Pra plus rPra2. These observations suggest that, though we have successfully reconstituted a portion of the protective efficacy that the full-length divalent vaccine (rAg2/Pra plus rPra2) provides C57BL/6 mice, the current immunization protocol and/or design of our divalent chimeric vaccines may not be optimal.

INTRODUCTION

*Coccidioides spp.* are dimorphic fungal respiratory pathogens which grow saprobically in the dry alkaline soils of the desert regions of North America, Mexico, and scattered areas of South America (28, 30). Disruption of the soil in these areas causes aerosolization of arthroconidia, which when inhaled initiate the parasitic life cycle of this fungus. *Coccidioides spp.* are primary pathogens capable of causing serious pulmonary and sometimes fatal disseminated disease in immunocompetent people visiting or living in endemic regions. Based on data from skin test surveys, it is believed that 25,000 to 100,000 infections are acquired annually in the United States alone (10, 31). Although most cases of pulmonary coccidioidomycosis are asymptomatic or present as a mild self-limiting flu-like illness, a number of patients develop persistent pulmonary or chronic progressive pulmonary coccidioidomycosis, and in rare instances life threatening extrapulmonary dissemination occurs (30, 35, 36). Currently it is estimated that the annual cost of health care for management of patients with coccidioidomycosis in the United States is 60 million dollars (www.valleyfever.com). It is important to note that individuals who develop coccidioidomycosis and recover are resistant to future infections with *Coccidioides* (32, 35, 36). On this basis it is argued that a human vaccine against
coccidioidal infections can be produced (6, 7). The geographic limitation of the disease, cost of patient treatment, and evidence that natural coccidioidal infection provides lifelong immunity has encouraged investigators to pursue the development of a vaccine against coccidioidomycosis. The current belief is that a successful vaccine against coccidioidomycosis will be composed of multiple recombinantly expressed Coccidioides proteins, which can provide the necessary antigenic diversity needed to induce protective immunity against Coccidioides in a heterogeneous population. Progress towards this goal has been made by the discovery and subsequent evaluation of several recombinantly expressed proteins of Coccidioides as monovalent vaccines. These studies have identified recombinant vaccine candidates that confer varying degrees of protection against coccidioidal challenge dependant on the strain of inbred mouse, route of challenge, and size of inoculum used (1, 9, 15, 20, 21, 23, 25, 27, 34). The evaluation of combinations of the monovalent candidates is a logical next step in the development of a vaccine against coccidioidomycosis. We have begun this work by demonstrating that compared to vaccination with either of the single antigens, a combined rAg2/Pra plus rPra2 vaccine provides enhanced protection to both C57BL/6 and BALB/c mice against a lethal intranasal challenge with Coccidioides (14, 15). Subsequent mapping of the Th and continuous B cell epitopes of Ag2/Pra and Pra2 induced by the immunization of C57BL/6 (H-2b) or BALB/c (H-2d) mice with either rAg2/Pra or rPra2 revealed significant differences in the fine specificities of immune responses to these antigens (14). These data support our hypothesis that immunization with multiple antigenically diverse recombinant Coccidioides proteins results in the induction of a larger Coccidioides specific immune repertoire, which is able to provide a higher level of
protection than immunization with any known single antigen. In the current study, we have attempted to extend our understanding of the ability of rPra2 to complement the protective capacity of rAg2/Pra in a multivalent vaccine by designing three divalent chimeric antigens composed of various combinations of previously identified immunologically reactive domains of each antigen. These immunologically reactive domains of Ag2/Pra and Pra2 were previously identified by epitope mapping studies and are known to harbor ($H-2^b$) restricted Th and continuous B cell epitopes. These divalent chimeric antigens were recombinantly expressed and evaluated for their ability to protect C57BL/6 mice from an intranasal challenge with \textit{Coccidioides}. Due to the extreme level of susceptibility of BALB/c mice to intranasal coccidioidal challenge we have focused our efforts in this study on the evaluation of divalent chimeric antigens designed expressly to protect intermediately susceptible C57BL/6 mice (22).

**MATERIALS AND METHODS**

**Mice.**

Eight- to 10-wk-old female C57BL/6 ($H-2^b$) mice from the National Cancer Institute (Bethesda, MD) were used for all of the following experiments. Animals were maintained in an American Association for the Accreditation of Laboratory Animal Care-approved animal facility.

**Strains, media, and growth conditions.**

\textit{Coccidioides posadasii} isolate C735 was used throughout this study, and was originally designated as \textit{C. immitis} (12). The strain was isolated from a patient with disseminated coccidioidomycosis. The saprobic phase of the fungus was grown \textit{in vitro} on GYE agar
(1% glucose, 0.5% yeast extract, 1.5% agar) at 30°C for 3 to 4 weeks prior to harvesting arthroconidia used in animal challenge experiments.

**Engineering chimeric PRA expression constructs.**

Three chimeric PRA expression constructs were generated using an overlap extension polymerase chain reaction (PCR) strategy (16). Each of these chimeric constructs is composed of three discrete nucleotide domains that have been joined by overlap extension PCR. The first of these domains is common to all three constructs and is derived from the pET32b expression plasmid (Novagen, Madison, WI). This nucleotide domain was designated as (UN) and was amplified by PCR using empty pET32b as template and Platinum Pfx high fidelity polymerase (Invitrogen, Carlsbad, CA) with primers #1 and #3 (Table 1). The amplified product is 538 bp in length and contains the T7 promoter, and nucleotide sequences encoding the thioredoxin domain, and thrombin cleavage site of pET32b. The second of the nucleotide domains used in all three of the chimeric constructs is derived from the coding sequence of AG2/PRA. The first chimeric construct (C1) contains a domain we have designated 1A, it is 412 bp and was amplified using AG2/PRA cDNA as template and Platinum Pfx polymerase with primers #4 and #5 (Table 1). The second and third chimeric constructs (C2 and C3) contain an AG2/PRA domain which we have designated 1B. This domain is 184 bp and was also amplified using AG2/PRA cDNA as template and Platinum Pfx polymerase utilizing primers #4 and #7 (C2) or #9 (C3) (Table 1). The reverse primers used to amplify 1B (#7 and #9) possess different 5’ overhangs for utilization in our overlap PCR strategy but both amplify identical product when paired with primer #4. The third nucleotide domain used in all three of the chimeric constructs is derived from the coding sequence of PRA2. The
first and second chimeric constructs (C1 and C2) contain a domain we have designated 2B, it is 371 bp and was amplified using PRA2 cDNA as template and Platinum Pfx polymerase with primers #6 (C1) or #8 (C2) and #12 (Table 1). The forward primers used to amplify 2B (#6 and #8) have different 5’ overhangs for utilization in our overlap PCR strategy, but both amplify identical product when paired with primer #12. The third chimeric construct (C3) contains a PRA2 domain which we have designated 2A. This domain is 256 bp and was also amplified using PRA2 cDNA as template and Platinum Pfx polymerase utilizing primers #10 and #12 (Table 1). Once all of the individual PCR products required to generate the three chimeric PRA expression constructs were amplified they were all gel purified and used as components of the overlap extension PCR reactions for C1, C2, and C3. Briefly, nested primers specific for the 5’ end of the common pET32b amplicon (#2) and for the 3’ end of the PRA2 amplicons (#11) were used in combination with the appropriate gel purified products generated above in overlap PCR reactions for C1, C2, and C3. The overlap PCR reaction for C1 contained approximately 100 ng each of UN, 1A, and 2B (amplified with #6 and #12) and 5 µl 10X amplification buffer, 1.5 µl 10 mM dNTP’s, 13 µl of 5M betaine, 0.5 µl of Takara ExTaq (Invitrogen), as well as 10 µM of primers #2 and #11. The overlap PCR reaction for C2 was identical to C1 except that 100 ng of 1B (amplified with #4 and #7) and 2B (amplified with #8 and #12) were used in combination with UN. The overlap PCR reaction for C3 was identical to C2 except that 100 ng of 1B (amplified with #4 and #9) and 2A were used in combination with UN. All overlap PCR reactions were subjected to the following thermocycling conditions, an initial denaturation step at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 10 s, annealing at 55°C for 15 s, and
extension at 72°C for 2 min followed by a final extension at 72°C for 5 min. The overlap PCR amplicons were gel purified, ligated into the pGEM T-easy cloning vector (Promega, Madison, WI), and the nucleotide sequences of the chimeric constructs was determined as reported previously (17). Error free inserts were removed from pGEM sequencing vector by digestion with XbaI and XhoI, gel purified, and ligated into pET32b to yield the pET32C1, pET32C2, and pET32C3 expression plasmid constructs. These constructs were transformed into *E. coli* BL21(DE3)slyD⁻ (33) (kindly provided to us by Ry Young, Texas A&M University).

**Expression and purification of recombinant proteins.**

Recombinant Ag2/Pra and rPra2 were expressed and purified as previously described (15). Chimeric transformants (C1, C2, and C3) were grown at 37°C with shaking at 225 rpm, until the absorbance at 600 nM was 0.6 (about 3 to 4 h), at which time the expression of rC1, rC2, and rC3 was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and the cells were incubated for another 4 h at 37°C. Histidine-tagged rC1, rC2, and rC3 were purified from the supernatant by batch metal affinity chromatography under denaturing conditions on Ni-nitrilotriacetic acid (Ni-NTA) agarose per manufactures instructions (Novagen). The purified recombinant protein preparations were concentrated and subjected to endotoxin removal on a E-TOX column (Sterogene Bioseparations, Carlsbad, CA). The total protein content of the preparations was determined by bicinechoninic acid using bovine serum albumin as a standard (Pierce, Rockford, IL), and the level of endotoxin contamination was determined with a *Limulus* amebocyte lysate QCL-1000 kit (Biowhittaker, Walkersville, MD). The recombinant antigens used in this study had ≤1.27 IU of endotoxin per µg of protein. In order to
assess purity and identity of these recombinant chimeric proteins, surface enhanced laser desorption/ionization time-of-flight mass spectrometry SELDI-TOF MS and tandem mass spectrometry (LC-MS/MS) analysis were performed as described below.

**Surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) and tandem mass spectrometry (LC-MS/MS).**

SELDI analysis was performed using an aliphatic reverse phase chip (H4 Protein Chip™, Ciphergen, Palo Alto, CA). The bait surfaces on the chip were pretreated with 2 µl of acetonitrile (Sigma, St. Louis, MO). Shortly before the acetonitrile completely evaporated, 1 µl of purified recombinant antigen in 0.1 M phosphate-buffered saline (PBS; pH 7.4) was applied to the bait surface. The analyte was allowed to concentrate by air-drying. The unbound material was removed by washing three times with 5 µl of high-pressure liquid chromatography-grade water (Fisher Scientific, Pittsburgh, Pa.). Five hundred nl of a saturated solution of 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid, Sigma), the energy-absorbing molecule of choice, was applied to the washed surface of the chip and allowed to crystallize. The was then subjected to mass analysis in a PBSII laser desorption/ionization mass spectrometer (Ciphergen) using the Ciphergen ProteinChip® software (Ciphergen). Data were collected by averaging 30-50 laser shots with an intensity of 280 and a detector sensitivity of 10.

**Mass spectrometry.**

Recombinant chimeric proteins (rC1, rC2, rC3) were separated by SDS-PAGE as described above. The Coomassie blue-stained protein bands (33 kDa, 18 kDa, and 12 kDa) were excised from the gel and subjected to in-gel trypsin digestion at 37ºC overnight using sequencing grade, modified trypsin (Promega) in 100 mM ammonium
bicarbonate. Peptides were then extracted from the gel with 60% acetonitrile: 0.1% TFA and speed-vac concentrated. The concentrated samples were separated on a reverse phase column (75 µm id x 5 cm x 15 µm Aquasil C18 Picofrit column, New Objectives), eluted from the column using a binary gradient of 1% acetic acid/acetonitrile (5-95% acetonitrile in 35 min) and then directly introduced into an ion-trap tandem mass spectrometer (LCQ Deca XP Plus, ThermoFinnigan) equipped with a nanospray source. 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 collision induced dissociation (CID) spectrum of the most abundant ion from the full MS scan was obtained. The CID spectra were manually interpreted using the TurboSEQUEST software package version 3.0 (Finnigan). The amino acid sequences of peptides matching corresponding CID spectra were then compared with the amino acid sequences of rC1, rC2, and rC3.

**Immunization and animal challenge.**

Immunoprotection experiments were conducted with C57BL/6 mice (females, 8 weeks old) supplied by the National Cancer Institute (Bethesda, MD). Mice were immunized subcutaneously (s.c.) with one of the following: phosphate buffered saline, 45 picomoles of rAg2/Pra (1 µg), 75 picomoles of rPra2 (1 µg), 45 picomoles of rAg2/Pra plus 75 picomoles of rPra2 (1 µg of each), 75 picomoles of rC1 (1.62 µg), 45 picomoles of rC1 (0.97 µg), 75 picomoles of rC2 (1.06 µg), 45 picomoles of rC2 (0.64 µg), 75 picomoles of rC3 (0.74 µg), 45 picomoles of rC3 (0.44 µg) plus CpG/IFA adjuvant using essentially the same protocol as previously described (27). The unmethylated CpG dinucleotides present in a synthetic oligodeoxynucleotide (ODN) preparation was used as an adjuvant.
(Integrated DNA Technologies, Inc., Coralville, Iowa). The CpG ODN sequence used to immunize mice was TCCATGACGTTCCTGACGTT (CpG motifs are underlined). Mice were first immunized s.c. with either adjuvant alone in PBS (10 µg of CpG prepared in 50 µl of PBS plus 50 µl of incomplete Freund’s adjuvant [Sigma, St.Louis MO]) or with CpG adjuvant plus either rAg2/Pra, rPra2, rAg2/Pra plus rPra2, rC1, rC2, or rC3. The mice were then boosted by s.c. immunization 14 days later with the same amount of immunogens plus adjuvant. The animals were subsequently challenged with either 47 or 90 viable arthroconidia from *C. posadasii* strain C735 by the i.n. route 4 weeks after the last immunization. Mice were scored for survival over a 45- or 90-day period post challenge. Survival differences between groups of i.n.-challenged mice were analyzed for statistical significance by the Kaplan-Meier method as previously reported (27). Fungal burden in the lungs and spleen of challenged mice were evaluated at the termination of the experiment on surviving animals. The CFU per organ were expressed on a log scale, and the independent-samples *t*-test was used to compare the means of the experimental groups. The lower limit of detection for the CFU assay is 10 colonies per organ homogenate (log_{10} CFU = 1).

**IFN-γ enzyme-linked immunospot assay (ELISPOT).**

C57BL/6 mice (*H-2b*) were primed and boosted s.c. with either PBS, or 450 picomoles of rAg2/Pra plus 750 picomoles of rPra2 (1 µg of each) plus adjuvant (CpG in IFA) as described above. Murine splenocytes from 5 mice per group were pooled and CD90- and CD4+ T-cells were isolated from immune animals for IFN-γ ELISPOT analysis. Immune and control CD4+ T cells were isolated by depletion of non-CD4+ T cells using a CD4+ T cell isolation kit (Miltenyi Biotec, Gladbach, Germany). CD90- splenocytes were
purified by depletion of CD90<sup>+</sup> cells using anti-CD90 (Thy 1.2) microbeads (Miltenyi Biotec). The purity of the isolated CD4<sup>+</sup> and CD90<sup>-</sup> cell preparations were verified by staining with FITC conjugated anti-CD4 and anti-CD90 mAbs (Miltenyi Biotec) followed by analysis of the labeled cells using an Epics Elite Flow Cytometer (Beckman Coulter, Fullerton, CA). Ninety-six well PVDF plates (Millipore, Bedford, MA) were coated overnight at 4°C with 50 µl/well of 15 µg/ml anti-murine IFN-γ mAb (AN18, Mabtech, Nacka Strand, Sweden). Wells were washed and unoccupied sites blocked with RPMI 1640 supplemented with 2% FCS (ATCC, Manassas, VA) for 2 h at 37°C under 5% CO<sub>2</sub>. Each well was seeded with 2x10<sup>5</sup> murine CD4<sup>+</sup> splenocytes from either C57BL/6 mice which were immunized and boosted with either rAg2/Pra plus rPra2 or PBS as described above, suspended in 50 µl of RPMI 1640 supplemented with 10% FCS and 1% penicillin/streptomycin (ATCC). CD90<sup>-</sup> splenocytes were used as APCs and isolated from naïve C57BL/6 mice and used at a concentration of 2.5x10<sup>5</sup> cells/well in 50 µl of RPMI 1640 supplemented with 10% FCS and 1% penicillin/streptomycin. Finally, ex vivo stimuli (recombinant proteins) were suspended in 50 µl of RPMI 1640 supplemented with 10% FCS and 1% penicillin/streptomycin and added to yield final concentrations of 750, 450, 250, 50, and 5nM. ELISPOT plates were incubated at 37°C for 24 hours under 5% CO<sub>2</sub>. Wells were washed five times with 200 µl of PBS, and then 100 µl/well of 1 ug/ml anti-murine IFN-γ biotinylated mAb (R4-6A2-biotin, Mabtech) was added and allowed to incubate for 2 hours at room temperature. Wells were washed as above and 100 µl/well of strepavidin-alkaline phosphatase [1:1000] was added for 1 hour (Mabtech). Wells were washed as above and 100 µl of BCIP/NBT substrate solution (Mabtech) was added to each well until development was complete. Developed
ELISPOT plates were allowed to dry overnight and were shipped to an ELISPOT plate evaluation service to be analyzed (Zellnet Consulting, Fort Lee, NJ). To control for non-specific background spots, all wells were corrected by the subtracting the number of spots detected in an identically treated well that contained PBS-immune CD4\(^+\) T-cells. The independent-samples \(t\)-test was used for statistical comparison of the means of each experimental group.

RESULTS

Design and construction of recombinant chimeric expression plasmids (pET32C1, pET32C2, and pET32C3).

Mapping of the Th and continuous B cell epitopes of Ag2/Pra and Pra2 induced by the immunization of C57BL/6 mice (\(H-2^b\)) with either rAg2/Pra or rPra2 revealed the presence of several immunogenic domains (black shaded boxes Figure 1) (14). The Ag2/Pra (\(H-2^b\)) restricted Th epitopes identified by the immunization of C57BL/6 mice with rAg2/Pra are localized to two central domains (amino acids 49-68 and 89-140). An additional domain within Ag2/Pra harbors continuous B-cell epitopes (amino acids 49-132), which were revealed by evaluation of rAg2/Pra-immune sera from C57BL/6 mice. To address the possibility that cytotoxic T cell (Tc) mediated immunity may play a significant role in the protection rAg2/Pra provides C57BL/6 mice, we examined the recall responses of rAg2/Pra-immune CD8\(^+\) T cells by IFN-\(\gamma\) ELISPOT analysis. No significant recall responses were detected when these cells were stimulated \textit{ex vivo} with the same range of concentrations of full-length rAg2/Pra or overlapping 20-mer peptides derived from the amino acid sequence of Ag2/Pra, which were identical to those used in
our previous CD4⁺ ELISPOT experiments (data not shown). These results are supported by a recent report by Kirkland et al. demonstrating that CD8⁺ T cells are not required for protective immunity against *Coccidioides* in rAg2/Pra-immune C57BL/6 mice (24).

Based on the locations of the Th and continuous B cell epitopes of Ag2/Pra we selected two domains (designated 1A and 1B) for inclusion in our recombinant chimeric constructs (Figure 1). The selection of the Pra2 chimeric domains (designated 2A and 2B) was based on the mapping of Th and continuous B cell epitopes induced by the immunization of C57BL/6 mice with rPra2 (14). The Pra2 (*H-2b*) restricted Th epitopes identified by the immunization of C57BL/6 mice with rPra2 are localized to two domains (amino acids 41-60 and 97-124). Two additional domains within Pra2 harbor continuous B-cell epitopes, (amino acids 49-68 and 81-100) which were revealed by evaluation of rPra2-immune sera from C57BL/6 mice. No significant recall responses were detected by IFN-γ ELISPOT analysis of rPra2-immune CD8⁺ T cells (isolated from C57BL/6 mice) stimulated *ex vivo* with several concentrations of full-length rPra2 or overlapping 20-mer peptides derived from the amino acid sequence of Pra2 (data not shown).

Oligonucleotide primers were designed to amplify the four selected cDNA fragments from the *AG2/PRA* and *PRA2* genes (1A, 1B, 2A, and 2B), which encode domains previously demonstrated to be immunogenic in C57BL/6 mice (14). These amplified cDNA fragments were joined together in several combinations using an overlap PCR strategy to yield the chimeric protein coding sequences of C1 (1A plus 2B), C2 (1B plus 2B), and C3 (1B plus 2A) (see Figure 2). These overlap PCR amplicons were initially ligated into pGEM T-easy plasmid for identification of clones with correct open reading frames and engineered restriction sites. The verified C1, C2, and C3 cDNA sequences
were sub-cloned into pET32b to yield the chimeric expression plasmids pET32C1, pET32C2, and pET32C3.

**Expression and analysis of recombinant chimeric Pra’s (rC1, rC2, and rC3).**

Individual bacterial clones harboring either pET32C1, pET32C2, or pET32C3 were grown in liquid media and induced with IPTG (isopropyl-β-D-thiogalactopyranoside). Induced chimeric proteins were purified by metal affinity chromatography under denaturing conditions, re-natured, and subjected to thrombin cleavage to remove vector encoded amino acids. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to examine the purified recombinant chimeric proteins (rC1, rC2, and rC3). Staining the polyacrylamide gel with coomassie blue reveals single protein bands with estimated molecular masses of 33 kDa, 18 kDa, and 12 kDa (Figure 3A). The migration patterns of the recombinant chimeric proteins is higher than their predicted molecular weights of 21.6 kDa (rC1), 14.1 kDa (rC2), and 9.9 kDa (rC3). This finding is consistent with previous reports, which demonstrate that rAg2/Pra, spherule-derived deglycosylated Ag2/Pra, and rPra2 migrate aberrantly on SDS-PAGE gels (13, 15, 23, 38). In order to more accurately determine the molecular masses of the recombinant chimeric proteins, we employed surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS). Small aliquots (approximately 0.2 µg of each) of purified rC1, rC2, and rC3 previously analyzed by SDS-PAGE above were bound to pre-treated bait surfaces of a H4 aliphatic reverse phase chip for SELDI-TOF MS analysis. Examination of the spectra revealed that the molecular weights of all the recombinant chimeric antigens are comparable to their calculated predicted molecular masses when evaluated by this technique (Figure 3B). To verify the presumptive
identities of the recombinantly expressed chimeric proteins, they were subjected to tandem mass spectrometry analysis (LC MS/MS). Coomassie blue-stained protein bands (33 kDa, 18 kDa, and 12 kDa) were excised from the gel (Figure 3A) digested with trypsin, separated on a reverse phase column, and then directly introduced into an ion-trap tandem mass spectrometer. Analysis of the collision induced dissociation (CID) spectra from the excised bands yielded multiple peptides from each of the samples. For each recombinant chimeric protein analyzed, the masses, relative amino acid positions, and sequences of all identified peptides are listed in table 2. The LC MS/MS analysis of rC2 and rC3 revealed a single peptide from each spanning the junctions between their Ag2/Pra and Pra2 domains (rC2-ISVSNIVVDQCEK and rC3-ISVSNIVVDQCVDQCSK). Since peptides for the LC MS/MS analysis were generated by trypsin digestion, the absence of a peptide spanning the junction between the Ag2/Pra and Pra2 domains of rC1 was expected due to the lack of proximal lysine or arginine residues flanking the junction site. All of the peptides isolated from the rC1 sample and the remaining peptides from rC2 and rC3 samples were identical to predicted sequences of the translated AG2/PRA and PRA2 genes.

Quantification of C1-, C2-, and C3-specific CD4+ T cell responses induced by the immunization of C57BL/6 mice with the combination of rAg2/Pra plus rPra2.

C57BL/6 mice were immunized with full-length rAg2/Pra [1 µg] plus rPra2 [1 µg] and CD4+ T cells were isolated from immune animals for ELISPOT analysis. The number of CD4+ T cells secreting IFN-γ in response to ex vivo stimulation with several different recombinant antigens at concentrations of 750, 500, 250, 50, and 5 nM was quantified (Figure 4). These data indicate that compared to the media control, significant numbers
rAg2/Pra plus rPra2-immune CD4\(^+\) T cells from C57BL/6 mice secrete IFN-\(\gamma\) in response to *ex vivo* stimulation with all concentrations of the multiple antigens tested (*P* \(\leq 0.004\)). Stimulation of rAg2/Pra plus rPra2-immune CD4\(^+\) cells *ex vivo* with either rAg2/Pra (black bars) or rPra2 alone (white bars) revealed significantly decreased recall responses in comparison to stimulation of identical cells with equivalent amounts of rAg2/Pra plus rPra2 (cross hatched bars) at all concentrations tested (*P* \(\leq 0.026\)). The recall responses of rAg2/Pra plus rPra2-immune CD4\(^+\) T cells to *ex vivo* stimulation with 750, 500, and 250 nM of rAg2/Pra plus rPra2 (cross hatched bars) were not significantly different than responses to stimulation with rC1 (dark gray bars) at the same concentrations (*P* \(\geq 0.068\)). However, at lower concentrations (50 and 5 nM) the recall responses of rAg2/Pra plus rPra2-immune CD4\(^+\) T cells to stimulation with rAg2/Pra plus rPra2 were significantly greater than those of rC1 (*P* \(\leq 0.002\)). Predictably, the recall responses of rAg2/Pra plus rPra2-immune CD4\(^+\) T cells to *ex vivo* stimulation with rC2 or rC3 (medium and light gray bars) were significantly less than observed responses to stimulation with either rC1 or rAg2/Pra plus rPra2 at all concentrations tested (*P* \(\leq 0.036\)).

**Evaluation of fungal burden in rC1-, rC2-, and rC3-immune C57BL/6 mice following a sub-lethal intranasal challenge with *C. posadasii*.

Eight-week old female C57BL/6 mice were immunized and boosted s.c. with one of the following: PBS, rAg2/Pra [45 pM], rPra2 [75 pM], the combination of rAg2/Pra[45 pM] plus rPra2 [75 pM], rC1 [45 or 75 pM], rC2 [45 or 75 pM], or rC3 [45 or 75 pM] in adjuvant (CpG in IFA). Four weeks after the last immunization the mice were challenged by the i.n. route with 47 viable arthroconidia from *C. posadasii* strain C735. The protective efficacy of the recombinant immunogens was measured by determination of
fungal burden in the lungs and spleen of mice sacrificed 45 days after challenge (Figure 5). With the exception of three PBS-control, two rPra2-immune, and two rChimeric-immune mice all animals survived until the termination of the experiment (45 days post-challenge). The splenic fungal burden in all surviving mice sacrificed at 45 days post-challenge was below the detection limit of the CFU assay (data not shown). In contrast, multiple mice from all experimental groups demonstrated significant pulmonary fungal burden at 45 days post i.n.-challenge (Figure 5). Pulmonary fungal burden in mice surviving until the end of the experiment are represented by a dot and bold X-symbols represent animals that survived less than 45 days. The mice that did not survive until the termination of the experiment were assigned the highest pulmonary fungal burden detected in a surviving mouse (6.45 log$_{10}$ colony forming units). Statistical comparison of the mean pulmonary fungal burdens between groups of PBS-immune (adjuvant control) and either rAg2/Pra-immune or rPra2-immune mice revealed that neither of these single full-length recombinant antigens provides better clearance than PBS ($P \leq 0.06$ and $P \leq 0.481$, respectively). Conversely, groups of mice immunized with either the combination of rAg2/Pra plus rPra2, rC1 [75 pM], rC2 [75 and 45 pM], or rC3 [45 pM] demonstrated significantly lower pulmonary fungal burdens than PBS-immune mice ($P \leq 0.025$). In addition, mice that were immunized with the combination of rAg2/Pra plus rPra2 demonstrated significantly reduced pulmonary fungal burden compared to PBS-, rAg2/Pra-, rPra2-, rC1 [45 pM]-, and rC3 [75 pM]-immune mice ($P \leq 0.029$). However, no statistically significant differences in pulmonary clearance were observed between groups of mice immunized with the combination of rAg2/Pra plus rPra2 versus rC1 [75 pM], rC2 [75 and 45 pM], or rC3 [45 pM] ($P \geq 0.083$).
Additional statistical analyses were conducted to compare the means of experimental groups in which mice that did not survive until the termination of the experiment were assigned a value equal to the mean of the group instead of the highest pulmonary fungal burden detected in a surviving mouse. Our previous statistical analyses in which deceased mice were assigned the highest detected pulmonary fungal burden in a surviving mouse established that rAg2/Pra plus rPra2, rC1 [75 pM], rC2 [75 and 45 pM], and rC3 [45 pM] demonstrate a statistically significant decrease in pulmonary fungal burden compared to the PBS group. However, when mice that did not survive until the termination of the experiment were assigned a value equal to the mean of the group our statistical analyses revealed that in comparison to PBS only the combination of rAg2/Pra plus rPra2 demonstrated a statistically significant decrease in pulmonary fungal burden \((P \geq 0.001)\). This was the only inconsistency identified by comparison of the statistical analyses of the two methods for treatment of deceased mice.

**Evaluation of survival and fungal burden in rC1-, rC2-, and rC3-immune C57BL/6 mice following a lethal intranasal challenge with *C. posadasii*.**

Experimental groups of eight-week old female C57BL/6 mice were immunized and boosted in an equivalent manner as the previous protection experiment. The immunization protocol was followed by i.n. challenge with a larger inoculum of *C. posadasii* (90 viable arthroconidia). Survival was scored over the course of 90 days post-challenge, and fungal burden in the lungs and spleen was assessed in all surviving animals upon termination of the experiment (day 90) (Figure 6). Statistical comparison of the survival rates of PBS- (solid circles n=16), rAg2/Pra- (open circles n=16) and rAg2/Pra plus rPra2-immune (open downward triangles n=16) mice revealed that
immunization with either rAg2/Pra alone or in combination with rPra2 results in a highly significant enhancement of survival compared to PBS controls ($P \leq 0.0001$). The group of mice immunized with rPra2 alone (solid downward triangles n=16) demonstrates less dramatic but still significantly improved survival when compared to PBS-immune mice ($P \leq 0.042$). With the exception of rC1 [45 pM] (solid squares n=16) the survival rates of groups of mice immunized with recombinant chimeric antigens rC1, rC2, and rC3 (n $\geq 14$) were all significantly improved compared to PBS-immune mice ($P \leq 0.009$). As we have demonstrated previously with smaller inocula (63 and 76 arthroconidia), the survival curves of rAg2/Pra- and rAg2/Pra plus rPra2-immune mice are nearly superimposable over a 90 day period following challenge (15). Despite a visible reduction in the rates of survival, no significant differences were found between groups of mice immunized with either rC2 or rC3 [75 or 45 pM] when compared to mice immunized with the combination of rAg2/Pra plus rPra2 ($P \geq 0.081$). Upon conclusion of the survival experiment fungal burden was assessed in the lungs and spleen of all surviving animals (Figure 6B). The fungal burden in spleens of all surviving mice sacrificed at 90 days post-challenge were below the detection limit of the CFU assay (data not shown). In contrast, multiple surviving animals from several groups demonstrated pulmonary fungal burden at the time of sacrifice (Figure 6B). The majority of surviving mice immunized with 1 μg of rAg2/Pra had detectable fungal burden in their lungs at 90 days post-challenge (81%). Although the final percentages of surviving mice in the rAg2/Pra- and the rAg2/Pra plus rPra2-immune groups were identical, the pulmonary fungal burden in the group of mice that received the combined vaccine was significantly lower (27% versus 81%) ($P \geq 0.007$). The pulmonary fungal burdens in rPra2-, rC1-, rC2-, and rC3-
immune survivors was 50% or less in all groups but the rate of survival for all these groups was less than or equal to 40% at the termination of the experiment.

**DISCUSSION**

Previously we have demonstrated that, compared to vaccination with either of the single antigens, a combined rAg2/Pra plus rPra2 vaccine provides enhanced protection to C57BL/6 and BALB/c mice against a lethal intranasal challenge with *Coccidioides* (14, 15). Subsequent mapping of the Th and continuous B cell epitopes of Ag2/Pra and Pra2 induced by the immunization of C57BL/6 (*H-2b*) or BALB/c (*H-2d*) mice with either rAg2/Pra or rPra2 revealed significant differences in the fine specificities of immune responses to these antigens (14). These data support our hypothesis that immunization with multiple antigenically diverse recombinant *Coccidioides* proteins results in the induction of a larger *Coccidioides* specific immune repertoire, which is able to provide a higher level of protection than immunization with any known single antigen. In an attempt to extend our understanding of the ability of rPra2 to complement the protective capacity of rAg2/Pra in a multivalent vaccine we have elected to design three divalent chimeric antigens to be recombinantly expressed and evaluated for their ability to protect mice from an intranasal challenge with *Coccidioides*. Due to the extreme susceptibility of BALB/c mice to intranasal coccidioidal challenge, we decided to focus our efforts on the evaluation of divalent chimeric antigens designed expressly to protect intermediately susceptible C57BL/6 mice (22). We have previously identified several immunologically reactive regions within Ag2/Pra and Pra2, which harbor both Th and continuous B cell epitopes induced by the immunization of C57BL/6 mice with either rAg2/Pra or rPra2
Evaluation of the locations and sizes of these immunologically reactive regions led to the selection of two domains from Ag2/Pra (1A and 1B) and two domains from Pra2 (2A and 2B) to be employed as subunits for generating the three chimeric vaccines. The chimeric domains 1A and 2B contain the complete array of Th and continuous B cell epitopes identified in Ag2/Pra and Pra2, respectively. However, chimeric domains 1B and 2A only contain a fraction of their total respective Th and continuous B cell epitopes.

The decision to include Th epitopes in the selected Ag2/Pra and Pra2 chimeric domains was based on considerable evidence that cell-mediated immunity is essential for resistance to coccidioidomycosis (2-5, 8, 11, 24). Conversely, only recently has there been any evidence that B cells and/or antibodies may also contribute significantly to vaccine-induced protection against *Coccidioides*. By using gene expression microarray analysis and B cell deficient MuMT mice, Magee et al. revealed that B cells and/or antibody play a role in the acquired protective immunity against *Coccidioides* induced by immunization of mice with a formalin-killed spherule (FKS) vaccine (29). Although effector splenocytes from rAg2/Pra-immune mice exhibit a Th1 phenotype *ex vivo* that has been associated with protection, these same mice paradoxically also produce very high titers of rAg2/Pra specific IgG1 (isotype associated with a Th2 response) (18, 19). It is not hard to imagine how these high titers of α-rAg2/Pra (IgG1) could enhance the survival of rAg2/Pra-immune mice by helping to facilitate a rapid reduction in fungal burden after coccidioidal challenge. This reduction may be driven by one or a combination of the following mechanisms: enhanced uptake of IgG1/coccidioidal complexes via Fc-mediated phagocytosis by macrophages and neutrophils, activation of
macrophages to a microbiocidal state by the same process, and initiation of the classical complement cascade by IgG coated pathogens leading to the production of soluble mediators of chemotaxis and inflammation (C5a) as well as further opsonization of the pathogen by binding of C3b. Hence, we appreciate that B cell mediated immunity might also play a significant role in the rAg2/Pra plus rPra2 induced protection we have observed in C57BL/6 mice. Therefore in addition to Th epitopes, we have also elected to include continuous B cell epitopes in the selected Ag2/Pra and Pra2 chimeric domains which we have employed as subunits of C1, C2, and C3. The possibility that CD8+ T cell-mediated immunity may contribute to the ability of the rAg2/Pra plus rPra2 vaccine to protect C57BL/6 mice from an intranasal challenge with Coccidioides has also been considered. However, data from other investigators as well as our own observations, indicate that CD8+-mediated immunity does not play a significant role in the protection that the combination of rAg2/Pra plus rPra2 affords C57BL/6 mice (24).

The chimeric domains 1A, 1B, 2A, and 2B were combined in several ways using an overlap PCR strategy to yield the divalent chimeric expression constructs encoding C1, C2, and C3. The most inclusive divalent chimeric vaccine construct that we generated is C1 (1A plus 2B); its translated product contains all of the Th and continuous B cell epitopes induced by immunization of C57BL/6 mice with rAg2/Pra and rPra2 (Figure 2). The translated products of the chimeric vaccines C2 (1B plus 2B) and C3 (1B plus 2A) were each respectively less inclusive (Figure 2). After expressing and purifying rC1, rC2, and rC3 we took considerable precautions to confirm their presumptive identities by SELDI-TOF MS and tandem mass spectrometry analysis prior to their use as vaccines in our protection experiments (Figure 3B and Table 2). We also demonstrated that the
divalent recombinant antigens are capable of inducing recall responses from rAg2/Pra plus rPra2-immune CD4+ T cells equivalent to (rC1) or proportionally less than (rC2 and rC3) recall responses induced by \textit{ex vivo} stimulation with equal molar amounts (750 nM, 500 nM, and 250 nM) of the combination of rAg2/Pra plus rPra2 (Figure 4).

We chose to use molar quantities for all the immunogens that we tested to enable a more standardized evaluation of the protective efficacy of the chimeric and full-length antigens. Previous experiments have demonstrated that immunization of C57BL/6 mice with 45 pM (1 µg) of rAg2/Pra is sufficient to provide enhanced survival following a lethal intranasal challenge with \textit{Coccidioides}, and that immunization with 45 pM (1 µg) of rAg2/Pra plus 75 pM (1 µg) of rPra2 imparts enhanced survival and pulmonary clearance (14, 15). Therefore we decided to use 75 pM and 45 pM doses of divalent chimeric antigen to immunize and boost mice prior to challenge.

Initially, the protective efficacy of the recombinant divalent chimeric antigens was measured by their ability to reduce pulmonary fungal burden 45 days after a sub-lethal intranasal challenge with \textit{Coccidioides} (47 arthroconidia). Comparison of the mean pulmonary fungal burdens between groups of mice immunized with PBS and those receiving either rAg2/Pra or rPra2 alone revealed that neither of these single full-length recombinant antigens provides significantly better clearance than the control. We have previously shown that although rAg2/Pra-immune C57BL/6 mice demonstrate enhanced survival following an intranasal challenge with larger inocula of \textit{Coccidioides}, the majority of these surviving mice (55-88%) maintain considerable pulmonary fungal burdens throughout the course of infection (15). Our current results indicate that a
comparable percentage of mice immunized with rAg2/Pra alone are not capable of clearing even a sub-lethal intranasal challenge with *Coccidioides* (62%).

In contrast to mice receiving monovalent vaccines (rAg2/Pra or rPra2 singly), groups of mice immunized with the combination of rAg2/Pra plus rPra2, rC1 [75 pM], rC2 [75 and 45 pM], or rC3 [45 pM] all demonstrated significantly lower pulmonary fungal burdens than PBS-immune mice. In addition, mice receiving rC1 [75 pM], rC2 [75 and 45 pM], or rC3 [45 pM] demonstrated no statistically significant differences in pulmonary clearance compared to the group of mice immunized with the combination of rAg2/Pra plus rPra2. These results indicate that compared to the combination of full-length rAg2/Pra plus rPra2, several of our chimeric antigens are capable of providing statistically equivalent protection against a sub-lethal intranasal challenge with *Coccidioides*. To determine whether the recombinant divalent chimeric antigens can provide protection against a lethal intranasal inoculum of *Coccidioides* we challenged groups of immunized mice with 90 viable arthroconidia and scored for survival over the course of 90 days post-challenge (Figure 6). The comparison of groups of PBS-, rAg2/Pra-, and rAg2/Pra plus rPra2-immune mice revealed that immunization with either rAg2/Pra alone or in combination with rPra2 results in a highly significant enhancement of survival compared to PBS controls. In addition, mice immunized with rPra2 or chimeric antigens rC1, rC2, and rC3 with the exception of rC1 [45 pM] all demonstrated less dramatic yet still significantly improved rates of survival compared to PBS-immune mice. Unexpectedly, the survival curves of rC1-immune groups [75 or 45 pM] were significantly decreased compared to mice immunized with the combination of rAg2/Pra plus rPra2. Although statistically there were no significant differences in the survival
curves of rAg2/Pra plus rPra2–immune in comparison to either rC2 or rC3-immune groups of mice, there were visible differences in their rates of survival at 90 days post-challenge (≥ 28%). As expected, the survival curves for rAg2/Pra- versus rAg2/Pra plus rPra2-immune mice were nearly identical and examination of the fungal burden of surviving animals upon conclusion of the experiment revealed dissimilar patterns of pulmonary clearance. (Figure 6B). This finding confirms our previous reports that rAg2/Pra plus rPra2-immune mice display significantly reduced pulmonary fungal burdens compared to rAg2/Pra-immune mice that survive a lethal intranasal challenge with *Coccidioides* (14, 15). Greater than 50% of all survivors from rPra2-, rC1-, rC2-, and rC3-immune groups were found to have no detectable pulmonary fungal burden, but the rate of survival for all these groups was less than or equal to 40% at the termination of the experiment.

The results of our sub-lethal and lethal intranasal challenge protection experiments provide evidence that the three divalent recombinant chimeric antigens we designed afford a level of protective efficacy that in some cases is no different (statistically) than the full-length rAg2/Pra plus rPra2 divalent vaccine. However, comparisons of pulmonary fungal burdens (sub-lethal challenge) and survival rates (lethal challenge) in our protection experiments indicate that all the chimeric antigens are noticeably (and in some cases statistically) less protective than the combination of rAg2/Pra plus rPra2. In addition, examination of data from the lethal challenge protection experiment revealed that the rAg2/Pra-immune survival curve is significantly higher than the survival curves of rC1-immune [75 pM and 45pM] and noticeably higher than the curves for rC2- and rC3-immune mice. These observations suggest that although we have successfully
reconstituted a portion of the protective efficacy that the full-length divalent vaccine (rAg2/Pra plus rPra2) provides C57BL/6 mice, the current immunization protocol and/or design of our divalent chimeric vaccines is not optimal.

In contrast to the inferior performance of rC2 and rC3 in our protection experiments, the failure of rC1 to consistently provide a level of protection similar to the full-length divalent vaccine was unexpected (survival curves of rC1 [75 pM and 45 pM] were statistically decreased compared to rAg2/Pra plus rPra2). There are several possible factors that could be contributing to the sub-optimal protection provided by rC1 when compared to the full-length divalent vaccine. One possibility is that rC1 does not contain the full repertoire of immunostimulatory epitopes that immunization with combination of full-length rAg2/Pra plus rPra2 provides. Based on our analyses of rC1 (Figures 3 and 4) and previous epitope mapping studies, we are confident that rC1 contains all of the continuous B cell and (H-2^b) restricted Th cell epitopes identified in both Ag2/Pra and Pra2. However, it is possible that by splicing together pieces of Ag2/Pra and Pra2 we may have eliminated one or more potentially immunologically relevant conformational B cell epitopes found in full-length Ag2/Pra and/or Pra2. The design of our chimeric antigens was based on the location of previously identified (H-2^b) restricted Th and continuous B cell epitopes to select chimeric domains (1A, 1B, 2A, or 2B) which were spliced together to form C1, C2, and C3. This strategy resulted in all three divalent chimeric antigens containing only a fraction of a highly conserved CFEM (conserved fungal extracellular matrix) domain found in both Ag2/Pra and Pra2 (Figure 1) (15, 26, 37). This domain has been previously shown to harbor continuous and conformational B cell epitopes using patient and hyper-immune goat sera (37). It is possible that the
exclusion of an intact CFEM domain from Ag2/Pra and/or Pra2 in rC1 has contributed to its reduced ability to protect C57BL/6 mice from a coccidioidal challenge in comparison to the divalent full-length vaccine.

Another related possibility is that rC1 contains all of the immunostimulatory domains necessary for providing a level of protection equivalent to the divalent full-length vaccine. However, the altered context of these epitopes in rC1 may result in the induction of immune responses different than those previously observed in response to immunization with the divalent full-length vaccine. An alteration in the efficiency of processing and presentation of Th epitopes in vivo could result in the requirement of higher doses of rC1 to induce an immune response as effective as the divalent full-length vaccine. We have observed that rC1 is capable of inducing recall responses from rAg2/Pra plus rPra2-immune CD4\(^+\) T cells equivalent to recall responses induced by ex vivo stimulation with equal molar amounts (750 nM, 500 nM, and 250 nM) of the combination of rAg2/Pra plus rPra2 (Figure 4). However, the ability of rC1 to induce recall responses comparable to equal molar amounts rAg2/Pra plus rPra2 is lost at lower concentrations (50 nM and 5 nM). By splicing chimeric domains together we may have added, removed, or masked natural protease sites important in the MHC class II presentation process which may have altered processing and presentation of rC1 Th epitopes by APCs to rAg2/Pra plus rPra2-immune CD4\(^+\) T cells. Since rC1 has its own unique hydrophilic / hydrophobic properties, the accessibility of continuous B cell epitopes (from Ag2/Pra and Pra2) to the immune system may also be altered in comparison to their status within their respective original molecules.
In future studies we propose to verify that immunization of C57BL/6 mice with rC1, rC2, or rC3 yields the expected continuous B cell and (H-2b) restricted Th cell repertoires by analyzing rC1-immune sera and CD4+ T cells as previously described (14). We also plan to create and evaluate several other divalent chimeric antigens that will include larger portions of Ag2/Pra and Pra2 (CFEM domain) as well as a chimeric antigen composed of the full-length Ag2/Pra and Pra2 spliced together.

This current study revealed that our recombinant divalent chimeric antigens are less effective than the full-length rAg2/Pra plus rPra2 vaccine. However, further evaluation of our current and novel chimeras designed by epitope mapping studies will be useful in advancing our knowledge about the specific immune responses (epitopes and types of epitopes) required for protection against coccidioidomycosis. More importantly, these findings suggest it is necessary to alter our strategy for the development of a multivalent recombinant vaccine against coccidioidomycosis. Although it is attractive from an economical standpoint to design chimeras to be as small as possible by restricting the domains to contain only known mapped epitopes, our current results suggest that this strategy will most likely not be as fruitful as including bigger pieces (full domains or full proteins). In addition to alleviating the problems that we have encountered in this study, inclusion of complete immunologically relevant (protective in one or more strain/s of mice) domains and / or full-length proteins will provide the added antigenic diversity necessary for protection of a polymorphic population. The current study has demonstrated that engineering a chimeric to be as protective as its full-length components is not easily accomplished even for a single strain of inbred mouse. Even though it would be more costly to produce, perhaps a strategy using multiple full-length
recombinantly expressed antigens will be more effective than trying to engineer a chimeric multivalent vaccine that can protect an outbred population against *Coccidioides*.
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### Table 1: Primers used to generate C1, C2, and C3 by an overlap PCR strategy

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<th>Primer #</th>
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<td>2</td>
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<tr>
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<td>5'-CAAGCGAgTGCAATCGATCGATCG-3'</td>
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* indicates a peptide which spans the junction between an Ag2/Pm and a Pm2 domain
FIGURES:

Figure 1: Graphical representation of Ag2/Pra and Pra2 proteins of *C. posadasii*, showing the presence of predicted signal peptides and conserved fungal extracellular membrane (CFEM) domains in each, as well as tandem TXX‘P repeats and predicted GPI-anchor unique to the C-terminal of Ag2/Pra. Below the graphical representations of Ag2/Pra and Pra2, the locations of the *H-2b* restricted Th and continuous B cell epitopes of each are highlighted in black. In addition, the locations and sizes of the four chimeric domains used as subunits for generating the divalent chimeric antigens C1, C2, and C3 are indicated below their respective full-length antigens.
Figure 2: Graphical representations of C1, C2, and C3 are shown directly above their respective amino acid sequences. The portion of each divalent chimeric antigen derived from Ag2/Pra is shaded in gray and its corresponding sequence is not underlined, while the underlined sequences correspond to the white portions which are derived from Pra2.
**Figure 3:** Assessment of purified rC1, rC2, and rC3 quality

(A) Purified rC1, rC2, and rC3 were electrophoresed on a 10-20% Tricine gel and the coomassie blue-stained protein bands (33 kDa, 18 kDa, and 12 kDa) were excised and subjected to LC-MS/MS analysis. (B) SELDI analysis was performed on purified rC1, rC2, and rC3. The low intensity peaks observed at 43334.1, 28346.4, and 19776.8 daltons represent dimers of rC1, rC2, and rC3 respectively.
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Figure 4: Quantification of *ex vivo* recall responses of rAg2/Pra plus rPra2-immune Pra2 CD4⁺ T cells harvested from C57BL/6 mice. Mice immunized with rAg2/Pra plus rPra2 were assayed for the presence of IFN-γ recall responses to *ex vivo* stimulation with equal molar amounts of recombinant antigens (750, 500, 250, 50, and 5 nM). Results are shown as number of spots per 2 X 10⁵ immune splenic CD4⁺ T-cells and are representative of two independent experiments.
Figure 5: Evaluation of protective efficacy of rC1, rC2, and rC3 in C57BL/6 mice challenged sub-lethally by the i.n. route with *C. posadasii*. C57BL/6 mice were immunized by the s.c. route with one of the following: PBS, 45 pM of rAg2/Pra (1 µg), 75 pM of rPra2 (1 µg), 45 pM of rAg2/Pra plus 75 pM of rPra2 (1 µg of each), rC1 (75 pM or 45 pM), rC2 (75 pM or 45 pM), or rC3 (75 pM or 45 pM) plus adjuvant (n ≥ 13 mice per group). Immunized mice were challenged by the i.n. route with *C. posadasii* (47 viable arthroconidia), and pulmonary fungal burden was determined at day 45 post-challenge. The CFU per organ were expressed on a log scale, and the independent-samples *t*-test was used to compare the means of the experimental groups.
Figure 6: Evaluation of protective efficacy of rC1, rC2, and rC3 in C57BL/6 mice challenged by the i.n. route with a lethal inoculum of *C. posadasii*. C57BL/6 mice were immunized by the s.c. route with one of the following: PBS, 45 pM of rAg2/Pra (1 µg), 75 pM of rPra2 (1 µg), 45 pM of rAg2/Pra plus 75 pM of rPra2 (1 µg of each), rC1 (75 pM or 45 pM), rC2 (75 pM or 45 pM), or rC3 (75 pM or 45 pM) plus adjuvant (n ≥14 mice per group). Immunized mice were challenged by the i.n. route with *C. posadasii* (90 viable arthroconidia). (A) Mortality was determined at days 1 through 90 post-challenge for each group. Survival differences between groups of i.n.-challenged mice were analyzed for statistical significance by the Kaplan-Meier method. (B) Pulmonary fungal burden in the survivors was determined at day 90 post-challenge. The CFU per organ were expressed on a log scale, and the independent-samples T test was used to compare the means of the experimental groups.
Figure 6: Evaluation of protective efficacy of rC1, rC2, and rC3 in C57BL/6 mice challenged by the i.n. route with a lethal inoculum of *C. posadasii*. C57BL/6 mice were immunized by the s.c. route with one of the following: PBS, 45 pM of rAg2/Pra (1 µg), 75 pM of rPra2 (1 µg), 45 pM of rAg2/Pra plus 75 pM of rPra2 (1 µg of each), rC1 (75 pM or 45 pM), rC2 (75 pM or 45 pM), or rC3 (75 pM or 45 pM) plus adjuvant (n ≥ 14 mice per group). Immunized mice were challenged by the i.n. route with *C. posadasii* (90 viable arthroconidia). (A) Mortality was determined at days 1 through 90 post-challenge for each group. Survival differences between groups of i.n.-challenged mice were analyzed for statistical significance by the Kaplan-Meier method. (B) Pulmonary fungal burden in the survivors was determined at day 90 post-challenge. The CFU per organ were expressed on a log scale, and the independent-samples T test was used to compare the means of the experimental groups.
DISCUSSION/SUMMARY

*Coccidioides* is a unique fungus that has a complex parasitic life cycle. At any given point in time during an infection the host’s immune system is exposed to immature spherules, mature spherules, rupturing spherules, and newly released endospores. These various stages of the pathogen are visibly unique at the morphological level and it is reasonable to assume that they demonstrate equal if not greater variation in their surface expressed and secreted antigenic profiles. Thus, it is unlikely that a single vaccine candidate would be able to induce a completely protective immune response against *Coccidioides*. This supposition is given further support by the fact that there is considerable evidence that cell-mediated immunity is essential for resistance to coccidioidomycosis (Ampel et al., 1993; Beaman et al., 1977, 1979; Cohen et al., 1982; Cox and Magee, 1998; Fish et al., 1990; Kirkland et al., 2006). This means that it is even less likely that a single vaccine candidate would provide the necessary antigenic diversity needed to induce protective immunity against *Coccidioides* in a heterogeneous population. Not surprisingly, to date there have been no reports providing clear evidence of the ability of any single recombinant vaccine candidate to adequately protect both C57BL/6 and BALB/c mice from intranasal challenge with *Coccidioides*. In direct contrast to these findings, multiple investigators have demonstrated that immunization of all strains of inbred mice tested, as well as outbred Swiss-Webster mice, can be protected from intranasal challenge by immunization with either a whole killed cell (FKS) or a complex cell extract from parasitic *Coccidioides* cultures (27K fraction) (Cox et al., 1988; Cox and Magee, 2004; Jiang et al., 1999b; Levine et al., 1961; Zimmermann et al., 1998). The superior protection that these undefined multicomponent immunogens
provide is primarily due to the complex array of \textit{Coccidioides} antigens that the immune system is exposed to by immunization with these vaccines. Presumably, these vaccines provide the antigenic diversity necessary for the induction of comprehensive immune responses (to all relevant parasitic stages) against \textit{Coccidioides} by all individuals in a heterogeneous population.

Therefore, it is believed that a successful recombinant vaccine against coccidioidomycosis will have to be composed of numerous \textit{Coccidioides} proteins, which provide the necessary antigenic diversity to induce protective immunity against multiple parasitic stages of \textit{Coccidioides} in a heterogeneous human population. Progress towards this goal has been made by the discovery and subsequent evaluation of several recombinantly expressed proteins of \textit{Coccidioides} as monovalent vaccines. These studies by multiple investigators have identified recombinant vaccine candidates that confer varying degrees of protection against coccidioidal challenge dependent on the strain of inbred mouse, route of challenge, and size of inoculum used (Abuodeh et al., 1999; Delgado et al., 2003; Jiang et al., 1999b; Kirkland and Cole, 2002; Kirkland et al., 1998a, b; Li et al., 2001; Shubitz et al., 2002). The evaluation of combinations of the monovalent candidates is a compulsory next step in the development of a multivalent vaccine against coccidioidomycosis. We have begun this work by demonstrating that compared to vaccination with either of the single antigens, a combined rAg2/Pra plus rPra2 vaccine provides enhanced protection to C57BL/6 and BALB/c mice against a lethal intranasal (i.n.) challenge with \textit{Coccidioides}. Subsequently we provided evidence that diverse immune responses are induced by these two vaccine candidates in both C57BL/6 and BALB/c mice, providing these animals with a more comprehensive
Coccidioides immune repertoire than immunization with either of the single antigens. We also assessed the ability of rPra7 singly and in combination with the rAg2/Pra plus rPra2 vaccine to protect C57BL/6 mice against a lethal intranasal challenge with Coccidioides. Although rPra7 did not significantly enhance the protection that the rAg2/Pra plus rPra2 provides C57BL/6 mice, we appreciate that this finding may be due to the inability of these mice to present Pra7 derived epitopes due to their genetic restriction (H-2^b). This is an important point since we realize that the epitopes we have identified within Ag2/Pra and Pra2 also may be genetically restricted (not promiscuous), and it is probable that mice with different haplotypes (other than H-2^b and H-2^d) will differ in their ability to present these or other epitopes of these two antigens.

The use of inbred mice as models of coccidioidomycosis in vaccine efficacy studies has facilitated the identification of several promising vaccine candidates and provided a better understanding of the requirements of protective immunity to Coccidioides. However, the adoption of an outbred model of murine coccidioidomycosis for further evaluation of promising monovalent and multivalent candidates is a requisite if we are serious about eventually producing a vaccine to protect an outbred population of humans. To date there have been no reports of the evaluation of any recombinant Coccidioides vaccine candidates in outbred mice. This is in direct contrast to the presence of multiple reports in the literature describing the evaluation of whole cell and cell extract fractions of Coccidioides in outbred animals (Levine et al., 1961; Pappagianis et al., 1961; Zimmermann et al., 1998). It would seem obvious that prior to the advancement of a coccidioidal vaccine candidate to expensive non-human primate trials or even human clinical trials it should demonstrate the ability to protect an outbred population of mice.
However, recently a chimeric antigen composed of Ag2/Pra (1-106) and Csa was tested (and failed) in non-human primates at great expense even though the chimeric provided significant protection against coccidioidal challenge to C57BL/6 mice (Shubitz et al., 2006). In an attempt to extend our understanding of the ability of rPra2 to complement the protective capacity of rAg2/Pra in a multivalent vaccine, we designed three divalent chimeric antigens (based on our epitope mapping experiments) to be recombinantly expressed and evaluated for their ability to protect C57BL/6 mice against an intranasal challenge with *Coccidioides*. Unfortunately, immunization with these chimeric antigens provided only a portion of the protective efficacy that the full-length divalent vaccine (rAg2/Pra plus rPra2) afforded C57BL/6 mice. Although it is attractive from an economical standpoint to design chimeras to be as small as possible by restricting the domains to contain only known mapped epitopes, the inclusion of complete immunologically relevant (protective in one or more strain/s of mice) domains and / or full-length proteins would provide added antigenic diversity necessary to help protect more individuals in a diverse population. Even though it would be more costly to produce, perhaps a strategy using multiple full-length recombinantly expressed antigens will be more effective than trying to engineer a chimeric multivalent vaccine capable of protecting an outbred population against *Coccidioides*. At this point it is impossible to ascertain the optimal number of recombinant proteins needed to provide a heterogeneous population with the antigenic diversity necessary for the induction of a protective immune response against *Coccidioides*. It is very likely that future investigations will have to focus on the identification of non-genetically restricted epitopes as well as
alternative methods of antigen delivery to make the production of an effective multivalent vaccine against \textit{Coccidioides} economically feasible.
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of mice against lethal respiratory infection with *Coccidioides posadasii* using two recombinant antigens expressed as a single protein. IN PRESS (Vaccine).


ABSTRACT

Evaluation of recombinant proteins of *C. posadasii* for their protective efficacy in a murine model of coccidioidomycosis has revealed several potential vaccine candidates. One of the most promising is a proline rich-antigen (Ag2/Pra). We have demonstrated that vaccination of C57BL/6 and BALB/c mice with the combination of rAg2/Pra plus rPra2 enhanced protection against a lethal intranasal challenge with *Coccidioides*, compared to vaccination with either of the single antigens. Enhanced protection was based both on higher percentage of surviving mice (as demonstrated by the BALB/c strain) or significantly better clearance of the fungal burden (C57BL/6). Despite the related primary structures of Ag2/Pra and Pra2, we have identified differences in their helper T cell (Th) and continuous B cell epitope profiles using immune CD4$^+$ T cells and sera from both C57BL/6 and BALB/c mice. The mapping of the Th and continuous B cell epitopes of Ag2/Pra and Pra2 induced by the immunization of C57BL/6 (*H-2^b*) mice with either rAg2/Pra or rPra2 facilitated the rational design of three divalent chimeric antigens composed of immunoreactive domains selected from both antigens. The three recombinantly expressed and purified divalent chimeric antigens were evaluated for their ability to protect C57BL/6 mice from both sub-lethal and lethal intranasal challenges with *Coccidioides* (47 and 90 arthroconidia, respectively). The results of these protection experiments provide evidence that the three divalent recombinant chimeric antigens we designed afford a level of protective efficacy that in some cases is statistically no different than the full-length rAg2/Pra plus rPra2 divalent vaccine. However, comparisons of pulmonary fungal burdens (sub-lethal challenge) and survival rates (lethal challenge) indicate that the chimeric antigens in all cases are noticeably (and in some
cases statistically) less protective than the combination of rAg2/Pra plus rPra2. These observations suggest that, though we have successfully reconstituted a portion of the protective efficacy that the full-length divalent vaccine (rAg2/Pra plus rPra2) provides C57BL/6 mice, the current immunization protocol and/or design of our divalent chimeric vaccines may not be optimal.