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Identification of a *Coccidioides immitis* Antigen 2 Domain That Expresses B-Cell-Reactive Epitopes

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Antigen 2 (Ag2), a major immunoreactive component of *Coccidioides immitis* mycelium- and spherule-phase cell walls, was recently cloned in our laboratory and was shown to elicit T-cell responses in *Coccidioides*-immune mice. In this investigation, we evaluated recombinant Ag2 (rAg2) and PCR-generated Ag2 truncations for expression of B-cell-reactive epitopes in enzyme-linked immunosorbent and immunoblot assays with sera from patients with active coccidioidomycosis, a hyperimmune goat anti-Ag2 serum, and a murine anti-Ag2 monoclonal antibody that recognizes a conformational epitope. The results established that rAg2 expresses both linear and conformational B-cell-reactive epitopes which are localized to a domain comprised of amino acids 19 through 96 (designated A19–96). Truncations designed to identify epitopes within the A19–96 domain yielded fragments that either were nonreactive (A62–194, A19–61, and A49–79) or showed reduced reactivity (A19–79). Hence, A19–96 was the shortest domain expressing epitopes recognized by the panel of antibodies. The prevalence of antibodies to the A19–96 domain was evaluated in enzyme-linked immunosorbent assays of sera from 28 coccidioidomycosis patients. Antibody reactivity was detected in 79% of the patients' sera, and the level of antibody reactivity was directly correlated with disease severity. Whereas patients with pulmonary disease showed a mean response (A_{405}) of 0.16 ± 0.04, patients with disseminated coccidioidomycosis showed a mean response of 0.69 ± 0.17 ($P < 0.05$). No reactivity was detected with sera from histoplasmosis or blastomycosis patients. The production of a recombinant peptide that expresses *C. immitis*-specific Ag2 epitopes provides a useful reagent for examining the role of anti-Ag2 antibodies in coccidioidomycosis.

Coccidioides immitis, the causative agent of coccidioidomycosis, is a dimorphic fungus which grows in the soil in the arid and semiarid regions of the Western Hemisphere (21). The disease is acquired by inhalation of arthroconidia, borne by the saprobic mycelia, which in host tissue undergo a morphologic conversion into a spherule-endospore phase (16). The majority of infections are asymptomatic. However, approximately 5% of patients develop acute or chronic pulmonary disease, ranging from a single nodule or cavity to a progressive fibrocavitary process (16). Dissemination to extrapulmonary sites occurs in approximately 1% of primary infections, with an increased incidence in persons of Asian, African-American, and, to a lesser extent, Hispanic ancestry (16, 21).

Host defense against *C. immitis* is associated with the acquisition of delayed-type dermal hypersensitivity and the production of the T helper 1 cytokines (1, 2, 6, 19). In studies to identify T-cell-reactive components of *C. immitis*, Cox et al. (5, 8, 10, 18, 25) isolated an alkaline-soluble, water-soluble extract from mycelia and spherules and showed that the extract, designated C-ASWS, elicited T-cell responses in coccidioidomycosis patients and experimentally infected animals and protected mice against challenge with arthroconidia. The C-ASWS extract was shown to be comprised of a large polymeric antigen, designated antigen 2 (Ag2), and a polysaccharide which has serological reactivity in the classical tube precipitin (TP) assay (8, 9).

We recently cloned Ag2 by using a *C. immitis* cDNA-lambda ZAP expression library and showed that the recombinant Ag2 (rAg2) protein elicited footpad hypersensitivity responses and induced T helper-1-associated cytokine expression in *Coccidioides*-immune mice (26, 28). In this investigation, we sought to identify and characterize B-cell-reactive epitopes by evaluating the reactivity of the rAg2 and Ag2-derived truncations in immunoblot and enzyme-linked immunosorbent assays (ELISAs).

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MATERIALS AND METHODS

Antibodies. Goat antiserum to Ag2 and a murine immunoglobulin G2a (IgG2a) monoclonal antibody (MAb), which recognizes a conformational peptide epitope on Ag2, were previously described (8, 11). Normal goat serum and an irrelevant murine IgG1 MAb (13) were included as negative controls. Goat anti-Ag2 and normal goat serum were preadsorbed with *Escherichia coli*-phage lysate (Stratagene) before use.

The 28 coccidioidomycosis serum samples were obtained from patients being treated at the Texas Center for Infectious Disease and the Brooke Army Medical Center, Fort Sam Houston, San Antonio, Tex. All but two of the serum samples were positive for complement-fixing (CF) antibody titers to coccidioidin, as measured by the quantitative immunodiffusion assay for CF antibody. Sera from 20 histoplasmosis patients, all of whom were serologically reactive in CF assays with histoplasmin and *Histoplasma capsulatum* yeast-phase cells, were kindly provided by L. Joseph Wheat (Wishard Memorial Hospital, Indianapolis, Inc.). Blastomycosis patients' sera were generously provided by Bruce Klein (University of Wisconsin—Madison Medical School, Madison) and were reported to be serologically reactive when assayed by a radioimmunoassay with *Blastomyces dermatitidis* WI-1 antigen (17). Normal control sera were obtained from 14 healthy subjects who were recruited from the staff at the Texas Center for Infectious Disease.

Plasmid construction. DNA fragments encoding overlapping regions of Ag2 were generated from Ag2 cDNA by PCR cloning. In brief, oligonucleotide primers containing *Eco*RI (sense primers) and *Xho*I (antisense primers) were designed according to the sequences shown in Table 1. The cDNAs were amplified in a PTC-100 thermocycler (MJ Research Inc., Watertown, Mass.) programmed for 30 cycles at 94°C for 90 s, 71°C for 45 s, and 72°C for 90 s, followed by a final cycle at 72°C for 10 min, except where noted in Table 1.

The amplified cDNA fragments were cloned into a pCR-II TA cloning vector (Invitrogen, San Diego, Calif.), released with *Xho*I and *Eco*RI, and subcloned into pGEX-4T-3 vector (Pharmacia) in frame with the plasmid-encoded glutathione *S*-transferase (GST) gene (23). The parental pGEX-4T-3 plasmid served as a control for GST peptide alone.

Expression and purification of GST fusion peptides. Details of the procedure for the expression and purification of the GST fusion proteins have been published elsewhere (23, 26). Briefly, *E. coli* TG-1 cells were transformed with the

TABLE 1. PCR primers used in the generation of Ag2 and Ag2 truncations

Truncation[a]	Primer sequence[b]	Nucleic acid starting site[c]	Predicted size (kDa)
A1–194	S, 5′-gaattcgaattcaATGCAGTTCTCTCACG-3′	174	50
	A, 5′-ctcgagctcgagTCATTTCGCCTTCCAAT-3′	864	
A1–18	S, 5′-tgaattcAATGCAGTTCTCTCACGCT-3′	174	30
	A, 5′-tGTCTGGGAGCTGctcgagGGCACTGGC-3′	220	
A19–194	S, 5′-tgaattcCCAGCTCCCAGACATCCC-3′	227	48
	A, 5′-tctcgagTTACAGGTAGGCAGCGAG-3′	742	
A19–96	S, 5′-tgaattcCCAGCTCCCAGACATCCC-3′	227	37
	A, 5′-tctcgagGGTGTCAACTGGTGGGAT-3′	448	
A19–79	S, 5′-tgaattcCCAGCTCCCAGACATCCC-3′	227	35
	A, 5′-tctctgagGACGATGTTGGACGAGGAGAT-3′	411	
A19–61	S, 5′-tgaattcCCAGCTCCCAGACATCCC-3′	227	33
	A, 5′-tctcgagAGGAGTGATTCTGTCCTGGGAG-3′	337	
A49–79	S, 5′-tgaattcACACTGCTCCAAGCCTGAGCT-3′	319	32
	A, 5′-tctcgagGACGATGTTGGAGACGGAGAT-3′	411	
A62–194	S, 5′-tgaattcACCTTGCGTTGAGGAGGCCTGC-3′	355	43
	A, 5′-tctcgagTTACAGGTAGGCAGCGAG-3′	742	

[a] Annealing temperatures for A1–194 and A1–18 were 68 and 67°C, respectively. All other truncations were annealed at 71°C.
[b] S, sense primer; A, antisense primer. Lowercase letters denote modified sequences: lowercase letters with single underline, *Eco*RI site; lowercase letters with double underline, *Xho*I site.
[c] Nucleotide starting numbers are based upon the cDNA sequence (26).

pGEX-4T-3 fusion plasmid and, for a negative control, the parental pGEX-4T-3 plasmid. The transfected host cells were grown in 400 ml of 2× YTA medium (1% yeast extract, 1.6% tryptone, 100 μg of ampicillin per ml in 0.9% NaCl, adjusted to pH 7.0) at 37°C in a shaking incubator until the optical density of the culture medium reached 0.6 to 0.8 at 600 nm. Fusion proteins were induced by the addition of isopropyl-β-D-thiogalactopyranoside (final concentration, 0.5 mM), and 3 h later, the cells were harvested by centrifugation, washed with ice-cold phosphate-buffered saline (pH 7.2), and lysed by sonication. Fusion proteins and the GST peptide control were affinity purified by adsorption on glutathione-Sepharose 4B beads according to the manufacturer's directions (Pharmacia) and stored at −80°C until assayed.

SDS-PAGE and Western blot analysis. Fusion proteins were analyzed for molecular size and homogeneity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and for immunoreactivity by Western blotting according to published methods (26). Briefly, 4 μg of the GST recombinant proteins and the GST peptide control were loaded onto 4 to 20% polyacrylamide gradient SDS-PAGE gels and electrophoresed in parallel with molecular size markers (Novel Experimental Technology, San Diego, Calif.). The gels were stained for protein with Coomassie blue, or for immunoblot analyses, the bands were electrophoretically transferred onto nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, N.H.), and the membranes were blocked overnight in Tris-buffered saline containing 0.5% Tween-20 (TBST buffer) and 1% bovine serum albumin (BSA). Individual lanes were incubated with goat anti-Ag2, normal goat serum, patient serum, normal human serum, anti-Ag2 MAb, or an irrelevant MAb. The goat and human antibodies were diluted 1:1,000 in TBST; the MAbs were diluted 1:100. Following a 1-h incubation, the membranes were washed and incubated with alkaline phosphatase-conjugated rabbit anti-goat IgG, goat anti-human Igs (IgG, IgM, and IgA [H + L]), or goat anti-mouse IgG, all obtained from Kirkegaard and Perry Laboratories (Gaithersburg, Md.) and used at a concentration of 1:1,000. After a 30-min incubation at room temperature, the reactions were visualized by the addition of 5-bromo-4-chloro-3-indolylphosphate–Nitro Blue Tetrazolium (Sigma Chemical Co., St. Louis, Mo.).

ELISA. Individual wells on a 96-well microtiter plate were coated overnight at 4°C with fusion proteins diluted in a carbonate-bicarbonate buffer (pH 9.6) to a concentration of 1.1 pmol/well for assays with goat or human serum and 2.7 pmol/well for assays with MAb. Wells were blocked overnight in TBST containing 1% BSA and then incubated for 1 h with serial dilutions of goat or human serum or overnight with a MAb. Alkaline phosphatase-labelled secondary antibodies (rabbit anti-goat IgG; goat anti-human IgG, IgM, and IgA [H+L]; and goat anti-mouse IgG) were added at 1:1,000 dilutions, and after a 1-h incubation at room temperature, the reactions were visualized by the addition of *p*-nitrophenylphosphate diluted in 1 M diethanolamine–0.5 mM buffer (pH 9.8). The absorbances were read at 410 nm 30 min later on an MR 700 microplate reader (Dynatech Laboratories) and are reported as means ± standard errors. Absorbance values that exceeded the mean plus 2 standard deviations of the mean in assays of the 14 healthy subjects were considered positive.

Statistical analyses. Differences in the responses among the patient groups were evaluated for statistical significance by the nonparametric Mann-Whitney test and Wilcoxon signed-rank test for unpaired and paired data, respectively. Correlation analyses were performed with Spearman's rank sum correlation. Probability values of <0.05 were considered significant.

RESULTS

Localization of B-cell-reactive epitopes. The molecular size and homogeneity of protein truncations expressed by the PCR-generated constructs were evaluated by SDS-PAGE (Fig. 1). Both full-length rAg2 (amino acids 1 to 194 [A1–194]) and the A19–194 truncation were expressed as two bands in *E. coli*, a result that is thought to be attributable to posttranslational modification of the peptides by *E. coli* or possibly proteolytic degradation (26). All other truncations showed a band approximating the predicted molecular size. We also noted the pres-

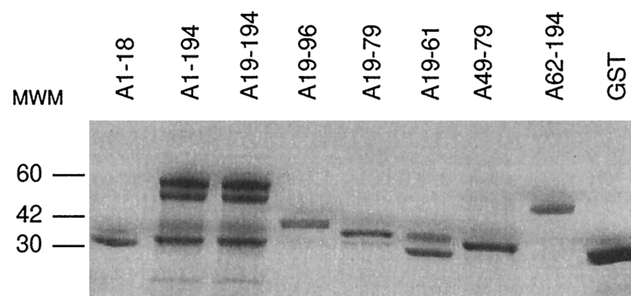

FIG. 1. SDS-PAGE analyses of rAg2 (A1–194) and PCR-generated truncations of Ag2. The recombinants were expressed as GST-fusion peptides. Molecular mass markers (MWM [kilodaltons]) are shown on the left.

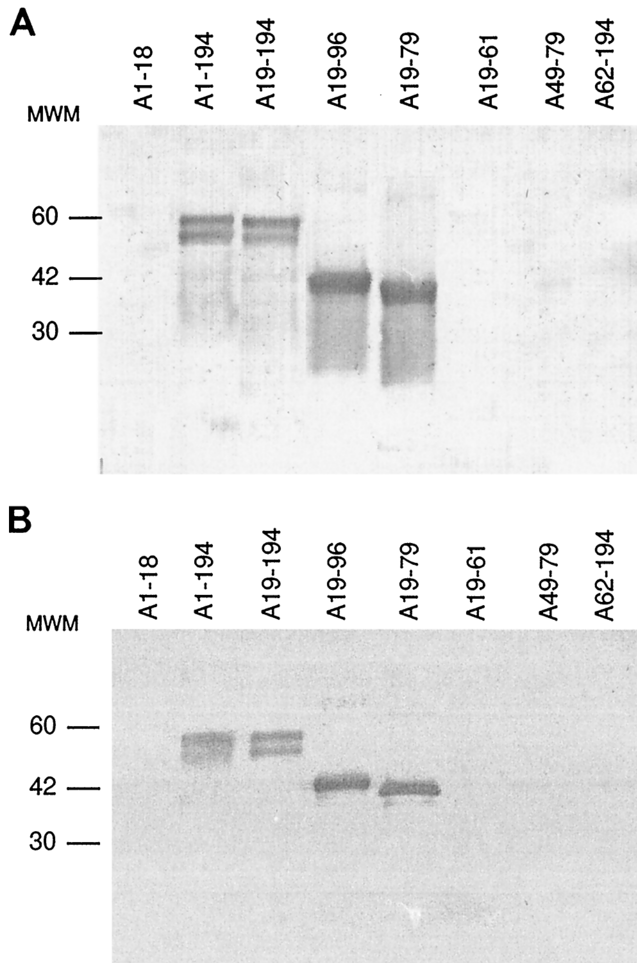

FIG. 2. Reactivity of rAg2 (A1–194) and Ag2 truncations in immunoblots probed with serum from a coccidioidomycosis patient (A) or hyperimmune goat anti-Ag2 (B). Molecular mass markers (MWM [kilodaltons]) are shown on the left.

ence of free GST peptide (33 kDa) in all preparations and at a particularly high level in A19–61.

The immunoreactivity of the bands obtained in SDS-PAGE gels was evaluated by immunoblot assays with serum from a patient with disseminated coccidioidomycosis, goat anti-Ag2 serum, and a murine anti-Ag2 MAb. The results showed that A1–194, A19–194, A19–96, and A19–79 were strongly reactive with the coccidioidomycosis patient serum (Fig. 2A) and goat anti-Ag2 serum (Fig. 2B), whereas the A62–194, A19–61, A49–79, and A1–18 truncations were nonreactive with these sera. Neither the full-length rAg2 nor the Ag2 truncations showed reactivity with the anti-Ag2 MAb, which is consistent with our previous report that the anti-Ag2 MAb recognizes a conformational epitope that is labile to denaturing SDS-PAGE (11).

The immunoreactivity of the rAg2 derivatives was further evaluated with ELISAs. The results, depicted in Fig. 3, showed that the A19–96 truncation had the highest level of reactivity when assayed against the coccidioidomycosis patient's serum, followed by A1–194, A19–194, and A19–79 (Fig. 3A). No reactivity was detected with A62–194, A19–61, A49–79, or A1–18 (data not shown). Similar results were obtained when the truncations were assayed against goat anti-Ag2 (Fig. 3B) and the anti-Ag2 MAb (Fig. 3C), with the notable exception that neither of these antibodies recognized the A19–79 truncation. The lack of reactivity of the goat polyclonal antiserum in the ELISA but not the immunoblot (Fig. 2) is surprising, but it could be explained on the basis that the goat antibody(s) recognizes one or more linear epitopes which are accessible only when assayed under denaturing conditions, such as SDS-PAGE.

Comparison of reactivities of rAg2 and A19–96 in detecting antibody in coccidioidomycosis patients' sera. Since the preceding data showed that A19–96 is the shortest truncation that expresses epitopes reactive with patients' sera, goat anti-Ag2, and the anti-Ag2 antibody, experiments were undertaken to examine and compare the reactivity of A19–96 with that of the full-length rAg2 protein in ELISAs of sera from 28 coccidioidomycosis patients. Seventeen (61%) of the serum samples re-

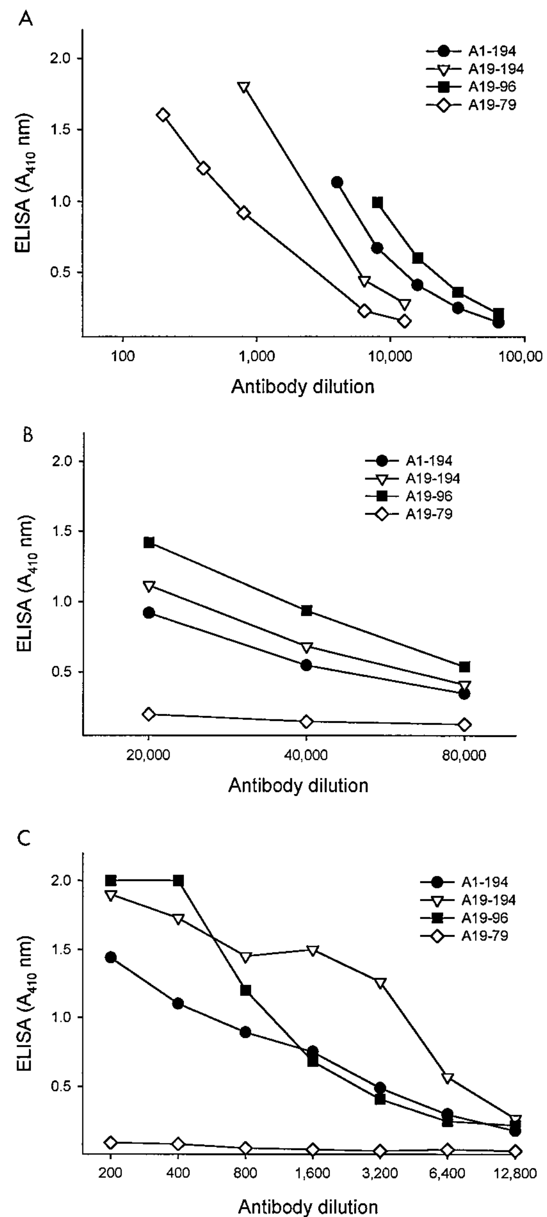

FIG. 3. Reactivity of rAg2 (A1–194) and Ag2 truncations in ELISAs with serum from a coccidioidomycosis patient (A), hyperimmune goat anti-Ag2 (B), or a murine anti-Ag2 MAb (C).

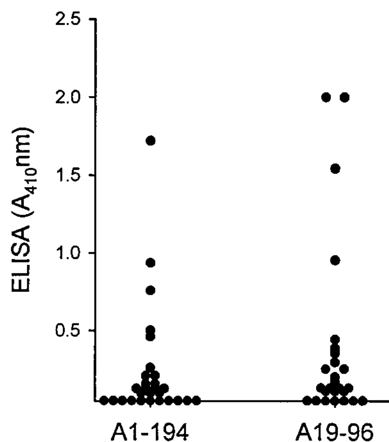

FIG. 4. Reactivity of rAg2 (A1–194) and A19–96 in ELISAs with sera from 28 patients with active coccidioidomycosis. The patients' sera were assayed at a 1:2,000 dilution.

acted with A1–194, with a mean response (A_{410}) of 0.25 ± 0.07 (Fig. 4). The A19–96 truncation detected antibody in 22 (79%) of the serum samples, with a mean response (A_{410}) of 0.43 ± 0.07. While A1–194 and A19–96 were comparable in reactivity in terms of the frequency of positive reactions, A19–96 was significantly more sensitive than A1–194 in terms of the magnitude of the responses ($P < 0.0001$ by Wilcoxon signed-rank test).

Since we had observed that goat anti-Ag2 serum showed reactivity in immunoblots, but not in ELISAs against A19–79, experiments were done to determine if sera from five patients who were negative to A1–194 and A19–96 in the ELISAs might show reactivity in immunoblots. Only one serum sample, which was obtained from a patient with multifocal disseminated disease, showed reactivity in blots but not the ELISA. The reverse situation was not observed. That is, patients who were reactive in the ELISA also showed reactivity to the peptides in immunoblots.

Relationship between disease severity and antibody reactivity to rAg2 and A19–96. Patients with multifocal coccidioidomycosis typically manifest proliferative B-cell responses, with increased levels of anti-*Coccidioides* IgG and IgE antibodies in serum (7). It was of interest, therefore, to compare the reactivities of the two antigens in ELISAs of sera from patients with disseminated versus those from patients with pulmonary disease. The results, depicted in Fig. 5, established that antibody levels to A1–194 and A19–96 directly correlated with disease severity. Seventeen of the 28 patients had disseminated coccidioidomycosis, and of these, 13 (76%) serum samples reacted with A1–194, with a mean response (A_{410}) of 0.35 ± 0.11. Only 4 (36%) of the 11 patients who had pulmonary disease showed reactivity to A1–194, and the mean response (A_{410}) of the group was 0.12 ± 0.02, which was significantly lower than the response of patients with disseminated disease ($P < 0.02$). A similar direct correlation was observed between antibody titers to A19–96 and disease severity. Fourteen (82%) of the serum samples from patients with disseminated disease and 8 (73%) of the serum samples from the patients with pulmonary disease showed reactivity, with mean responses (A_{410}) of 0.69 ± 0.17 and 0.16 ± 0.04, respectively ($P < 0.05$). In comparison, CF antibody titers, which are known to directly correlate with disease severity (22), were 1:32 and 1:8 in patients with disseminated and pulmonary disease, respectively ($P < 0.01$).

Specificity of epitopes expressed by rAg2 and A19–96. The specificity of the epitopes expressed by A1–194 and A19–96 was examined with ELISAs of sera from 20 patients with active histoplasmosis and 12 patients with active blastomycosis. None of the sera showed reactivity with either A1–194 or A19–96 (results not shown).

DISCUSSION

A number of investigations have established that the Ag2-enriched C-ASWS cell wall extract of mycelium- and spherule-phase cells induces cell-mediated immune responses to *C. immitis* and protects mice against lethal challenge with arthroconidia (10, 18, 25). We recently cloned Ag2 cDNA and showed that the rAg2 protein elicits T-cell responses in *C. immitis*-immune mice to a level comparable to that obtained with C-ASWS (26). In this investigation, we report that the rAg2 protein is reactive with sera from coccidioidomycosis patients, a hyperimmune goat anti-Ag2 serum, and a murine anti-Ag2 MAb. The epitopes recognized by these antibodies appear to be specific to *C. immitis* and are localized within the A19–96 domain.

The rAg2 protein and the A19–96 domain express linear and conformational B-cell-reactive epitopes, as judged by their reactivity with sera from coccidioidomycosis patients and goat anti-Ag2 in immunoblots and ELISAs and their reactivity with the murine anti-Ag2 in the ELISA. With two exceptions, sera that were reactive in immunoblots were also reactive in the ELISA, indicating that the serum samples, one from a patient with advanced disseminated disease and the other from a hyperimmune goat anti-Ag2 serum, recognize a strictly linear epitope. It bears emphasis, however, that the assignment of epitopes that are detected by the polyclonal sera as having a linear versus conformational structure is tentative, since linear (continuous) epitopes may in fact represent only a portion of a larger conformational (discontinuous) epitope (20, 24). It also bears emphasis that the recombinant peptides were produced in the procaryotic *E. coli* system and thus lack posttranslational modifications which may be required for the expression of conformational epitopes.

Neither we nor other investigators have succeeded in purifying native Ag2; hence, only a paucity of information is available about the composition and possible structural linkages of this cell wall antigen. Two-dimensional immunoelectrophoresis of coccidioidin showed that Ag2 has a polymeric precipitin

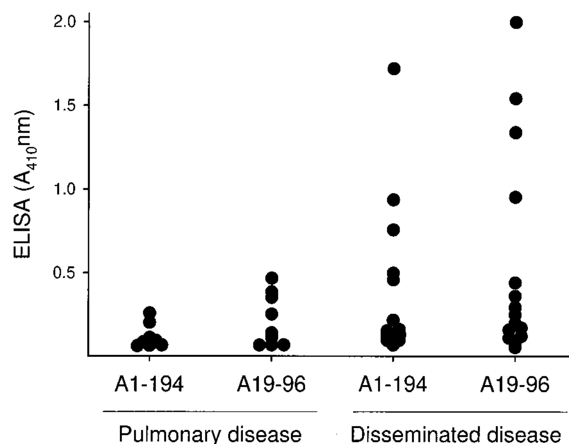

FIG. 5. Relationship between antibody reactivity to A1–194 and A19–96 and the severity of coccidioidal disease. The patients' sera were assayed at a 1:2,000 dilution.

pattern which is characterized by a large cathodal peak connected to a smaller anodal peak (8). The cathodal precipitin peak of the Ag2 polymer shows a diffusely staining pattern and reacts with anti-TP antibodies, including an anti-TP MAb (14), indicating the presence of the TP polysaccharide antigen. This feature, together with the fact that Ag2 and the TP polysaccharide coelute on molecular-sieve, ion-exchange, lectin affinity, and immunoaffinity columns (9), suggests that these two antigens represent a single complex rather than distinct components. In keeping with this possibility, the translated Ag2 gene contains several putative O-glycosylation sites (27), which could be involved in the linkage of the Ag2 protein with the 3-*O*-methylmannose-rich TP polysaccharide (3, 4, 29). The translated Ag2 DNA also showed several cysteine residues, suggesting intra- or interdisulfide bridges, which might confer tertiary structure. In addition, computerized algorithms showed that Ag2 has a high probability of having α-helices and β-sheets (27), which suggests secondary structure. These collective results predict that Ag2 is a highly complex antigen, comprised of secondary and tertiary domains, which have the potential for expressing both linear and conformational epitopes.

Dugger et al. (15) isolated a 33-kDa peptide by chromatographic fractionation of a chemically deglycosylated spherule extract and showed that the peptide fused with the anodal precipitin peak of the Ag2 polymer in tandem two-dimensional immunoelectrophoresis. When used as a target antigen in ELISAs, the 33-kDa peptide showed reactivity with sera from 35 (95%) of 37 patients with active coccidioidomycosis. This high level of sensitivity contrasts to our results with rAg2 and the A19–96 truncation, which detected antibodies in only 77 and 79% of the serum samples, respectively. One explanation to account for these differences is that the recombinant preparations lack epitopes that are expressed by the native antigen. It is noteworthy, however, that the molecular mass of the 33-kDa peptide exceeds the 19.4 kDa which is predicted by translated Ag2 cDNA (26) and the Ag2 gene (27), raising the possibility that the 33-kDa peptide preparation may not have been antigenically homogeneous.

The role of anti-Ag2 antibodies in host defense against *C. immitis* is not known. Passive immunization of mice with the anti-Ag2 IgG2a MAb does not have a discernible effect upon the course of the disease (12), but this result does not preclude the possibility that antibodies directed against other epitopes may play a protective role. Experiments are needed, therefore, to develop other MAbs to Ag2 and the recombinant derivatives and to evaluate their immunopotentiating effect on the course of the disease. It would also be of great interest to examine antibody responses in mice immunized with plasmids containing Ag2 cDNA and the PCR-generated DNAs and the effect of the immunizations on protection against challenge.

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