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Isolation and Morphology of an Immunoreactive Outer Wall Fraction Produced by Spherules of *Coccidioides immitis*

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A previously undescribed, immunoreactive, membranous spherule outer wall (SOW) fraction produced by *Coccidioides immitis* (strains 634 and 735) grown in culture was isolated. Both this fraction and intact spherules were reactive with sera from coccidioidomycosis patients, as demonstrated by immunofluorescence microscopy. The serological activity of SOW was also demonstrated by its reactivity with human anti-*C. immitis* tube precipitin in a standardized immunodiffusion assay. Extraction of SOW with the nonionic detergent *N*-octyl-β-D-glucopyranoside (OG) permitted the isolation of an OG-soluble fraction which was reactive in the immunodiffusion assay. Rabbit antisera raised against the OG-soluble fraction were used in immunofluorescence and immunoelectron-microscopic studies of the parasitic cycle to confirm that the immunoreactive components of the solubilized fraction of SOW were associated with the inner and outer layers of the spherule wall as well as with distinct cytoplasmic organelles observed in thin sections of spherules. The immunoreactivity of SOW with sera from patients suggested that infected individuals are exposed to this surface wall material isolated from in vitro-grown spherules.

Coccidioides immitis is a human respiratory pathogen which demonstrates an unusual morphogenetic transformation in vivo. In the first generation of the parasitic cycle, individual inhaled infectious cells, or arthroconidia (approximately 3 to 6 by 2 to 4 μm), grow to form large, spherical, multinucleate cells (approximately 60 μm in diameter). The latter, referred to as spherules (6), undergo segmentation by ingrowth of wall layers, a process which initially results in the formation of discrete, multinucleate compartments. The contents of these compartments soon differentiate into packets of small, uninucleate cells (approximately 2 to 4 μm in diameter) called endospores. The individual endospores, which at their earliest stage of development are small enough to be disseminated hematogenously, grow and differentiate into the second generation, endosporulating spherules.

The parasitic cycle of *C. immitis* has been reproduced in vitro by means of a defined medium in an atmosphere of 20% CO_2–80% air at an elevated temperature (21). Although the question of whether the in vivo and in vitro behaviors of the pathogen are identical cannot be completely answered as yet, morphological details of the fungus grown under these two conditions appear to be similar (18, 24). Evidently, at least some of the antigens produced by the in vitro-grown fungus are produced by in vivo forms, since patients demonstrate immunological responses to specific antigens prepared from the cultured pathogen (4, 6). In addition, antisera raised in rabbits against specific, wall-associated antigens derived from cultured cells have been shown by immunoelectron microscopy to react with cell wall components of the pathogen in lung tissue of intranasally infected BALB/c mice (unpublished data).

In this paper, we examine the morphology and immunoreactivity of a spherule outer wall (SOW) fraction which is released in large quantities into liquid culture medium. The isolated SOW reacts with sera obtained from coccidioidomycosis patients but not with control sera, as demonstrated by immunofluorescence, suggesting that infected individuals respond to the surface wall material of in vitro-grown spherules.

MATERIALS AND METHODS

Cultivation. Arthroconidia of *C. immitis* 634 and 735 (recent isolates from patients with disseminated coccidioidomycosis) were produced in plate cultures and isolated by vacuum harvesting (4). Conidia were suspended in liquid Converse medium (8, 21) at a concentration of approximately 10^5 cells per ml. Equal volumes of this suspension (about 0.5 ml) were added to the surfaces of sterile, 13-mm-diameter polycarbonate filters (Nuclepore Corp., Pleasanton, Calif.) which were laid over the surface of Converse medium containing 2% agar. The cultures, contained in petri plates (15 by 60 mm), were incubated at 39°C in a sealed Modular Incubator Chamber (Billups-Rothenberg, Del Mar, Calif.) purged with a filtered, premixed gas composed of 20% CO_2 and 80% air. Some cultures were incubated for 10 days, and then samples were removed from the filters for examination under a light microscope. Alternatively, filters and adherent fungal cells were transferred after 4 days of incubation to liquid Converse medium (100 ml) in 250-ml rubber-stoppered Erlenmeyer flasks (7). The same premixed gas was bled into each flask (7), and the cultures were placed in a shaking incubator at 39°C for 5 to 14 days. Additional CO_2-air was added to the flasks every 2 days after inoculation. Production of the sloughing, membranous SOW was monitored by phase-contrast microscopy. All steps of the inoculation and subsequent isolation procedure were performed in a Bioflow chamber (Germfree Laboratories Inc., Miami, Fla.) housed in our Biohazardous Materials Containment Laboratory.

Isolation of SOW. The liquid cultures were pooled and centrifuged (500 × *g*, 15 min, 4°C), and the supernatant was decanted onto sterile Whatman no. 1 filter paper and subjected to aspirator-vacuum filtration. The filtrate was centrifuged (10,000 × *g*, 15 min, 4°C), and the supernatant was

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discarded. The pellet was washed with distilled water and centrifuged five times (10,000 × *g*, 15 min, 4°C). The pellet was composed of a gellike upper layer (SOW) and a small, densely packed lower layer (endospores and inner spherule wall fragments). The upper layer of the pellet was carefully removed with a sterile Pasteur pipette, suspended in distilled water, and centrifuged (10,000 × *g*). Samples of this pellet were examined under light and electron microscopes for homogeneity of the isolated wall material (see Fig. 1D and E) and plated on glucose-yeast extract medium (4) for detection of residual, live cells. The remaining samples of this pellet were lyophilized and stored at −20°C.

Light and electron microscopy. Spherules grown on polycarbonate filters and in Converse liquid medium as well as the SOW fraction isolated from liquid cultures were examined directly by phase-contrast microscopy. Spherules were prepared for the scanning electron microscope as described previously (2, 5), except that dehydration was performed in four increasing concentrations of ethanol (10% to absolute, 5 min each) at 4°C, and the cells were critical point dried with liquid CO_2 on Nuclepore filters (0.45-μm pore size). The isolated SOW was sectioned and examined with the transmission electron microscope (TEM) by two procedures. In the first method (see Fig. 1E), the SOW was fixed in 1% glutaraldehyde–1% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4), washed with buffer, dehydrated in three increasing concentrations of ethanol (10, 50, and 95%; 5 min each) at 4°C, and embedded in K4M low-temperature resin (Polysciences, Warrington, Pa.). Thin sections were poststained with uranyl acetate and lead citrate (1) and examined in the TEM. Osmium tetroxide (OsO_4) cannot be used as a fixative or stain for tissue embedded in K4M plastic because it blocks the UV light used for polymerization of the low-temperature resin. In the second method (see Fig. 1F), the SOW was fixed as described above, dehydrated in increasing concentrations of *N,N*-dimethyl formamide, and embedded in K4M at 4°C (10). Thin sections were mounted on gold grids, poststained with uranyl acetate and lead citrate as described above, and stained to enhance the contrast of lipid-containing membranes (23). The mounted sections were exposed for 2 h to vapors of 2% OsO_4 heated to 50°C. After four to five rinses with hot (50°C) distilled water, the grids were floated on droplets of 1% thiocarbohydrazide (Sigma Chemical Co., St. Louis, Mo.) for 1 h at 50°C. After additional rinses with hot distilled water, the grids were exposed to OsO_4 vapors as described above for 1 h and examined directly in the TEM.

Immunofluorescence. Spherules grown in liquid medium and on polycarbonate filters as well as SOW isolated from liquid cultures were washed by centrifugation in sodium phosphate buffer (0.1 M, pH 7.6) and suspended in 50 μl of a 1:200 dilution of a panel of sera from coccidioidomycosis patients (sera were obtained from the Veterans Administration Hospital, San Antonio, Tex.). All sera from coccidioidomycosis patients used in this study were complement fixation (CF) positive in a standardized immunodiffusion (ID) assay described below (15–17). For immunofluorescence, the spherules and isolated SOW were each incubated in sera for 15 min at room temperature, washed five times in sodium phosphate buffer, suspended in a 1:30 dilution in sodium phosphate buffer of goat anti-human immunoglobulin G (IgG) (heavy [γ]-chain specific) conjugated to fluorescein isothiocyanate (FITC; Sigma), and incubated at room temperature (23°C) for 15 min. The samples were again washed five times in sodium phosphate buffer and mounted in buffered glycerol (9 parts of glycerol to 1 part of 0.2 M sodium phosphate buffer [pH 9.0] [vol/vol]). The preparations were examined by fluorescence microscopy with a FITC filter. Samples prepared as described above but reacted with FITC conjugate alone or with human control sera (3) and then FITC conjugate served as controls.

Spherules grown in liquid medium and isolated SOW were also reacted with specific rabbit antisera raised against a nonionic detergent extract of SOW, described below, and then with goat anti-rabbit IgG conjugated to FITC. Samples reacted with FITC conjugate alone or with rabbit preimmune sera and then FITC conjugate served as controls.

Extraction of SOW. Sodium phosphate buffer (0.05 M NaH_2PO_4 [pH 7.4]) containing 0.15 M NaCl was prepared, and 1.8% (wt/vol) *N*-octyl-β-D-glucopyranoside (Calbiochem-Behring, La Jolla, Calif.), a nonionic detergent, was added. After complete solubilization of the detergent, the solution was filter sterilized (0.2-μm-pore Nuclepore filter). The lyophilized SOW (50 to 100 mg) was suspended in this solution (1 mg/ml) and stirred at 4°C for 48 h. The suspension was centrifuged (26,000 × *g*, 30 min, 4°C). Both the pellet (*N*-octyl-β-D-glucopyranoside-insoluble fraction; OG-IF) and supernatant (*N*-octyl-β-D-glucopyranoside-soluble fraction; OG-SF) were reserved in an ice bath. The OF-IF was washed five times with buffer by centrifugation, and the supernatant from the first wash was pooled with the OG-SF. The OG-IF was suspended in sterile distilled water, both the OG-IF and OG-SF were dialyzed separately against distilled water (wet cellulose dialysis tubing; molecular weight cutoff, 1,000; Medical Industries Inc., Los Angeles, Calif.) at 4°C for 48 h (six changes of dialysate, 6 liters each), and the retained material of each was lyophilized. The OG-IF was stored at −20°C.

Production of rabbit antisera. New Zealand White rabbits (two females, 3 kg each) were immunized subcutaneously with the OG-SF (1.0 mg) in 0.5 ml of phosphate-buffered saline (PBS) (pH 7.4) plus 0.5 ml of complete Freund adjuvant, followed by booster injections of the same wall fraction (1.0 mg suspended in PBS plus incomplete Freund adjuvant) at 3-week intervals for 5 months. Rabbits were test bled 5 to 10 days after each immunization. Antibody titers against the OG-SF were monitored by an indirect enzyme-linked immunosorbent assay (12). Amounts of bound antibody in the wells of microdilution plates (Nunc Immunoplate II; Hazelton Research Products, Denver, Pa.) were determined with goat anti-rabbit IgG (heavy- and light-chain specific) conjugated to peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, Md.).

Immunoelectron microscopy. Spherules were isolated from liquid cultures at 4, 6, 9, and 14 days after inoculation and prepared for thin sectioning and immunoelectron microscopy as described previously (4), except that cells were embedded in Spurr low-viscosity resin (1) polymerized at 37°C for 72 h. Sections were incubated (4°C, 48 h) with either rabbit anti–OG-SF or coccidioidomycosis patient serum (both determined to be tube precipitin [TP] positive as described below) plus 1% ovalbumin (Sigma) prepared in Tris hydrochloride buffer (0.02 M, pH 8.2). Several dilutions of the reaction mixture (1:1,000 to 1:10,000) were tested. The secondary antibody reaction mixture consisted of either goat anti-rabbit or goat anti-human IgG conjugated to colloidal gold (15-nm-diameter particles; Janssen Pharmaceutica Inc., Piscataway, N.J.) plus 1% ovalbumin prepared in Tris hydrochloride buffer. Sections reacted with preimmune rabbit serum or human control serum and then with the secondary antibody-gold conjugate served as controls.

IEP. The antigen content of wall fractions of *C. immitis*

was analyzed in our earlier investigations (4, 5) by two-dimensional immunoelectrophoresis (IEP). The coccidioidin (CDN)–anti-CDN reference system used for identification of specific antigens was originally developed by Huppert and co-workers (14, 19) and has been reproduced in our laboratory with anticoccidioidin obtained from newly immunized burros. The procedure used for IEP examination in this study involved preparation of an intermediate gel (22) composed of 1 part rabbit serum (either preimmune serum or antiserum raised against OG-SF) to 1 part 2% agarose. The upper gel contained the burro anti-CDN immunoglobulin diluted 1:10 in electrophoresis buffer (14). The precipitin arcs are numbered according to the previously described reference system (5, 14). CDN was used as the reference antigen and was prepared as described previously (4, 5). This IEP procedure was used to test for the reaction of antibodies in the rabbit anti–OG-SF serum with specific antigens recognized in the reference system.

ID. ID tests were performed by the method of Huppert and Bailey (15–17) for the detection of antigens which react with CF and TP antibodies in reference sera from coccidioidomycosis patients. The CF and TP reference antigens are 97 and 73/67L, respectively. The human reference antibodies against the CF and TP antigens are CF26 and TPO414, respectively. The test antigen was OG-SF suspended in PBS (pH 7.4).

RESULTS

Morphology. Spherules produced by both strains of *C. immitis* had a membranous outer wall component which, on solid media, was confluent between adjacent cells (Fig. 1A). When grown in liquid cultures, spherules began to shed detectable amounts of membranous wall material into the medium after 1 to 2 days. The thin, hyaline sheets of wall material were easily observed by phase-contrast optics (Fig. 1B). Under the scanning electron microscope (Fig. 1C), the partly sloughed, thin outer wall layer of a young spherule was visible. The large membranous sheets tended to roll up when released from the surfaces of spherules grown in liquid cultures. This behavior resulted, under the light microscope, in the false impression of needlelike structures (Fig. 1D). The SOW continued to accumulate throughout the entire period of incubation and was isolated from intact cells by filtration, successive washes with distilled water, and centrifugation (Fig. 1D). When the isolated SOW was seeded onto glucose-yeast extract medium, no growth was noted after 2 weeks at 30°C. Both light and electron microscopic observations of the isolated SOW indicated a homogeneous preparation of membranous material. Under the TEM, transverse and tangential sections of the isolated SOW revealed thin membranous and apparently amorphous wall material (Fig. 1E). After exposure of the sections to OsO_4 vapors and thiocarbohydrazide, the membranous wall material became more electron dense (Fig. 1F), indicative of OsO_4 binding to lipid in the sample; note the tripartite nature of the transversely sectioned and stained SOW (arrows).

Immunofluorescence. After growth for 10 days on polycarbonate filters or for 14 days in liquid Converse medium, several generations of spherules were produced by both strains from the initial inoculum of arthroconidia. The same spherules examined with the light microscope with bright-field illumination in Fig. 2A were examined by indirect immunofluorescence microscopy after reaction with serum from a coccidioidomycosis patient (Fig. 2B). Note the absence of hyphal fluorescence in Fig. 2B and the variable amount of fluorescence at the perimeter of the spherules, which may be related to the variable thickness of the spherule envelope. Also note in Fig. 2B the fluorescent SOW fragments which were not visible when bright-field optics were used (Fig. 2A). The isolated SOW was also immunoreactive with sera from coccidioidomycosis patients (Fig. 2C). Control preparations of intact spherules and isolated SOW showed no detectable fluorescence.

Spherules and SOW isolated from liquid Converse medium were also examined by indirect immunofluorescence microscopy after reaction with rabbit antisera raised against the OG-SF of SOW (Fig. 3B, D, and E). The spherule wall and membranous SOW released from the surfaces of spherules showed positive reactions. In the case of endosporulating spherules (Fig. 3A to D), the ruptured spherule wall was clearly fluorescent, while the wall of newly released endospores was weakly fluorescent.

Immunoelectron microscopy. Figure 3F shows a thin section of a young spherule reacted with rabbit antiserum raised against the OG-SF of SOW and then with goat anti-rabbit IgG–colloidal gold conjugate. The majority of label was bound to the spherule wall and surface material. However, some label was also observed in the cytoplasm, specifically associated with electron-translucent, irregularly shaped organelles (Fig. 4A). When thin sections of spherules were reacted with TP-positive human serum and then with goat anti-human IgG–colloidal gold conjugate, a similar distribution of label was noted on the cell wall and cytoplasmic organelles (Fig. 4B).

IEP. Comparison of the intermediate-gel IEP plates in Fig. 4C and D demonstrated that the rabbit antiserum raised against the OG-SF of SOW contained antibodies against antigens 2 and CS of the reference system (4, 14, 19).

ID. The ID plate in Fig. 5A shows CF and TP precipitin lines between the corresponding human reference antibody- and antigen-containing wells. The OG-SF was solubilized in PBS and added to the lower central well (200 μg in 20 μl of buffer). Fusion of the TP reference precipitin line with a precipitin line formed between the test well and the well containing the TP antibody was visible. A second, fused precipitin line (arrows) was also faintly visible between the same three wells and was continuous with the precipitin line formed between the test well and the well containing the CF antibody. The latter crossed the CF reference precipitin line. No fusion with or distortion of the CF reference precipitin line was observed in the ID plate when the OG-SF was placed in the test well. This same pattern of precipitin lines in the ID plate was observed after the OG-SF in PBS was incubated at 60°C for 30 min and then added to the test well (data not shown).

In Fig. 5B, the TP precipitin line formed between the two wells containing the TP reference antigen and human reference antibody was fused with a precipitin line formed between the test well (lower right) and the TP antigen-containing well. The test well contained the rabbit antiserum raised against the OG-SF of SOW.

DISCUSSION

The membranous surface wall material of *C. immitis* spherules revealed in this study has not been reported previously in ultrastructural examinations of the pathogen, probably because of poor preservation methods. Conventional methods of chemical fixation for preparing spherules for the TEM involve extensive use of dehydrating agents (e.g., absolute alcohol and acetone) prior to final transfer to

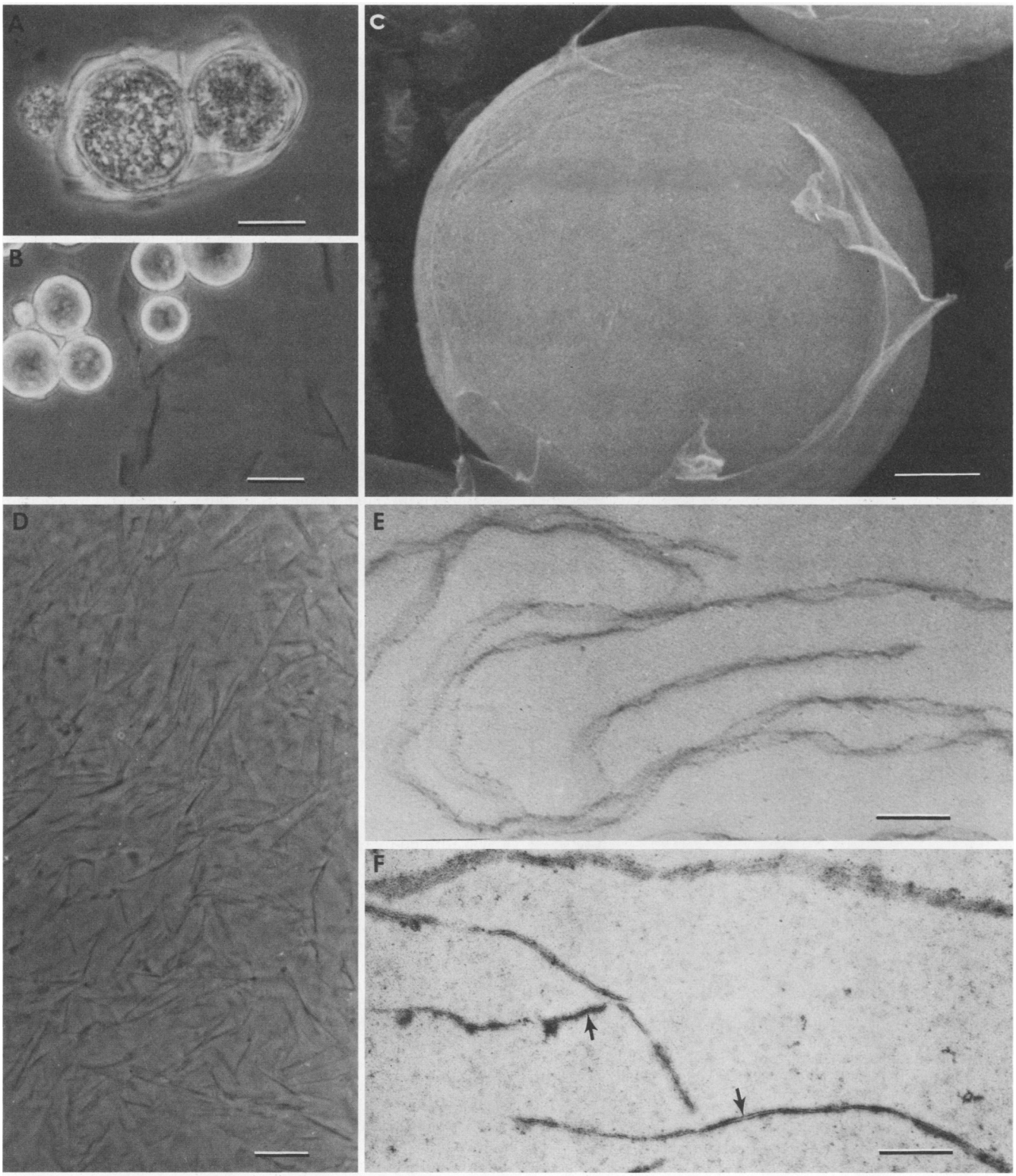

FIG. 1. (A, B, and D) Phase-contrast light micrographs of the SOW component of cells grown on a polycarbonate filter laid over the surface of Converse agar (A) and cells grown in liquid Converse medium (B) and the isolated SOW obtained from liquid cultures (D). Bars in panels A, B, and D, 10 μm. (C) Scanning electron micrograph of a young spherule showing partly released SOW. Bar, 2.0 μm. (E and F) TEM of isolated SOW revealing the thin, lightly stained membranous layer prior to reaction with OsO_4 vapors (E) and after reaction with OsO_4 vapors (F). Arrows in panel F indicate tripartite regions of the transversely sectioned SOW. Bars in panels E and F, 3.0 μm.

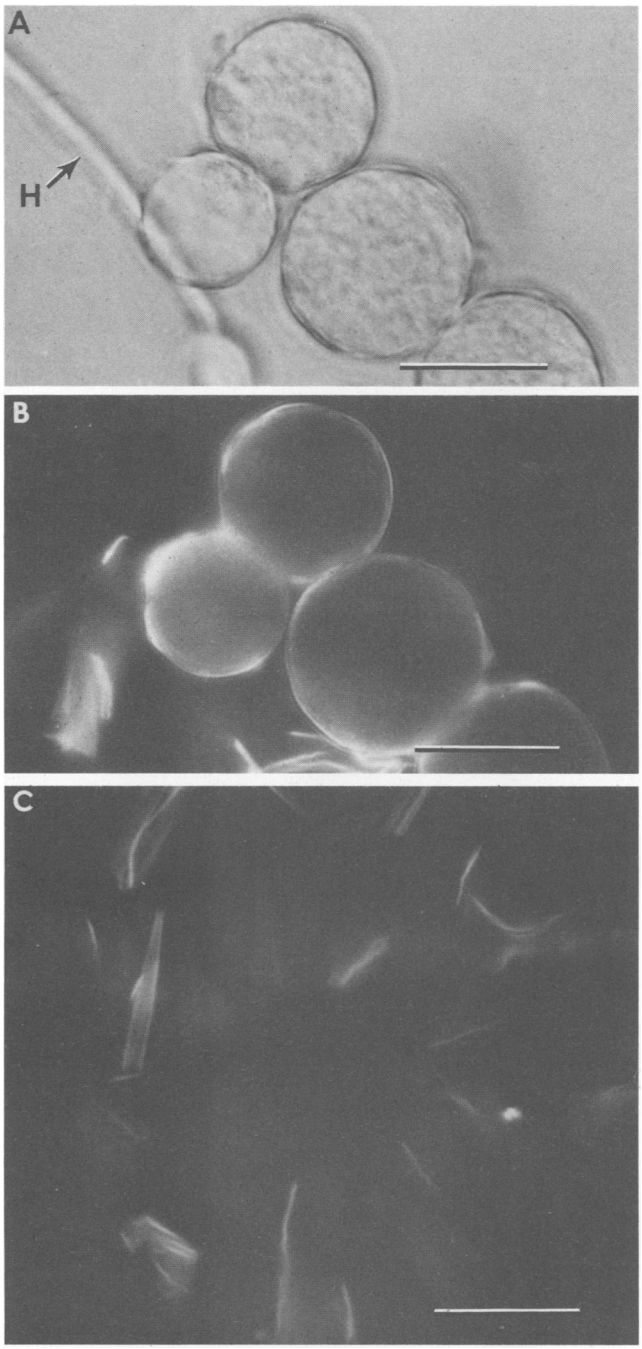

FIG. 2. Bright-field micrograph (A) and corresponding immunofluorescence micrograph (B) of spherules grown in liquid medium and immunofluorescence micrograph of isolated SOW (C). Samples were reacted with sera from coccidioidomycosis patients, washed, and then reacted with goat anti-rabbit IgG conjugated to FITC. Note the released SOW fragments in panel B which were not visible with bright-field optics (panel A). H, Hypha. Bars, 10 μm.

the embedding resin (24, 25). These organic solvents are capable of at least partially solubilizing the SOW, particularly the lipid components. Conventional fixation and dehydration methods were used in this study for preparing material for immunoelectron microscopy. Although the surface wall material of spherules still reacted with specific immunolabel, the SOW had the appearance of a fibrillar matrix, as described in an earlier investigation (11). Even the abbreviated dehydration steps and use of a water-miscible plastic for embedding the isolated SOW (Fig. 1E and F) resulted in some loss of structural components of the membranous outer wall layer. Only freeze-fracture preparation of spherules for TEM examination, which avoids exposure of cells to chemical fixatives and organic solvents (1), preserves the native outer wall structure (unpublished observations).

OsO_4 is a lipophilic heavy metal fixative or stain used in conventional electron microscopy (13). Thin sections of isolated SOW, chemically fixed in the absence of OsO_4 and embedded in low-temperature resin, demonstrated positive reactivity with OsO_4 vapors, especially when the sections were also exposed to the sulfur-containing, osmiophilic reagent thiocarbohydrazide (23). These results attest to the presence of a lipid matrix in SOW. In their pioneering histochemical studies of *C. immitis* spherules, Tarbet and Breslau (26) reported that the walls of mature spherules are rich in "lipid complexes," which they identified as phospholipids. The authors suggested that the lipid layer of the spherule wall may "resist the diffusion of large molecules" but "retain certain chemotactic substances within the cell and block or alter chemical interchange between parasite and tissues" of the host. The lipid-rich SOW layer may be a protective barrier which contributes to the survival of the pathogen in host tissue. Frey and Drutz (11) presented evidence that an extracellular matrix produced by the spherule may partly account for its survival in the presence of leukocytes from healthy donors. The authors suggested that the matrix may impede contact between polymorphonuclear neutrophils and the fungus and somehow reduce the efficiency of host attack against spherules. The SOW layer is also immunoreactive, as revealed by both immunofluorescence and ID tests with sera from coccidioidomycosis patients. Release of some of these immunoreactive macromolecules in vivo may be attributed to digestive activity by host cells (e.g., polymorphonuclear neutrophils) adjacent to the spherule envelope (9).

The immunoreactive components of the OG-SF of SOW identified in the ID assay were shown to be stable at 60°C. Although the heat-labile CF antigen (4) was absent from the OG-SF, as revealed by the ID assay, a distinct precipitin line was formed by reaction of the IDCF reference antibody with the test antigen. This precipitin line also appeared to be fused with one of the two continuous precipitin lines formed between the wells containing the IDTP reference antibody, the test antigen, and the IDTP reference antigen. Thus, the ID assay revealed that the detergent extract of SOW contains at least two immunoreactive, heat-stable components. One was common to both the IDCF and IDTP reference systems. Further studies are required to determine whether this antigen is the same as the heat-stable antigen reported by Kaufman and co-workers (20). The other component of the OG-SF of SOW was identified only in the IDTP reference system, a result which is interpreted as evidence for the presence of the TP antigen.

The location of antigenic components of the OG-SF was determined by immunofluorescence and immunoelectron microscopy with rabbit antiserum raised against the OG-SF. The majority of antigens were present in the inner spherule wall, while some apparently diffused to the cell surface and became incorporated into the membranous outer wall material (SOW). Detection of the immunogold label in vesicular bodies may be indicative of a mechanism of transport of antigen from the cytoplasm to the plasmalemma and cell wall. In a recent report of the localization of the TP antigen

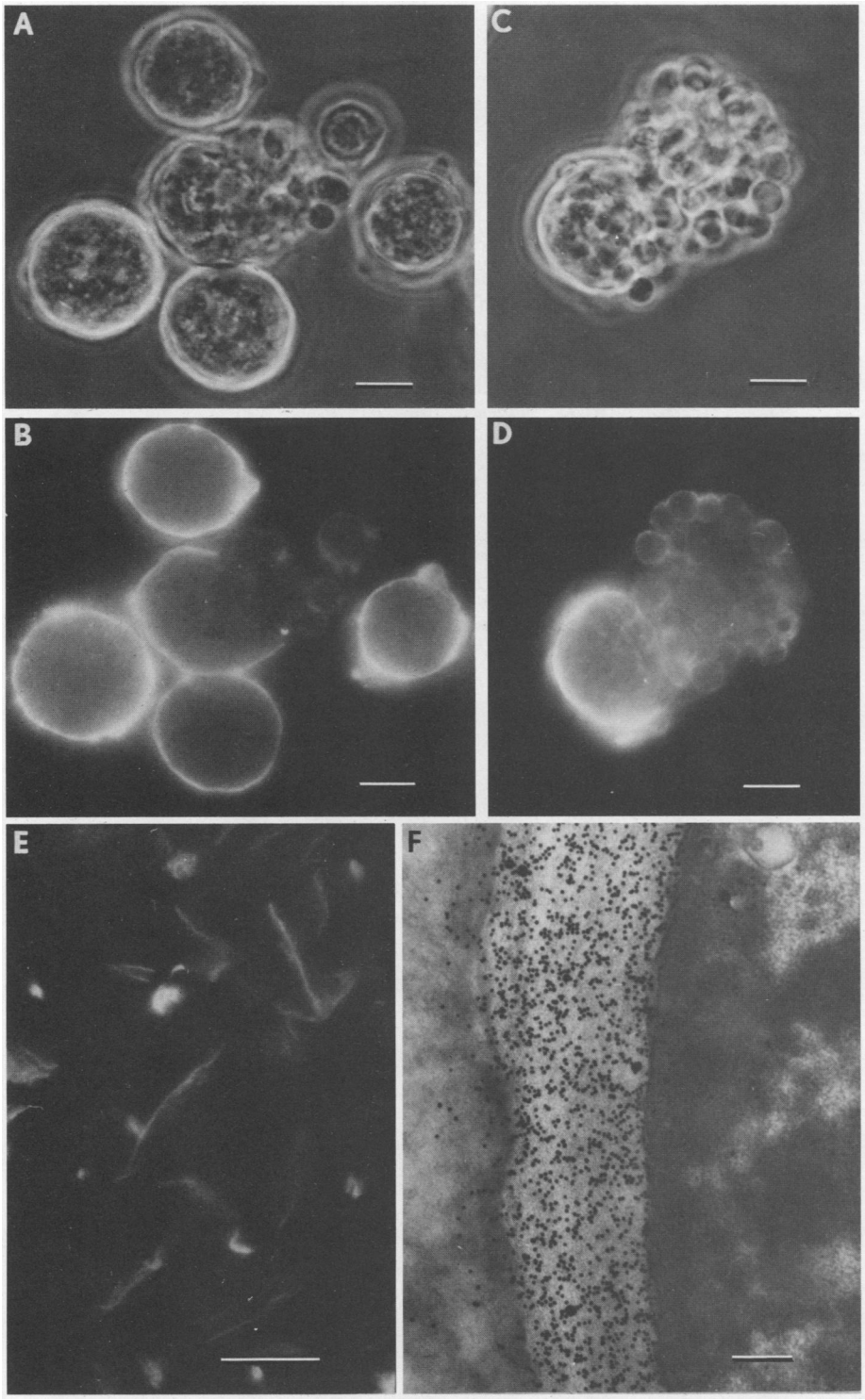

FIG. 3. (A to E) Phase-contrast micrographs (A and C) and corresponding immunofluorescence micrographs (B and D, respectively) of spherules grown in liquid medium and immunofluorescence micrograph of isolated SOW (E). Samples were reacted with rabbit antisera raised against the OG-SF of SOW, washed, and then reacted with goat anti-rabbit IgG conjugated to FITC. Note the endosporulating spherules with a fluorescent, ruptured outer wall and the low level of fluorescence associated with endospores in panels B and D. Bars in panels A to E, 10 μm. (F) Immunoelectron micrograph of a thin-sectioned spherule, which was reacted with the same primary rabbit antibody as in panels A to E, showing that most of the immunogold label (15-nm-diameter particles) was associated with the inner and outer wall layers. Bar in panel F, 2.0 μm.

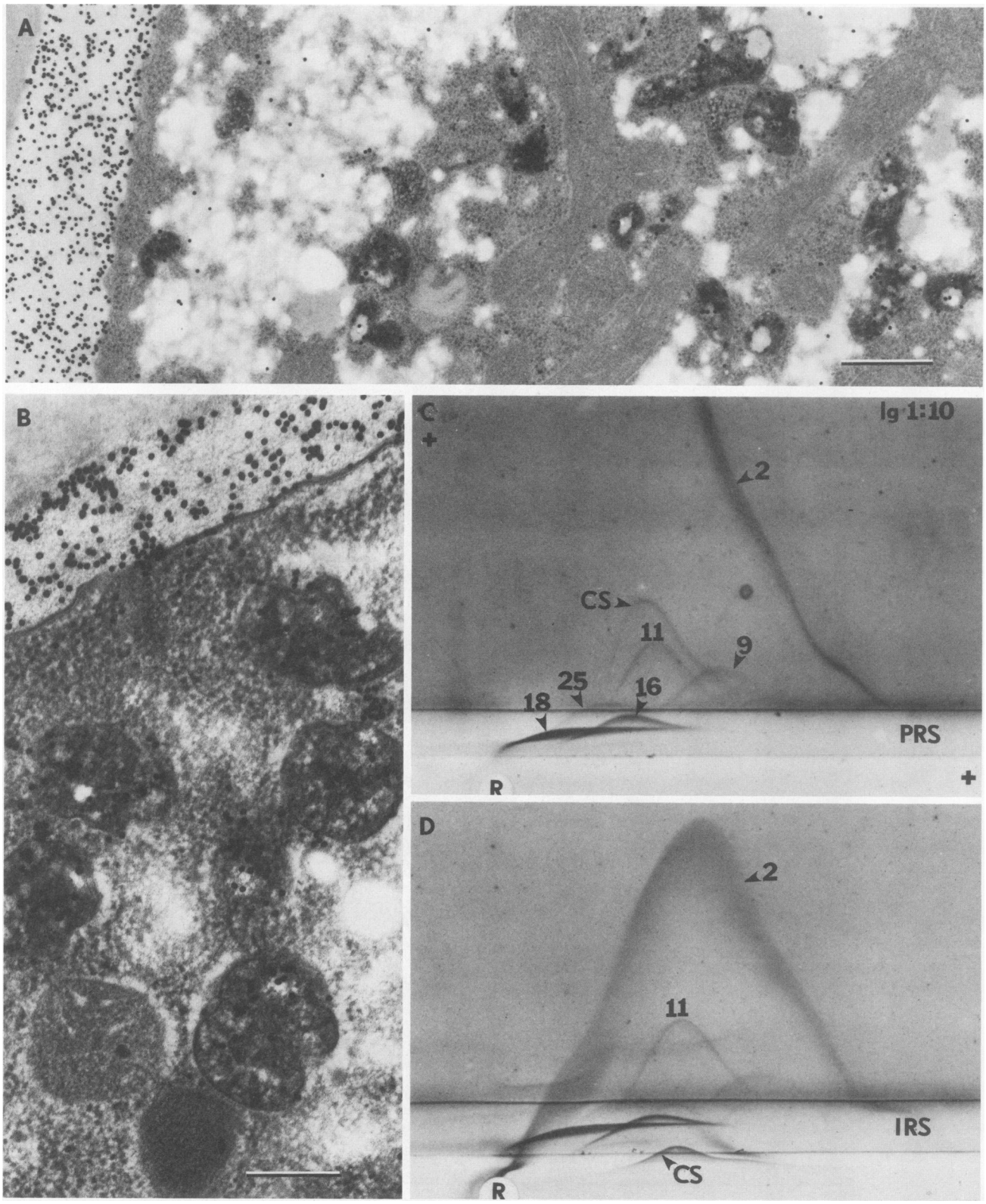

FIG. 4. (A and B) Immunoelectron micrographs of presegmented spherules reacted with rabbit antiserum raised against the OG-SF of SOW (A) or with TP-positive human (coccidioidomycosis patient) serum (B). Note the similar distribution of gold immunolabel on the cell wall and cytoplasmic vesicles. Bars, 4.0 μm (panel A) and 2.0 μm (panel B). (C and D) Intermediate-gel IEP plates showing precipitin peaks of the reference antigen (CDN, indicated by R) in the upper and intermediate gels, numbered according to the CDN–anti-CDN reference system. The intermediate gel contained either preimmune rabbit serum (PRS) or immune rabbit serum (IRS) raised against the OG-SF of SOW. Note the marked reduction in height of antigen 2 and the absorption of antigen CS in the intermediate gel in panel D. R, Reference antigen (25 μg in 20 μl of PBS). Ig, Burro immunoglobulin (1:10 dilution in electrophoresis buffer). The plus sign indicates the anode and the direction of migration.

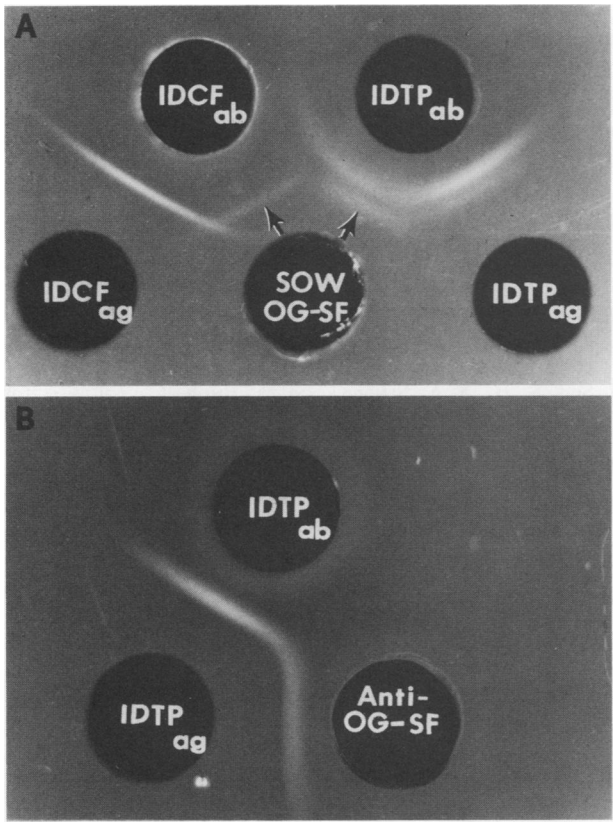

FIG. 5. (A) Reactivity of the OG-SF of SOW with human reference antibody (ab) in the ID assay for CF and TP reactions. The concentration of the OG-SF in the test well was 200 μg in 20 μl of PBS. The arrows indicate a minor fused precipitin band which was adjacent to a major fused TP precipitin band and which crossed the CF reference precipitin band. ag, Antigen. (B) Reactivity of rabbit antiserum raised against the OG-SF of SOW with the TP reference antigen in the standardized ID assay for TP reaction. Abbreviations are as in panel A.

in *C. immitis*, monospecific goat anti-TP antibody was shown to react with cytoplasmic organelles having morphologic and staining characteristics consistent with those of the Golgi apparatus (J. Harrison, S. H. Sun, and R. A. Cox, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, F53, p. 400). The rabbit antiserum raised against the OG-SF of SOW was shown to be reactive with the TP antigen in the ID assay and was associated with cytoplasmic vesicles when used as an immunologic probe for antigen localization on thin sections. The morphology of these vesicles was similar to that described by Harrison and co-workers. TP-positive human antiserum showed a comparable distribution of immunogold label in the cytoplasm and wall of spherules. The intermediate-gel IEP plates demonstrated that the rabbit antiserum also contained antibodies reactive with antigens 2 and CS of the reference system. These two antigens have been shown to be components of the OG-SF of SOW (3). Until monospecific antisera against antigens 2 and CS are available, the location of these antigens within the parasitic cell types of *C. immitis* will remain unknown.

In contrast to the high levels of immunolabel and immunofluorescence associated with the spherule wall, the envelope of freshly released endospores showed low levels of immunofluorescence. These observations support the suggestion that the antigenic components extracted from SOW and identified in our immunolocalization studies become progressively concentrated in the inner and outer spherule wall layers during cell maturation. The cell walls of spherules and endospores, like that of the saprobic phase of *C. immitis* (4), are reservoirs of immunoreactive macromolecules which may play important roles in fungus-host interactions.

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