INFECTION AND IMMUNITY, July 1992, p. 2627–2635
0019-9567/92/072627-09$02.00/0

Vol. 60, No. 7

An Arthroconidial-Spherule Antigen of *Coccidioides immitis*: Differential Expression during In Vitro Fungal Development and Evidence for Humoral Response in Humans after Infection or Vaccination

JOHN N. GALGIANI,[1,2]* SUNG H. SUN,[3] KRIS O. DUGGER,[1,2] NEIL M. AMPEL,[1,2] GREGORY G. GRACE,[1,2] JEFF HARRISON,[3] AND MARION A. WIEDEN[4]

Medical and Research Services[1] and Laboratory Service,[4] Veterans Affairs Medical Center, Tucson, Arizona 85723; Department of Medicine, College of Medicine, University of Arizona, Tucson, Arizona 85724[2]; and Research Service, Veterans Affairs Medical Center, San Antonio, Texas 78284[3]

Received 28 January 1992/Accepted 3 April 1992

A 33-kDa protein antigen purified from spherules of *Coccidioides immitis* was analyzed for ultrastructural localization and for binding to serum antibodies from infected or immunized humans. By using colloidal gold detection of affinity-purified anti-33-kDa protein antibodies, electron photomicrographs showed binding to the inner cell wall of arthroconidia and spherules and to the septa and glycocalyx surrounding endospores. Enzyme immunoassay measurements also demonstrated that the antigen was most abundant in mature spherules. Of 37 patients with coccidioidomycosis but without concurrent human immunodeficiency virus infections, all but 2 demonstrated immunoglobulin M (IgM) (usually with early infection) or IgG antibodies for the 33-kDa antigen. In contrast, only one of four HIV-infected patients with active coccidioidal infections demonstrated antibody. On the other hand, 107 of 108 patients without evident coccidioidomycosis and 15 of 16 patients with histoplasmosis did not have similar antibodies, indicating a high degree of specificity. Immunization of humans with a spherule vaccine produced IgM responses to this antigen that were not evident in placebo recipients.

The immune responses resulting from infection with *Coccidioides immitis* are diagnostically useful and critical to resolution of illness. Tests for serum antibodies and delayed-type dermal hypersensitivity with extracts of *C. immitis* have been in clinical use for more than 40 years (35). Moreover, a variety of observations have indicated the importance of T-cell immunity in controlling both experimental animal infections and naturally acquired human disease (2, 3, 19). These considerations have focused attention on defining the nature of the specific antigens against which the immune response is directed (7–9, 11–15, 24, 30, 31, 42, 43).

We have recently purified a 33-kDa protein from spherules of *C. immitis* (17). By immunoblotting, this protein was found to react with antibodies from several patients with coccidioidal infections, yet other evidence indicated that it was distinct from the specific antigens used in conventional complement fixation, tube precipitin, and immunodiffusion procedures. Additionally, this protein stimulated peripheral blood lymphocytes from patients with delayed-type dermal hypersensitivity to coccidioidal antigens. We have used an immunoelectron microscopy and an enzyme-linked immunosorbent assay (ELISA) to determine the ultrastructural location and concentration of the 33-kDa antigen in various phases of coccidioidal growth. ELISA was also used to measure the concentration of anti-33-kDa antigen antibodies in the serum of patients after naturally acquired infection and in human subjects who had received a killed-spherule vaccine. As detailed in this report, the 33-kDa antigen appears to be a cell wall constituent, against which specific serum antibodies are frequently detected after coccidioidal infection and with which human immunity may be stimulated.

MATERIALS AND METHODS

Fungal isolates. *C. immitis* strain Silveira (also known as strain 659), originally isolated from a patient with extrathoracic dissemination, was used for all studies.

Sources of sera. Human sera were obtained from several sources. Patients with active coccidioidomycosis were cared for at the Tucson Veterans Affairs Medical Center between 1980 and 1986. Information regarding the patients' clinical status was taken from hospital and personal records by one of the authors (J.N.G.). None of these patients are known to be infected with human immunodeficiency virus (HIV). Infection was classified as early if the serum sample was obtained after no more than several weeks of respiratory symptoms in conjunction with serologic or cultural evidence of *C. immitis* infection. Infections were classified as progressive if *C. immitis* was isolated from an extrathoracic site or from a pulmonary source and illness had been present for more than 6 months. Sera were obtained from additional patients as part of a prospective study of coccidioidomycosis in HIV-infected patients (1a, 1b). Control sera were available from patients who were hospitalized in 1991 at the Veterans Affairs Medical Center in Tucson for reasons unrelated to coccidioidal infection and who had no evidence of recent or active coccidioidomycosis.

Serum samples from patients living in regions where histoplasmosis is endemic were kindly provided by James Johnson (Veterans Affairs Serology Reference Laboratory, Lexington, Ky.). Complement-fixing antibodies to *Histoplasma capsulatum* yeast phase antigens (measured in Dr. Johnson's laboratory [23]) were detected at concentrations

* Corresponding author.

of 1:128 in four samples, 1:32 in three samples, 1:16 in one sample, and 1:8 in three samples and were undetectable in five samples. Samples were received coded, and the coccidioidal serologic studies were performed without knowledge of the samples' reactivity to histoplasmal antigens.

Serum samples were also obtained from healthy subjects participating in the Valley Fever Vaccine Project (39). Subjects between 18 and 60 years of age, in good health, and with no known prior coccidioidal infection were recruited between October 1980 and September 1984. They were included in the project only if dermal delayed hypersensitivity to both coccidioidin and spherulin was absent when each was applied at the usual and concentrated strengths. Subjects meeting the enrollment criteria were randomized to receive either placebo or 1.75 mg of a Formalin-killed particulate spherule vaccine (prepared from strain Silveira of *C. immitis*) on the day of the first serum sample collection and then repeated 1 and 7 weeks later. Discomfort was assessed after each injection by a subjective 10-point scale (0 indicating no discomfort and 9 indicating severe discomfort). One to three weeks after the third injection, a second serum sample was obtained from each subject, and skin tests were repeated. For the studies reported here, serum pairs were selected and assayed before the results were correlated with injection type.

All serum samples were stored at −70°C until testing was done.

Antigen purification. Purified 33-kDa antigen was prepared as described previously (17). Briefly, a toluene-induced spherule lysate was prepared from first-generation, 96-h spherules grown in liquid Converse medium (20), and the lyophilized extract was deglycosylated with anhydrous HF in a closed system under an approved fume hood (33). Chemical deglycosylation with anhydrous hydrogen fluoride cleaves all the linkages of neutral and acidic sugars while leaving peptide bonds intact (28, 37). However, N-linked glycopeptide bonds are not broken, and therefore a small number of sugar residues are likely to remain attached to the polypeptide. The resulting supernatant was dialyzed in phosphate-buffered saline (PBS) (pH 7.2), concentrated, and fractionated on a Sephacryl S-200 HR column (Pharmacia, Piscataway, N.J.). Fractions that reacted with anti-33-kDa antigen rabbit antibodies by dot blot were pooled and demonstrated a single band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver stains (17).

Purification of antiapoglycoprotein antibodies and immunoelectron microscopy. An affinity column was prepared with chromatographically purified apoglycoprotein by a slight modification of a previously described method (18). Briefly, 75 μg of protein was mixed with an equal weight of *N*-hydroxysuccinimido-biotin (Sigma) in 0.1 M $NaHCO_3$ for 3 to 4 h at room temperature. Unreacted *N*-hydroxysuccinimido-biotin was removed by dialysis, and the biotinylated protein was mixed with excess agarose avidin D (Vector Laboratories, Burlingame, Calif.) with rocking for 3 h. The agarose was placed in a 10-ml column and washed extensively with PBS to remove unbound protein. Immune or nonimmune human serum was circulated over the column for 30 to 60 min to obtain human affinity-purified antibody. Bound antibody was eluted from the column with 3 M $MgCl_2$, desalted, and concentrated by ultrafiltration. Affinity-purified antibodies demonstrated specific binding to the 33-kDa antigen by immunoblot techniques, performed as described previously (17).

The following developmental stages of *C. immitis* were studied: arthroconidia harvested after 6 to 8 weeks of growth on GYE agar at 28°C; mycelia removed after 24, 48, or 72 h of growth in GYE liquid medium at 28°C; first-generation spherules recovered from Converse agar medium after 24, 48, 72, 96, and 144 h of growth at 40°C; and endospores released from 144-h first-generation spherules after medium replenishment. Methods for fixing, embedding, and sectioning fungal preparations have been described elsewhere (40). Staining and immunoelectron microscopy were done as described by Coalson et al. (5), except that the primary (human affinity-purified anti-33-kDa antigen) antibody was diluted 1:50 in Tris buffer, pH 8.2, containing 1% ovalbumin, and 10-nm colloidal gold particles conjugated to goat anti-human immunoglobulin antibody (Amersham) were used.

The density of colloidal gold particles bound to various fungal preparations was determined by enumeration of particles and measurement of cell wall and whole-cell areas. For each fungal growth preparation, six contiguous images were analyzed.

Measurement of human anti-33-kDa antigen antibodies in patients' sera. Microtiter wells were coated with 200 μl of purified 33-kDa antigen (50 ng/ml). Serum samples from infected or uninfected patients were serially diluted with PBS containing 0.05% Tween from 1:80 in twofold steps and added to precoated, washed wells, and the plates were incubated at 37°C for 1 h. Horseradish peroxidase-linked goat anti-human immunoglobulin G (IgG) or IgM (Sigma, St. Louis, Mo.) was added to each well and further incubated for 1 h. After the plates were washed, bound enzyme-linked second antibody was allowed to react with substrate and *o*-phenylenediamine dihydrochloride as an indicator for measurement of the A_{490}. Wells which received no serum produced an optical density of less than 0.05, and results were expressed as the highest serum dilution that produced an optical density of greater than 0.1. To allow comparison of results from several different days' studies, a reference serum of known titer was included in all studies, and the endpoint result for this serum was always within twofold.

Detection of 33-kDa antigen in fungal extracts. Various soluble extracts of *C. immitis* were assayed for the presence of the 33-kDa antigen. Toluene-induced lysates of 24-, 48-, and 96-h growths of developing spherules were prepared as described previously (20). Spherulin (25) was the generous gift of Tom Greene (Berkeley Biologicals, Berkeley, Calif.). Filtrates of spent medium from mycelial growth and toluene-induced lysates of mycelia were prepared as described previously (29, 35). Both were found to possess complement fixing-type and tube precipitin-type antigens by standard agar double-diffusion techniques (22, 38).

Antigen was measured by an inhibition ELISA modified from previously described methods (21, 36). Microtiter plates were prepared as described above. Coccidioidal extracts or various concentrations of the purified 33-kDa antigen in phosphate buffer for constructing standard curves were combined in equal parts with rabbit anti-33-kDa antigen antiserum (1:500 dilution), and the mixture was added in triplicate to microtiter plates for 1 h. After being washed, the wells received enzyme-conjugated goat anti-rabbit immunoglobulin antibodies, and binding was detected as described above. Standard curves demonstrated antigen detection at concentrations as low as 0.1 ng of protein per ml.

RESULTS

Differential expression of antigen in different phases of fungal development. Colloidal gold particles conjugated to anti-human IgG or IgM antibodies detected significant dif-

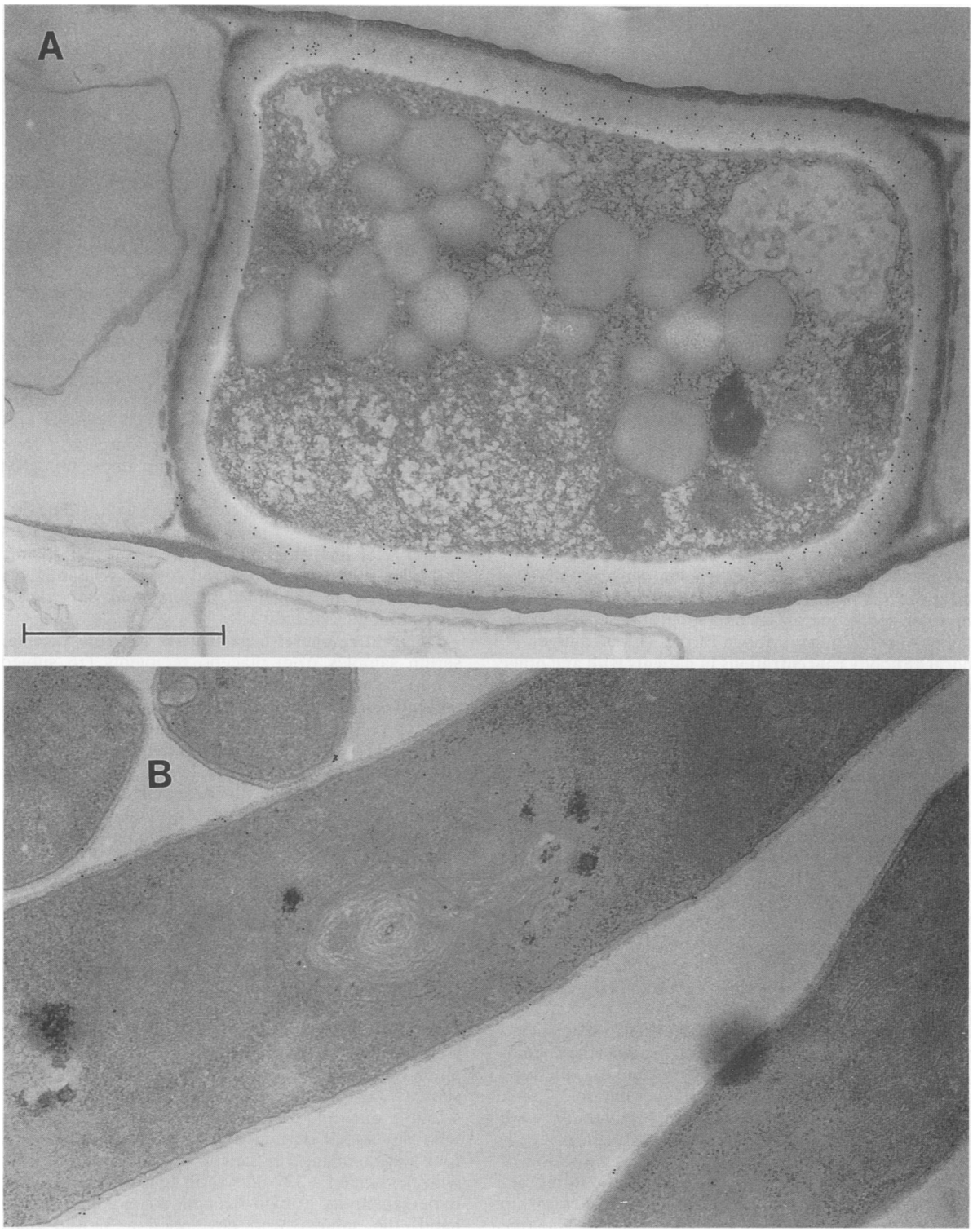

FIG. 1. Colloidal immunogold particles bound to the 33-kDa antigen in mycelial preparations. (A) Arthroconidium, with binding evident to the inner conidial wall; (B) 48-h growth of mycelia, in which binding of colloidal gold is relatively sparse. Bar, 1 μm.

ferences in the binding of human affinity-purified anti-33-kDa antigen antibodies within the fungal cells and at different stages of coccidioidal growth. Colloidal gold was evident in the inner conidial wall of arthroconidia (Fig. 1A), whereas virtually no binding was observed to either the hyphal outer wall layer (HOWL) that surrounds the inner conidial wall or within the cytoplasm. In preparations in which arthroconidia had germinated and mycelial growth had proceeded for 24, 48 (Fig. 1B), or 72 h, the amount of 33-kDa antigen appeared to be considerably reduced. On the other hand, spherules

that had developed from arthroconidia in Converse medium demonstrated progressively more 33-kDa antigen within their cell walls and internal septa (Fig. 2A and B). As the spherules matured, binding became evident on the periphery of the cell wall. In endospore preparations (Fig. 2C), antigen appeared to be freely exposed on the wall surface and the interconnecting glycocalyx, which dispersed into the surrounding milieu.

Quantitation of gold particles at the various stages of development corroborated the visual impressions (Fig. 3). The density of colloidal immunogold particles was lowest in young (48-h) mycelia and increased with maturation to arthroconidia. Similarly, particle density increased with spherule maturation. These differences are attributable in part to the proportion of the cell constituted by cell wall, which also increased with maturation in both phases. However, since the densities in cell wall areas were much higher than in whole-cell areas, the concentration of the antigen in the cell wall actually increased as well. Binding of immunogold was specific for the 33-kDa antigen, since substituting nonimmune serum produced 97.3% less binding for all phases of growth combined.

Quantitation of the 33-kDa antigen in different soluble preparations by ELISA also showed differences in the amount of antigen related to the phase of growth (Table 1). Antigen was detected in toluene-induced lysates of 24-, 48-, and 96-h growths of first-generation spherules, and antigen concentration increased with maturation. In contrast, two mycelium-derived preparations and spherulin demonstrated very low or absent concentrations of the 33-kDa antigen.

Humoral antibody concentrations in patients with and other persons without coccidioidal infections. Anti-33-kDa antigen antibodies were measured by ELISA in serum samples from 11 patients with early coccidioidal infections (Table 2). All had tube precipitin-type serum antibodies, as determined by the double immunodiffusion technique (22). In addition, titers of complement fixing-type antibodies ranged from undetectable to 1:16, and *C. immitis* was recovered in cultures from three patients. All but one patient had either an anti-33-kDa antigen IgM antibody titer greater than 1:160 or an IgG titer greater than 1:80 by ELISA. The IgM titer was as high as 1:20,480 in one patient who died of fulminant coccidioidal pneumonia. The single patient without elevated IgM or IgG anti-33-kDa antigen antibodies had pleuritic pain, cough, and fever of several days' duration. Radiographs of his chest, however, showed nothing remarkable, and illness resolved without specific antifungal therapy.

Anti-33-kDa antigen antibody titers were also determined for serum samples from 26 patients with progressive coccidioidomycosis who had conventional complement-fixing-type serum antibody titers ranging from undetectable to 1:2,048. Anti-33-kDa antigen IgG antibody titers generally were above 1:320, and some were greater than 1:81,920. A significant relationship was found between the concentration of complement-fixing and anti-33-kDa antigen IgG antibodies ($P < 0.001$, paired t test; Fig. 4). However, the relationship is very loose ($r = 0.541$, Pearson correlation). The one patient with a low IgG titer also had an IgM titer of 1:160. The serum from this patient had been obtained after intravenous amphotericin B therapy for a chronic pulmonary infection and during an interval in which the infection appeared to be inactive.

Serum samples from four HIV-infected patients (all with AIDS) with pulmonary coccidioidal infection detected during prospective surveillance were available for testing (Table 2). Three patients had unilateral infiltrates, and one had bilateral reticular infiltrates. ELISA-detectable anti-33-kDa antigen antibodies were found in only one of these patients. This patient had a nodule identified on chest radiographs 9 months previously, and complement-fixing-type antibodies had been detected 6 months previously. Of the remaining three patients without ELISA-detectable anti-33-kDa antigen antibodies, two had detectable coccidioidal antibodies by conventional tests.

To define nonspecific activity, serum antibodies to the 33-kDa antigen were measured in serum samples from 108 persons without evidence of coccidioidal infection. Over 95% of the samples from these subjects had anti-33-kDa antigen IgM titers of ≤1:160 and IgG titers of ≤1:80 (Table 2). In this survey, one healthy subject had an IgG titer of 1:1,280. This 29-year-old male had been born in New York and had lived in regions where coccidioidomycosis was endemic for 6 years. At the time that the serum sample was obtained, he was nonreactive to all coccidioidal skin tests. Serum samples obtained four times over the next 2 years demonstrated similar IgG anti-33-kDa antigen antibodies. However, the tests were uniformly nonreactive for conventional tube precipitin-type and complement fixing-type antibodies in the same samples.

Of 16 serum samples from subjects residing in regions where histoplasmosis is endemic, IgM titers were ≤1:80 in 15 and 1:160 in 1, and IgG titers were ≤1:80 in 15 and 1:1,280 in 1. The subject with elevated anti-33-kDa antigen IgG had an antibody titer against *Histoplasma capsulatum* yeast phase antigens of only 1:8.

Humoral response to particulate spherule vaccine. Paired serum samples from placebo recipients (16 subjects) and spherule vaccine recipients (20 subjects) were tested for IgM and IgG anti-33-kDa antigen antibodies (Table 3) by ELISA. Placebo recipients showed no change in antibodies of either isotype. However, 12 of the vaccine recipients showed a change. The differences in the IgM responses of placebo and vaccine recipients were highly significant ($P < 0.003$, Mann-Whitney U test). Comparing vaccine and placebo recipients for the proportion of subjects manifesting a fourfold or greater rise in titer, a significant difference is again apparent ($P < 0.03$, Fisher's exact test). IgM responses occurred in five of seven male subjects and 6 of 13 female subjects ($P < 0.37$, Fisher's exact test).

Seroconversion appeared to be unrelated to either discomfort from vaccine injections or the appearance of delayed-type dermal hypersensitivity after receipt of the spherule vaccine. For example, four subjects reported subjective discomfort of 7 or 8 on our 10-point scale; two of these subjects had no change in their IgM anti-33-kDa antigen antibody concentration. Of nine subjects with a discomfort score of 3 or less, IgM concentrations changed 2-fold in four, 4-fold in one, and 32-fold in one. Similarly, of nine subjects who developed dermal hypersensitivity after vaccination, four had no change in their IgM concentration. The subject who developed a 32-fold rise in IgM also developed dermal hypersensitivity to high-strength coccidioidin. On the other hand, the subject who developed a 32-fold rise in IgG remained nonreactive to all four coccidioidal skin tests.

DISCUSSION

In this study, electron microscopy used immunogold to locate affinity-purified antibody binding predominantly to the cell wall. As in a previous report, which described studies with a different strain of *C. immitis* (17), the affinity-purified antibodies appeared to be specific for the 33-kDa antigen,

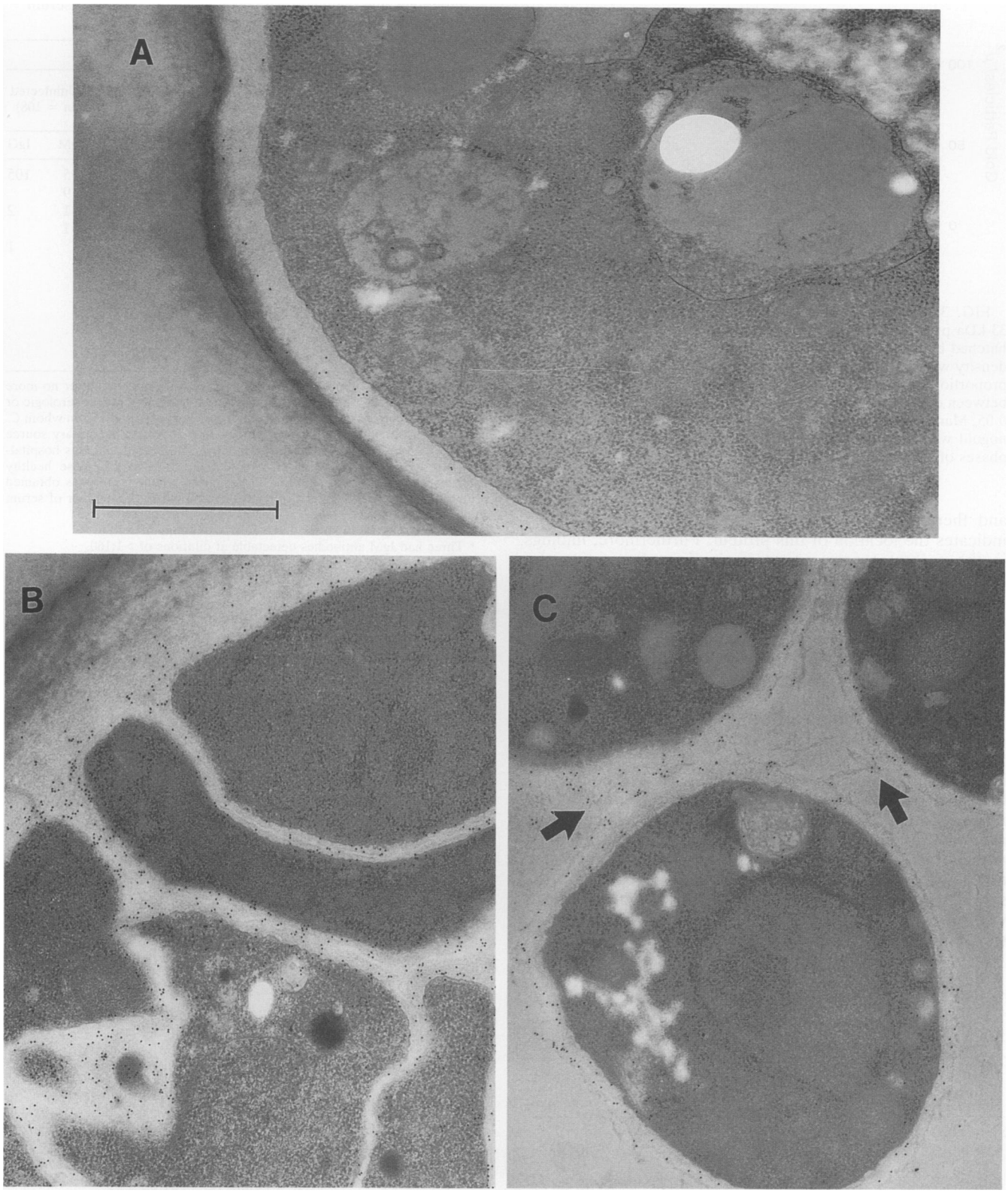

FIG. 2. Colloidal immunogold particles bound to the 33-kDa antigen in spherule preparations. (A) Developing spherule after 72 h, demonstrating moderate immunogold particle binding to the inner portions of the wall; (B) segmented spherule after 144 h of growth, with considerable binding to the inner wall and septa and also some binding evident on the periphery of the wall as well; (C) endospores and interconnecting glycocalyx (arrows), with immunogold particles bound to the wall and dispersing into the medium. Bar, 1 μm.

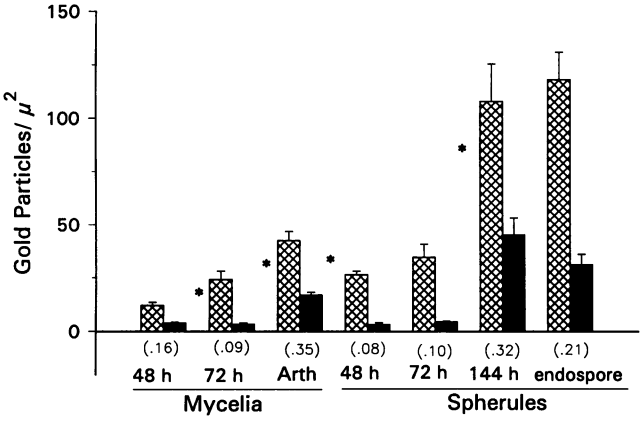

FIG. 3. Density of colloidal immunogold particles bound to 33-kDa protein in different phases of growth of *C. immitis*. Cross-hatched bars indicate density within cell walls; solid bars indicate density within the entire cell. Numbers in parentheses indicate the proportion of surface area that was cell wall. Asterisks are placed between cell wall density results that are significantly different ($P < 0.05$, Mann-Whitney U test). Arth, arthroconidia. Binding of immunogold with nonimmune serum reduced binding by 97.3% for all phases of growth combined.

and therefore binding of the immunogold most probably indicates the location of this protein. Furthermore, findings in the current report extend our earlier observations by demonstrating phase specificity. The amount of immunogold binding was scant in young mycelia, greater in arthroconidia, and densest in spherules and endospores. Within arthroconidia, the antigen appears to be a constituent of the inner conidial wall which is interior to the HOWL. Mechanically removing the HOWL (also termed the outer conidial wall [10]) has resulted in enhanced phagocytosis and antibody-dependent killing by human neutrophils (16). Just as these observations suggest that the HOWL is an impediment to host defenses, it is also conceivable that the 33-kDa antigen is inaccessible by host immunity until the HOWL is shed. On the other hand, after endospores have been released from ruptured spherules, the 33-kDa antigen appears to be readily available to stimulate host responses. The tube precipitin antigen, which frequently reacts with antibodies from patients with coccidioidomycosis, is also detectable on the spherule surface. It has been suggested for that antigen as well that its presentation to host cells may involve the sloughing component of the spherule envelope (9, 41).

It is of interest to compare the differences in 33-kDa antigen content of different phases of coccidioidal growth with the relative immunogenicity of vaccines, which differed when prepared by Levine and coworkers from spherules, arthroconidia, or mycelia. For example, in studies that employed an intranasal infection with *C. immitis* sufficient to result in 75% mortality after 90 days in unvaccinated mice, mortality was reduced to 69% after mycelial vaccination, to 48% after arthroconidial vaccination, and to 7% after spherule-endospore vaccination (27). In other studies, mature spherules were found to be more protective than immature

TABLE 1. ELISA-detectable 33-kDa antigen in various extracts of *C. immitis*

Growth phase	Method of extraction	33-kDa antigen (μg/mg of total protein)
Spherules		
96 h	Toluene lysis (source of 33-kDa antigen)	50–100
48 h	Toluene lysis	4.2–50
24 h	Toluene lysis	0.22–0.57
Spherulin	Autolysate	<0.05
Mycelia		
Filtrate	Spent medium	0.02–0.06
Lysate	Toluene lysis	0.001–0.003

TABLE 2. ELISA-detectable IgM and IgG anti-33-kDa antigen antibodies and conventional coccidioidal antibodies in serum samples from different groups of subjects

Highest reactive serum dilution	No. of samples[a]							
	Early (n = 11)		Progressive (n = 26)		HIV infected (n = 4)		Uninfected (n = 108)	
	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
≤1:80	4[b]	4[c]	11	1	3	3	95	105
1:160		2	7		1		9	
1:320	1		2	1		1	1	2
1:640	2	1	1	3			1	
1:1,280	1	4	3	8				1
1:2,560	1		2	3				
1:5,120	1			2				
1:10,240				2				
1:20,480	1			1				
1:40,960				3				
>1:81,920				2				

[a] Early, patients from whom serum samples were obtained after no more than several weeks of respiratory symptoms in conjunction with serologic or cultural evidence of *C. immitis* infection; progressive, patients from whom *C. immitis* was isolated from an extrathoracic site or from a pulmonary source and who had been ill for more than 6 months. Uninfected, patients hospitalized for reasons unrelated to coccidioidal infection or otherwise healthy participants in the Valley Vaccine Project from whom serum was obtained prior to vaccination. Numbers in parentheses indicate the number of serum samples tested.

[b] Three had IgG antibodies detectable at dilutions of >1:80.

[c] Three had IgM antibodies detectable at dilutions of >1:160.

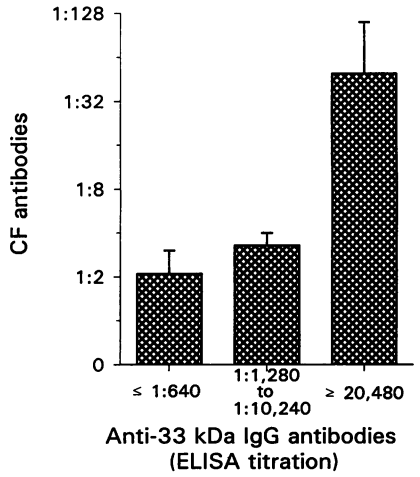

FIG. 4. Relationship between complement-fixing and anti-33-kDa antigen IgG antibody concentrations in patients with coccidioidal infections. Bars represent geometric mean ± standard error of the mean. $P < 0.001$, paired t test of all results combined.

TABLE 3. Change in ELISA-detectable IgM and IgG anti-33 kDa antibodies after injection of killed particulate spherule vaccine or placebo[a]

Increase in antibodies (fold)	No. of samples			
	Placebo recipients (n = 16)		Vaccine recipients (n = 20)	
	IgM	IgG	IgM	IgG
None	16	16	9	18
2			5	1
4			4	
8			1	
16				
32			1	1

[a] Subjects received injections of either placebo or 1.75 mg of killed spherule vaccine on the day of the first serum sample collection and again 1 and 7 weeks later. The second serum sample was drawn 1 to 3 weeks after the last injection. Data are expressed as the change in titer between the two results. Differences between IgM responses for placebo and vaccine recipients are significant ($P < 0.003$, Mann-Whitney U test). Differences of fourfold or greater in IgM response for placebo and vaccine recipients are significant ($P < 0.03$, Fisher's exact test).

spherules (26). Although our studies did not examine cellular responses to the 33-kDa antigen, the concordance of the quantitative particle densities in analogous preparations in our studies is consistent with the possibility that the 33-kDa antigen is involved in protective immunity as well as with humoral responses.

ELISA measurement of the 33-kDa antigen in soluble extracts of different coccidioidal growths generally corroborated the differences in concentration in various morphologic phases of growth. Toluene-induced lysis of mature spherules produced preparations in which 5 to 10% of the protein could be accounted for as the 33-kDa antigen. Toluene-induced lysates of younger spherules and mycelia as well as autolysis of spherules (spherulin) and filtrates collected after mycelial growth all contained smaller proportions of reactive antigen. Both spherulin and mycelial preparations possess the conventionally used complement-fixing antigen and the tube precipitin antigen (4, 29, 32, 35), and the lack of 33-kDa antigen in these preparations further indicates that the 33-kDa antigen is distinct from both of these well-established and clinically useful antigens. Mycelial antigens and spherulin are also used commonly to test for delayed-type dermal hypersensitivity in persons with prior coccidioidal infection (16, 25). Therefore, the dermal hypersensitivity evoked by these preparations does not appear to be due to the 33-kDa antigen either. Although neither the lysate nor the purified antigen has been used as a skin-testing reagent, studies reported elsewhere have demonstrated that the 33-kDa antigen can stimulate peripheral blood lymphocytes from persons who react in skin tests with spherulin (17) and that the toluene-induced lysate from which the 33-kDa antigen has been purified can stimulate gamma interferon release in vitro (1). Delayed-type dermal hypersensitivity to coccidioidal extracts has generally correlated with a competent host response to coccidioidal infections (35), but whether the mechanisms that evoke the dermal response are the same as those that afford protection is not clear. A full understanding of the relationship of these observations will require further study.

Of the serum samples from patients with coccidioidal illness, 95% showed a humoral response to the 33-kDa antigen, whereas 98% of those from persons without evidence of active coccidioidal infection did not. IgM anti-33-kDa antigen antibodies were found more frequently in patients with relatively recent infections, and IgG anti-33-kDa antigen antibody levels were highest in patients with protracted and extensive infections. IgM anti-33-kDa antigen antibodies were also noted in a few patients with progressive disease. However, by our methods, we cannot exclude the possibility that some of the apparently specific IgM was due to rheumatoid factor binding to specific IgG (34). In addition, only one of four HIV-infected patients demonstrated anti-33-kDa antigen antibodies, underscoring earlier observations that HIV-infected and other immunosuppressed patients may not be as likely to develop a detectable humoral response as normal individuals (6, 30). These findings suggest the possibility that this protein may prove useful as a new diagnostic reagent, but further study will be needed to determine whether detection of anti-33-kDa antigen antibodies will supplement conventional methods. Although an association was found between the concentration of complement-fixing-type and anti-33-kDa antigen IgG antibodies, the correlation was rough. At present, no ELISA with any antigen has been sufficiently correlated with patient status to be used as a substitute for the complement fixation test.

Serum samples from subjects enrolled in a field trial of a killed-spherule particulate vaccine demonstrated an increase in anti-33-kDa antigen antibodies not seen in those from placebo recipients. Although the vaccine itself was not assayed for 33-kDa antigen, the vaccine was made from the same strain of *C. immitis* that was used in the other studies reported here and was made from mature spherules, a phase of growth that our studies indicate should have had high concentrations of the 33-kDa antigen. It has not been established that the 33-kDa antigen is an important stimulus for protection against coccidioidomycosis. However, should that be the case, it is of interest that the degree of antibody response did not appear to correlate with cutaneous reactions at the site of injection, suggesting that it may be possible to dissociate the untoward reactions evident with the particulate vaccine from its immunogenicity.

Thus far, it has not been technically feasible to study the ability of the 33-kDa antigen to protect against experimental infection, as has been done for the particulate vaccine. If the primary sequence of the 33-kDa antigen were known or if its gene were isolated, synthetic oligopeptides could be synthesized or the protein could be expressed in sufficient quantities by recombinant genetic approaches. We believe that the findings in this report provide additional encouragement to proceed in these directions to make such direct studies possible.

ACKNOWLEDGMENTS

Rosemary Hayden provided valuable technical assistance during the course of our studies. We thank James Johnson for providing serum samples from patients with histoplasmosis.

This work was supported in part by the Department of Veterans Affairs and by the Arizona Disease Control Research Commission, grant 82-0689. Some of the patients' sera were collected in conjunction with treatment protocols carried out by the NIAID Mycoses Study Group and the Valley Fever Vaccine Study Group. This study was conducted with the approval of the Human Subjects Committee of the University of Arizona.

REFERENCES

1. **Ampel, N. M., G. C. Bejarano, S. D. Salas, and J. N. Galgiani.** 1992. In vitro assessment of cellular immunity in human coccidioidomycosis: relationship between dermal hypersensitivity, lymphocyte transformation, and lymphokine production by pe-

ripheral blood mononuclear cells from healthy adults. J. Infect. Dis. **165:**710–715.

1a. **Ampel, N. M., C. L. Dols, and J. N. Galgiani.** Submitted for publication.

1b. **Ampel, N. M., C. L. Dols, J. N. Galgiani, L. Hood, and J. J. Rohwedder.** 1991. Coccidioidomycosis (coccy) during HIV infection: a continuing prospective study in the coccidioidal endemic area, abstr. WB2259. Program and Abstracts of the Seventh International Conference on AIDS.

2. **Beaman, L., D. Pappagianis, and E. Benjamini.** 1977. Significance of T cells in resistance to experimental murine coccidioidomycosis. Infect. Immun. **17:**580–585.

3. **Beaman, L. V., D. Pappagianis, and E. Benjamini.** 1979. Mechanisms of resistance to infection with *Coccidioides immitis* in mice. Infect. Immun. **23:**681–685.

4. **Calhoun, D. L., E. O. Osir, K. O. Dugger, J. N. Galgiani, and J. H. Law.** 1986. Humoral antibody responses to specific antigens of *Coccidioides immitis*. J. Infect. Dis. **154:**265–272.

5. **Coalson, J. J., V. T. Winter, H. M. Martin, and R. J. King.** 1986. Colloidal gold immunoultrastructural localization of rat surfactant. Am. Rev. Respir. Dis. **133:**230–237.

6. **Cohen, I. M., J. N. Galgiani, D. Potter, and D. A. Ogden.** 1982. Coccidioidomycosis in renal replacement therapy. Arch. Intern. Med. **142:**489–494.

7. **Cole, G. T., T. N. Kirkland, M. Franco, S. Zhu, L. Yuan, S. H. Sun, and V. M. Hearn.** 1988. Immunoreactivity of a surface wall fraction produced by spherules of *Coccidioides immitis*. Infect. Immun. **56:**2695–2701.

8. **Cole, G. T., D. Kruse, and K. R. Seshan.** 1991. Antigen complex of *Coccidioides immitis* which elicits a precipitin antibody response in patients. Infect. Immun. **59:**2434–2446.

9. **Cole, G. T., D. Kruse, S. Zhu, K. R. Seshan, and R. W. Wheat.** 1990. Composition, serologic reactivity, and immunolocalization of a 120-kilodalton tube precipitin antigen of *Coccidioides immitis*. Infect. Immun. **58:**179–188.

10. **Cole, G. T., L. M. Pope, M. Huppert, S. H. Sun, and P. Starr.** 1985. Wall composition of different cell types of *Coccidioides immitis*, p. 112–129. *In* H. E. Einstein, and A. Catanzaro (ed.), Coccidioidomycosis: Proceedings of the 4th International Conference. The National Foundation for Infectious Diseases, Washington, D.C.

11. **Cole, G. T., K. R. Seshan, M. Franco, E. Bukownik, S. H. Sun, and V. M. Hearn.** 1988. Isolation and morphology of an immunoreactive outer wall fraction produced by spherules of *Coccidioides immitis*. Infect. Immun. **56:**2686–2694.

12. **Cox, R. A., and L. A. Britt.** 1987. Antigenic identity of biologically active antigens in coccidioidin and spherulin. Infect. Immun. **55:**2590–2596.

13. **Cox, R. A., L. A. Britt, and R. A. Michael.** 1987. Isolation of *Coccidioides immitis* F antigen by immunoaffinity chromatography with monospecific antiserum. Infect. Immun. **55:**222–232.

14. **Dolan, M. J., and R. A. Cox.** 1991. Production and characterization of a monoclonal antibody to the complement fixation antigen of *Coccidioides immitis*. Infect. Immun. **59:**2175–2180.

15. **Dolan, M. J., R. A. Cox, V. Williams, and S. Woolley.** 1989. Development and characterization of a monoclonal antibody against the tube precipitin antigen of *Coccidioides immitis*. Infect. Immun. **57:**1035–1039.

16. **Drutz, D. J., and M. Huppert.** 1983. Coccidioidomycosis: factors affecting the host-parasite interaction. J. Infect. Dis. **147:** 372–390.

17. **Dugger, K. O., J. N. Galgiani, N. M. Ampel, S. H. Sun, D. M. Magee, J. Harrison, and J. H. Law.** 1991. An immunoreactive apoglycoprotein purified from *Coccidioides immitis*. Infect. Immun. **59:**2245–2251.

18. **Estes, D. M., J. W. Templeton, D. M. Hunter, and L. G. Adams.** 1990. Production and use of murine monoclonal antibodies reactive with bovine IgM isotype and IgG subisotypes (IgG1, IgG2a, and IgG2b) in assessing immunoglobulin levels in serum of cattle. Vet. Immunol. Immunopathol. **25:**61–72.

19. **Fish, D. G., N. M. Ampel, J. N. Galgiani, C. L. Dols, P. C. Kelly, C. H. Johnson, D. Pappagianis, J. E. Edwards, R. B. Wasserman, R. J. Clark, D. Antoniskis, R. A. Larsen, S. J. Englender, and E. A. Petersen.** 1990. Coccidioidomycosis during human immunodeficiency virus infection. A review of 77 patients. Medicine (Baltimore) **69:**384–391.

20. **Galgiani, J. N., K. O. Dugger, N. M. Ampel, S. H. Sun, and J. H. Law.** 1988. Extraction of serologic and delayed hypersensitivity antigens from spherules of *Coccidioides immitis*. Diagn. Microbiol. Infect. Dis. **11:**65–80.

21. **Galgiani, J. N., G. M. Grace, and L. L. Lundergan.** 1991. New serologic tests for early detection of coccidioidomycosis. J. Infect. Dis. **163:**671–674.

22. **Huppert, M., J. W. Bailey, and P. Chitjian.** 1967. Immunodiffusion as a substitute for complement fixation and tube precipitins in coccidioidomycosis, p. 221–225. *In* A. Ajello (ed.), Coccidioidomycosis: the second symposium on coccidioidomycosis. University of Arizona, Tucson.

23. **Johnson, J. E., and N. L. Goodman.** 1985. Variation in complement fixation test results with three *Histoplasma capsulatum* yeast phase antigens. J. Clin. Microbiol. **22:**1066–1067.

24. **Kruse, D., and G. T. Cole.** 1990. Isolation of tube precipitin antibody-reactive fractions of *Coccidioides immitis*. Infect. Immun. **58:**169–178.

25. **Levine, H. B., J. M. Cobb, and G. M. Scalarone.** 1969. Spherule coccidioidin in delayed dermal sensitivity reactions of experimental animals. Sabouraudia **7:**20–32.

26. **Levine, H. B., J. M. Cobb, and C. E. Smith.** 1961. Immunogenicity of spherule-endospore vaccines of *Coccidioides immitis* for mice. J. Immunol. **87:**218–227.

27. **Levine, H. B., Y.-C. M. Kong, and C. E. Smith.** 1965. Immunization of mice to *Coccidioides immitis*: dose, regimen and spherulation stage of killed spherule vaccines. J. Immunol. **94:**132–142.

28. **Mort, A. J., and D. T. A. Lamport.** 1977. Anhydrous hydrogen fluoride deglycosylates glycoproteins. Anal. Biochem. **82:**289–309.

29. **Pappagianis, D., C. E. Smith, G. S. Kobayashi, and M. Saito.** 1961. Studies of antigens from young mycelia of *Coccidioides immitis*. J. Infect. Dis. **108:**35–44.

30. **Pappagianis, D., and B. L. Zimmer.** 1990. Serology of coccidioidomycosis. Clin. Microbiol. Rev. **3:**247–268.

31. **Resnick, S., B. Zimmer, D. Pappagianis, A. Eakin, and J. McKerrow.** 1990. Purification and amino-terminal sequence analysis of the complement-fixing and precipitin antigens from *Coccidioides immitis*. J. Clin. Microbiol. **28:**385–388.

32. **Scalarone, G. M., H. B. Levine, D. Pappagianis, and S. D. Chaparas.** 1974. Spherulin as a complement-fixing antigen in human coccidioidomycosis. Am. Rev. Respir. Dis. **110:**324–328.

33. **Shively, J. E.** 1986. Reverse-phase HPLC isolation and microsequencing, p. 60–64. *In* J. E. Shively (ed.), Methods of protein microcharacterization: a practical handbook. Humana Press, Clifton, N.J.

34. **Siegel, J. P., and J. S. Remington.** 1983. Comparison of methods for quantitating antigen-specific immunoglobulin M antibody with a reverse enzyme-linked immunosorbent assay. J. Clin. Microbiol. **18:**63–70.

35. **Smith, C. E., E. G. Whiting, E. E. Baker, H. G. Rosenberger, R. R. Beard, and M. T. Saito.** 1948. The use of coccidioidin. Am. Rev. Tuberc. Pulm. Dis. **57:**330–360.

36. **Wack, E. E., K. O. Dugger, and J. N. Galgiani.** 1988. Enzyme-linked immunosorbent assay for antigens of *Coccidioides immitis*: human sera interference corrected by acidification-heat extraction. J. Lab. Clin. Med. **111:**560–565.

37. **Wagh, P. V., and O. P. Bahl.** 1981. Sugar residues on proteins. Crit. Rev. **10:**307–377.

38. **Wieden, M. A., J. N. Galgiani, and D. Pappagianis.** 1983. Comparison of immunodiffusion techniques with standard complement fixation assay for quantitation of coccidioidal antibodies. J. Clin. Microbiol. **18:**529–534.

39. **Williams, P. L., and the Valley Fever Vaccine Study Group.** 1985. Further assessment of the morbidity associated with the killed *Coccidioides immitis* spherule vaccine in humans, p. 339–346. *In* H. E. Einstein and A. Catanzaro (ed.), Coccidioidomycosis: Proceedings of the 4th International Conference on Coccidioidomycosis. The National Foundation for Infectious Diseases,

Washington, D.C.
40. **Yuan, L., G. T. Cole, and S. H. Sun.** 1988. Possible role of a proteinase in endosporulation of *Coccidioides immitis*. Infect. Immun. **56:**1551–1559.
41. **Zimmer, B. L., and D. Pappagianis.** 1986. Comparison of immunoblot analyses of spherule-endospore-phase extracellular protein and mycelial-phase antigen of *Coccidioides immitis*. Infect. Immun. **53:**64–70.
42. **Zimmer, B. L., and D. Pappagianis.** 1988. Characterization of a soluble protein of *Coccidioides immitis* with activity as an immunodiffusion-complement fixation antigen. J. Clin. Microbiol. **26:**2250–2256.
43. **Zimmer, B. L., and D. Pappagianis.** 1989. Immunoaffinity isolation and partial characterization of the *Coccidioides immitis* antigen detected by the tube precipitin and immunodiffusion-tube precipitin tests. J. Clin. Microbiol. **27:**1759–1766.