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Serology of Coccidioidomycosis

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INTRODUCTION

The terricolous fungus *Coccidioides immitis* is well ensconced in the soil of the New World. The expanding human and nonhuman populations of the Sun Belt area of the southwestern United States and of other endemic regions provide a continuing supply of individuals who are susceptible to infection with *C. immitis*.

Serologic tests have served for several decades as aids in the diagnosis and management of coccidioidomycosis. Among the serologic tests available for the mycoses, those for coccidioidomycosis have been the most reliable (139, 141). These have proved useful in veterinary as well as human medicine (101, 122).

The following serologic tests have been used for diagnosing coccidioidomycosis: complement fixation (CF); precipitin reaction in several versions, i.e., tube precipitin (TP), immunodiffusion TP (IDTP), IDCF, and quantitative IDCF (QIDCF), agar gel precipitin-inhibition test, and counterimmunoelectrophoresis (CIE); latex particle agglutination (LA); fluorescent antibody (FA); radioimmunoassay (RIA); and, more recently, enzyme-linked immunosorbent assay (ELISA).

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NATURE OF *C. IMMITIS*

C. immitis exists in nature and in the usual culture media in its saprobic form: septate hyphae 2 to 4 μm in diameter which, in 5 to 7 days, yield a chain of multinucleate arthroconidia which usually alternate with smaller, nonviable, brittle cells (Fig. 1). The latter degenerate, thus releasing the arthroconidia, which readily become airborne. The arthroconidia can germinate to yield new hyphae or can serve as the form infecting humans and other hosts. When inhaled, the arthroconidia, in the presence of the phagocytic cells (42) and increased CO_2 (80), convert into a different morphologic form. Shedding an outer wall layer and all but one nucleus, the arthroconidia round up and enlarge to produce an immature spherule. The nucleus undergoes division, which is followed by partitioning of the cytoplasm by inward extension of the cell wall. Completion of the nuclear division and segmentation produce a mature spherule with endospores within 48 h in vivo and at 30 to 96 h in vitro. Following the segmentation, the endospores become

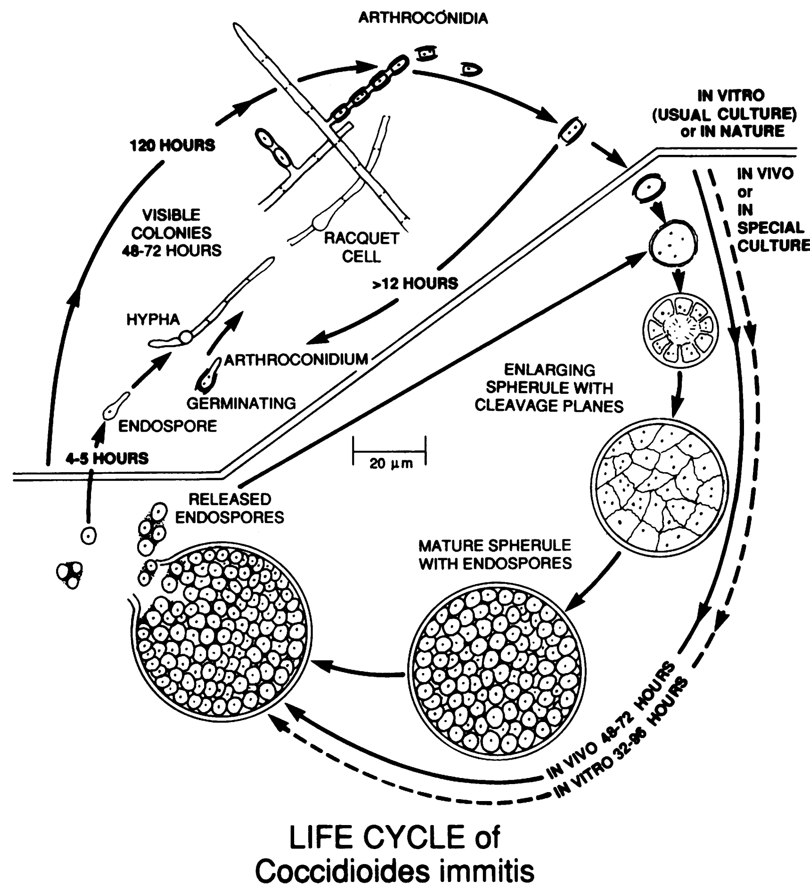

FIG. 1. Various morphologic forms of *C. immitis*.

rounded within the mature spherule. The spherule wall contains glucans and chitin, which are also found in the endospore wall (50), as well as polymers containing mannose, 3-*O*-methylmannose, and galactose, as described by Wheat et al. (150) and Cole et al. (19). The spherule then opens to release the endospores, which, in vivo, enlarge to form endosporulating spherules repeating the cycle: spherule → endospore → spherule. The mechanism of release of the endospores from spherules has not been established; however, in cultures, chitinase and glucanases become detectable at about the time that endospores are released (R. F. Hector, B. L. Zimmer, and D. Pappagianis, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, F61, p. 374; B. L. Zimmer and D. Pappagianis, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, F48, p. 397). Shortly thereafter, protease(s) becomes detectable in culture medium and increases as the chitinase and glucanases diminish in concentration (102, 123) and could therefore influence the morphologic evolution of *C. immitis*. This phenomenon has not yet been demonstrated. Cole et al. (23) proposed that the protease(s) serves a role in morphogenesis.

On occasion, the hyphal form and even arthroconidia have been observed in vivo. For example, hyphae are sometimes seen in coccidioidal pulmonary cavities and infrequently in tissues other than lung (94, 152).

At least two kinds of antibody (discussed later) are produced in response to infection with *C. immitis*: an early antibody detected by TP, IDTP, and LA; and a later antibody detected by CF and IDCF.

SPECTRUM OF DISEASE

Coccidioidomycosis may be manifest or occult. It may be without apparent detriment to the host, or it may progress to death in an insidious or fulminating manner. No case of respiratory human-to-human transmission has been reported.

In the noncompromised host, infection is followed by symptoms in about 40% of patients. The approximately 60% of persons who are without acknowledged symptoms can be detected by a positive skin test with coccidioidal antigen; in some, detection of prior coccidioidal infection results when a pulmonary lesion is detected by routine chest roentgenogram, and subsequent laboratory tests confirm its etiology. Smith et al. (139) found only 7% seropositivity in a small sample of asymptomatic skin test converters without evidence of disease.

In those who develop symptoms, onset of illness occurs 7 to 28 days (usually 10 to 16 days) after exposure to arthroconidia of *C. immitis*. The disease can be roughly categorized as (i) primary acute (pulmonary) disease, (ii) disseminated (metapulmonary) disease, and (iii) pulmonary residua.

Early in the course of disease various rashes may occur, the most prominent of which is erythema nodosum. This transient eruption lasts only a few days and may be the result of formation of immune complexes present for only a short period of time. Its occurrence usually is associated with a good prognosis, but disseminated coccidioidomycosis has followed erythema nodosum. Some rashes appear as ur-

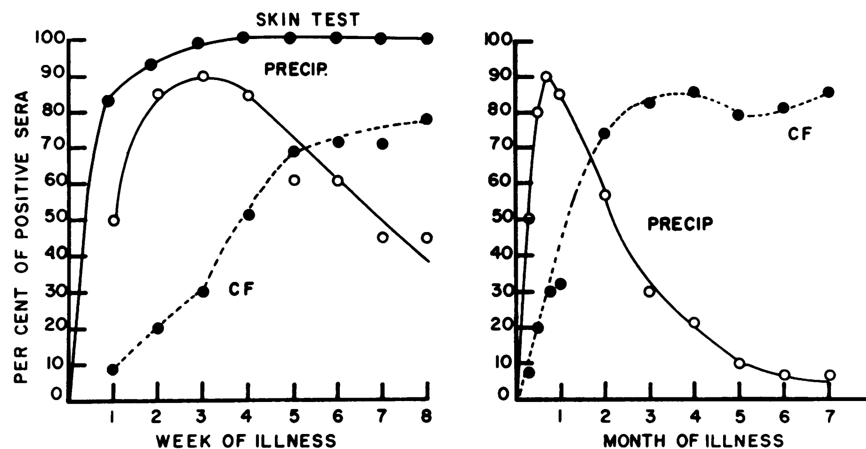

FIG. 2. Temporal sequence of immunologic changes in primary nondisseminating coccidioidomycosis. Precip, Tube precipitins; CF, complement-fixing antibodies. After Smith (135) and Smith et al. (100, 139, 141), with permission of the publishers.

ticaria, suggesting a histamine-releasing mechanism; however, an immunoglobulin E (IgE)-mediated reaction has not been demonstrated with these early brief rashes. Cox et al. (27) have related increased anticoccidioidal IgE to more severe coccidioidal disease. Peripheral eosinophilia may be seen: the higher the eosinophilia, the worse the prognosis; pulmonary eosinophilia also is seen (90).

The usual primary pulmonary disease can present in a variety of forms, often with hilar lymphadenopathy, and sometimes with pleural or pleuropericardial effusion. Occasionally no pulmonary lesions are detected by standard roentgenography (newer computerized axial tomography (CAT) and magnetic resonance imaging (MRI) examination may reveal them), and hilar lymphadenopathy alone may be detected. Complete resolution of the primary pulmonary disease usually occurs; however, some 5 to 10% of symptomatic primary pulmonary coccidioidomycosis is followed by persistence of a pulmonary residual (cavity, solitary nodule, or bronchiectasis). In a few patients the primary pulmonary infection may be sufficiently extensive or severe to cause death (56, 126). Because recovery from infection usually results in resistance to exogenous second infection, vaccines have been developed which have a protective effect in some species (89), but these have not yet been shown to protect humans (D. Pappagianis and the Vaccine Study Group, Program Abstr. 26th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 784, 1986).

In a small fraction of patients with symptomatic disease, *C. immitis* spreads outside the thorax. Dissemination occurs within weeks of the primary pulmonary infection but may be delayed (or manifestation of spread may be delayed) for some months, infrequently for years. Metapulmonary spread is usually evident from the development of some metastatic lesion, but occult dissemination has also been reported (6, 117). The endocardium and intestinal mucosa are rarely affected (149); virtually all other tissues can be affected. Metastasis may be represented by a single pustular lesion in the skin or by extensive involvement of many organs and tissues. The meningeal form of coccidioidomycosis represents the worst form of the disease, always leading to death if untreated; therapy for this form is often only partially effective.

The frequency of dissemination is increased by certain risk factors: ethnic background (Filipino and other Asians and African Americans appear at greater risk than other groups); acquisition of a primary acute infection during the later stages of pregnancy; and immunosuppressive states (iatrogenic as in renal transplantation; or acquired immunodeficiency state, such as acquired immunodeficiency syndrome (AIDS) or lymphoma). While complete recovery from primary infection is usually followed by lasting resistance to exogenous reinfection, such resistance may be diminished by a superimposed immunosuppressive condition leading to exacerbation and dissemination of the disease previously considered healed (35, 99). In the immunocompromised host, progression of the disease is often more rapid than in the noncompromised host.

Certain forms of disseminated disease, e.g., osseous or joint involvement, appear to undergo partial resolution only to become clinically evident later, and such improvement and worsening may occur repeatedly for many years.

In most cases of coccidioidomycosis, serologic findings are of help in diagnosis and prognosis. Occasionally, serologic failures are recognized in patients with certain immunocompromising states or when progression of disseminated disease is very rapid and antibody is not detected.

HOST RESPONSE TO *C. IMMITIS*

Allergy

The host generates delayed cutaneous hypersensitivity to coccidioidin or spherulin (see below) within 3 days to 3 weeks after onset of symptoms (10 days to 6 weeks after exposure). A positive skin test indicates current or prior infection, but only conversion from negative to positive demonstrates the former. Some individuals produce an immediate wheal and erythema reaction but this may be either to the coccidioidal antigen or to the preservative thimerosal. Smith et al. (142) could show no passive transfer (Prausnitz-Kustner test) of immediate-type hypersensitivity from five individuals with current and prior coccidioidal infections and positive coccidioidin skin tests to coccidioidin-negative recipients. In these studies (142), it was apparent that, in primary nondisseminating coccidioidomycosis, skin reactivity to coccidioidin usually preceded by a few days the formation of early antibody (detected by TP test), which in turn was followed by development of CF antibodies (139, 141) (Fig. 2). In recent years, for unknown reasons, early

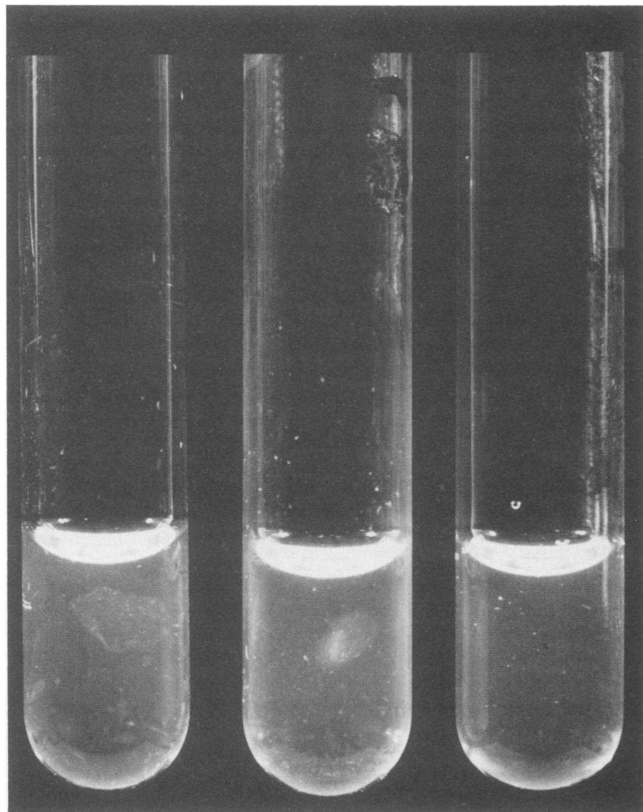

FIG. 3. TP test. Left tube contains human serum plus undiluted coccidioidal antigen, middle tube contains serum plus coccidioidal antigen diluted 1:10, and right tube contains serum plus culture medium control. The button of precipitate has been dislodged from the bottom of the tube for photographic clarity. Reprinted from reference 159 with the permission of the publisher.

antibody has often been detected in the serum at a time when the skin test has been negative. In part, this may be due to improvement in tests such as LA and ID that are more sensitive in detecting antibody than the TP test (60, 61, 65). A negative skin test does not rule out coccidioidomycosis and should not preclude serologic and other tests (biopsy or culture) to detect the disease. Ordinarily, if a patient has had a positive coccidioidal skin test for several months or longer and develops an illness accompanied by negative serology, the illness is not likely coccidioidal except in the immunosuppressed patient whose skin test reactivity has faded and in whom serologic reactivity never developed. Administration of a coccidioidal skin test does not induce or increase the titer of coccidioidal antibody (34, 113, 142).

Antibody

Over a 15-year period, the serologic studies of Smith et al. (139, 141) involved some 39,500 tests on patients, including 5,579 with primary nondisseminating coccidioidal infections, 722 with disseminated infections, 271 with noncavitating pulmonary residuals, and 577 with pulmonary cavities. The temporal sequence of antibody detected by TP (Fig. 3) and CF tests has been described above. Rarely, the CF antibody is detected before TP. The antigens differ in that the TP antigen is heat stable, retaining potency even after autoclaving, whereas the CF antigenic activity is destroyed by heat. Subsequent studies making use of immunoelectrophoresis indicated that the major antibodies involved are IgM in the TP and IgG in the CF reactions (109, 128).

In 1958, Heiner (51) showed that, when coccidioidin and serum from a patient with coccidioidomycosis diffuse toward each other in an agar gel immunodiffusion test, a band of precipitate forms. Various fractions derived from coccidioidin yielded multiple bands when diffused toward human patients' sera (111). Schubert and Hampson (132) demonstrated an ID reaction between human serum and coccidioidin. Huppert and Bailey (60, 61) refined the ID test and demonstrated that, in the gel milieu, reactions corresponding to the TP and CF reactions (IDTP and IDCF, respectively) could be detected (Fig. 4). This observation posed the seeming paradoxes that the CF antigen-antibody reaction, which produces no precipitate in liquid, forms a precipitate in a gel (complement is not required for this); nor do the TP and IDTP reactions involving IgM require intact complement despite the usual description of IgM as more efficient than IgG in binding complement. The ability of CF antibody to form a precipitate in a gel has created some confusion because the reaction has been referred to correctly as a "precipitin" reaction; however, the clinical and temporal definition of a precipitin (139, 141) has been that of the early IgM antibody response detected by TP test. Therefore, some laboratories may report the presence of precipitins detected by ID and mean a reaction produced by IgG (Fig. 4, bottom) from a patient with a longstanding coccidioidal infection rather than IgM produced during a recent primary coccidioidal infection.

The data depicted in Fig. 2 indicate that it is important to test for IgM antibody as this may be the only means of readily confirming the presence of an early acute coccidioidal infection. Smith et al. (134, 136) found that during the first week of illness 53% of patients with primary nondisseminating coccidioidomycosis had detectable antibody by TP (of a subgroup who had erythema nodosum, 75% were TP positive within week 1 of illness); this percentage increased to a maximum of 91% during weeks 2 and 3 of illness. The IDTP and LA tests showed a greater sensitivity than the TP test in detecting early antibody (61, 62, 65; D. Pappagianis, S. Lindsay, and S. Beall, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, F19, p. 400). Of 24 specimens tested simultaneously by TP and IDTP, 18 were reactive by both methods, but 6 (25%) were reactive only by IDTP. Of 36 specimens positive by IDTP, 21 were reactive by both IDTP and TP but 15 (42%) were reactive only by IDTP (62). It is likely, therefore, that the IgM antibody is detectable earlier, and in a greater fraction of patients than shown by Smith et al., using the TP test alone (139, 141). The greater sensitivity of the IDTP than the TP is also inferred from our findings of persistence (or "reappearance") of IgM antibody in patients with coccidioidal pulmonary cavities (97). Smith et al. (139, 141) had pointed out that TP reactivity may reappear after coccidioidal hydropneumothorax resulting from rupture of a coccidioidal pulmonary cavity. Our studies have indicated that chronic coccidioidal pulmonary cavities are often accompanied by antibody detectable by IDTP. Thus, the apparent reappearance of IgM noted by Smith et al. (139, 141) is likely the result of an increase in already persisting precipitins that are detectable by IDTP but below the threshold of detection by TP. TP and IDTP reactivity can also be demonstrated in the pleural effusion of acute pulmonary disease.

In the study by Smith et al. (141), TP reactivity was detected in 91% of patients with primary nondisseminating infections at week 3 of illness and declined to only 4% 7

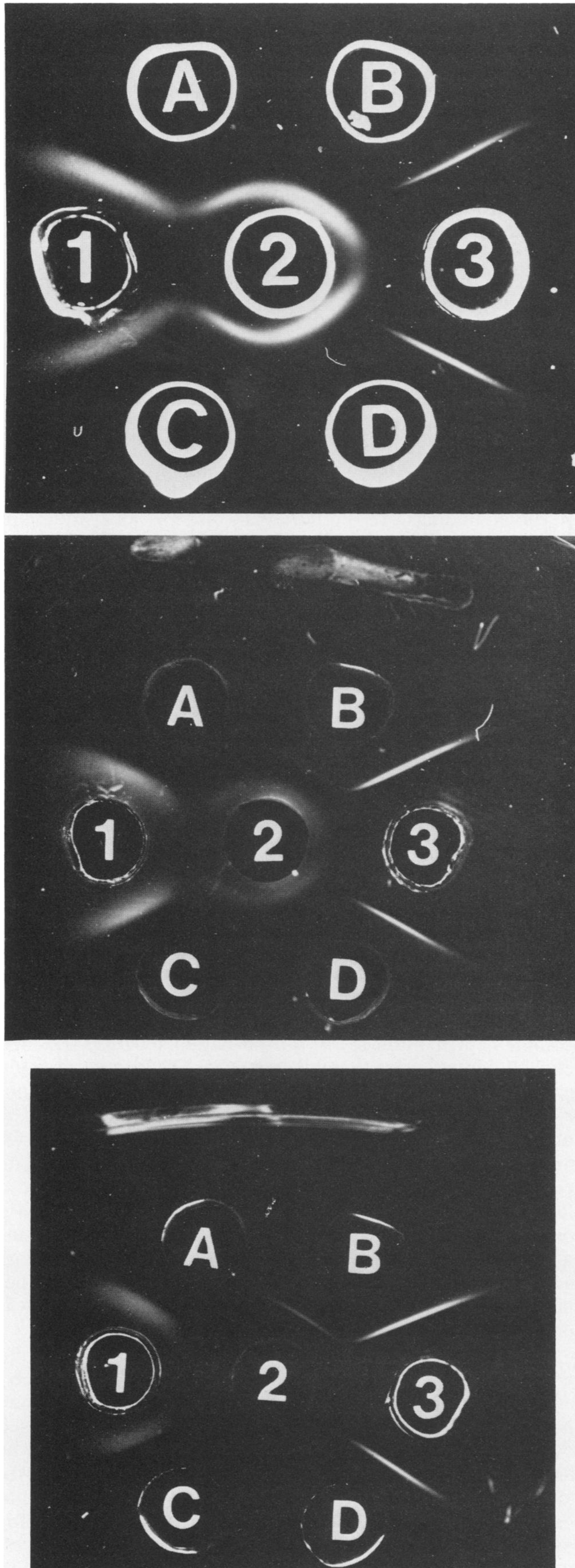

FIG. 4. ID of serum from a patient at different stages of coccidioidomycosis. (Top) Serum of a patient with acute primary coccidioidomycosis showing presence of IDTP (IgM) reactivity. (Middle) Serum of a patient still in a relatively early stage of disease showing presence of IgM (near serum well) and IDCF (IgG) reaction further from serum well each showing line of identity with respective control sera. (A similar pattern may accompany a chronic coccidioidal pulmonary cavity.) (Bottom) Serum of a patient in the course of coccidioidomycosis showing only IDCF (IgG) antibody. Wells A and C contained heated coccidioidal antigen reactive with IgM; wells B and D contained unheated antigen reactive with both IgM and CF (IgG) antibodies. Well 1, Control IDTP (IgM)-positive serum; well 2, patient serum; well 3, control IDCF (IgG)-positive serum.

months after onset of illness. However, TP-positive reactions persisted in 347 of 722 (48%) patients with disseminating infections, sometimes for several years; 8 patients were TP positive in the second year, 3 in the fourth year, and 1 was still positive during the ninth year after infection and dissemination. Thus, persistence of TP (and IDTP) antibody may be of partial assistance (in addition to CF titer) in judging severity of coccidioidal disease. Some workers have indicated that reactivation of disseminated coccidioidal disease is accompanied by reappearance of IgM; however, it is likely that by concentration of the serum, in most instances, persistence rather than de novo renewed production of IgM can be demonstrated. IgM can reappear in the serum when there is systemic reinfection from the meninges via a ventriculoperitoneal or ventriculoatrial shunt (101).

The persistence or reappearance of IgM may reflect spherule proliferation and release of endospores. In vitro, the antigen responsible for IDTP reactivity is liberated into the culture medium at a stage of the life cycle in which young endospores are present before their evolution to spherules (156).

The presence of IgM antibody has been noted infrequently in the cerebrospinal fluid (CSF). Smith et al. (141) obtained a positive TP with CSF in only 1 of 92 patients with coccidioidal meningitis. Our experience has been similar (103); for example, we have now detected IgM antibody by IDTP in the CSF of 4 of 311 patients with coccidioidal meningitis. This test may provide another marker for diagnosing meningitis when the CF test is negative.

Smith et al. (139, 141) detected CF antibody in cord blood of babies born to serologically positive mothers but made no comment regarding TP reactivity. We have recently received cord bloods from two babies (whose mothers had coccidioidomycosis during pregnancy) both of which gave positive IDTP reactions. We feared that this presence of IgM might indicate in utero infection; however, both infants have remained without clinical evidence of coccidioidomycosis through many months of postpartum follow-up. Both mothers had detectable IgM and IgG in their sera at the time of parturition. Whether the IDTP reactivity represented maternal IgM that had contaminated infant blood because of rents in the placenta or its separation from the uterus (95) or whether it was of fetal origin is unknown. Its presence in these cases had no connotation of in utero infection with *C. immitis* as indicated by the subsequent clinical course.

If IgG antibody alone is detected and it yields a low CF titer (e.g., 2 to 8), it is important to know whether the patient has had a recent acute illness. Such a low titer may persist in patients with a well-focalized coccidioidal pulmonary residual that has been present for many months or even years. A subsequent serum sample should provide evidence (appear-

ance of IgM or change in CF titer) that would indicate a recent coccidioidal infection. A stable, low CF titer and no IgM detected in a subsequent serum sample provides evidence that the coccidioidal infection had been acquired at least many weeks earlier. Smith et al. (139), however, had noted that a few patients had TP-reactive sera persisting even after the CF titer had reverted to negative.

Detection of CF antibody is useful in diagnosing acute coccidioidomycosis and offers supportive information to that provided by detection of IgM. Detection of the IgG by ID and its quantitation by the CF or quantitative ID test are important in ascertaining the course followed by the disease. CF reactivity at any titer is significant provided it has been confirmed as coccidioidal by prior detection of coccidioidal IgM or by positive IDCF.

There is generally a reliable correlation between the CF titer in serum and the severity (extent) of coccidioidal disease (Fig. 5). CF titers of above 16 in the serum should alert one to the possibility of disseminated coccidioidomycosis. This level is based on the studies of Smith et al. (139, 141), who used a Kolmer CF test with complement binding for 2 h at 37°C. We routinely use 2-h binding of complement at 37°C for sera because of the extensive clinical correlation established by these workers. As pointed out by Smith et al. (140, 141), overnight (18-h) binding of complement at 4 to 5°C yields higher titers. This greater sensitivity prompted us to adopt binding of complement at 4 to 5°C to detect antibody in the CSF. Several other methods make use of binding of complement in the cold, including that of the Laboratory Branch Complement Fixation Test (LBCF) of the Centers for Disease Control (98). (The trade-off is greater sensitivity with low-temperature binding of complement for more rapid results and probably better clinical correlation with 2-h binding at 37°C.) (See below regarding serologic failures.) When dissemination is extensive, the titer generally is >128 unless the infection is so fulminating that there is insufficient time to mount an antibody response or the host has an immunocompromising limitation of humoral response.

If the disease is limited to a portion of lung or even a limited focus of extrapulmonary dissemination, e.g., the wrist joint or even the meninges, the serum CF titer may be in the low range of 2 to 8. On the other hand, with involvement of lungs or mediastinal lymph nodes or both, serum CF titers may be 128 to 256, which usually denotes dissemination. When sera are tested, the usual starting dilution is 1:2, with subsequent twofold dilutions. However, to increase sensitivity when testing CSF, we and some other laboratories begin the CF antibody titration with undiluted CSF when sufficient specimen is provided. However, to our knowledge no correlation has been established between fixation of complement by undiluted CSF and activity of the meningitis. Indeed, when concentrated by evaporation, even CSF from patients with nonmeningeal coccidioidomycosis may yield an IDCF reaction (112). Therefore, CF reactivity by undiluted CSF does not establish the presence of meningitis. However, in the patient known to have meningitis, decrease in CF antibody and reversion of the CF test to negative provide proportionately favorable information.

With the presence of coccidioidal antibody established by TP, IDTP, or IDCF, there is little to be gained clinically by repeating these qualitative tests. These tests may be repeated when the patients who have had positive serologic findings, e.g., CF, that become equivocal or negative as a result of healing subsequently are believed to have relapsed or to determine the reappearance of IgM antibody in a patient with a ventriculoperitoneal or ventriculoatrial shunt (indicative of systemic reinfection).

Thus, quantitation of antibody by CF or quantitative IDCF provides guidance on the course of the disease. Specimens should be tested at 3- to 4-week intervals as there is usually no significant serologic change in less time. Positive specimens should be frozen at −15 to −20°C and thawed for retesting when a subsequent specimen from the same anatomical source (e.g., serum, CSF, or pleural fluid) is obtained. Thus, two sequential specimens should be tested simultaneously to control any test variations that may occur in the CF or quantitative IDCF. A fourfold change in titer is significant, with an increase indicating worsening of disease and a decrease indicating improvement. In the usual nonimmunocompromised patient, the titer continues to rise with worsening disease even in the moribund patient who has developed anergy to the coccidioidal skin test.

Negative serologic findings in the face of cultural or histopathologic confirmation of coccidioidal disease have been infrequent. Smith et al. (141) indicated that, of 722 patients with disseminating disease, only 2 had negative serologic findings. Two others with disseminated infections were CF negative when complement binding was carried out for 2 h at 37°C but positive when binding was carried out at 4°C for 18 h. The authors speculated that their standard antigen may have lacked antigenic components present in the patients' own *C. immitis* isolates. The isolate from one patient was used to prepare "autogenous" coccidioidin. However, that patient's serum exhibited an even lower CF titer with the autogenous antigen than with the standard multiple-strain coccidioidin.

In general, coccidioidal serologic tests are highly sensitive. We have noticed two patient groups in particular for whom the potential for serologic failure is great: patients undergoing organ transplants, and patients with AIDS. In the former group, for example, 3 of 13 with disseminated coccidioidomycosis had negative serum CF tests and 1 of 5 with pulmonary disease only had a negative serum CF test (18). In another report, three of seven renal transplant patients with coccidioidal lesions were negative by CF test, but two of these were positive by ID (the third was not tested serologically) (9). One of four patients who had coccidioidomycosis complicating a cardiac transplant was negative by complement fixation (8, 9). Of 48 patients with AIDS and coccidioidomycosis (18 published cases, 30 from our serodiagnostic work), 4 have been seronegative. Also, in our experience, the number of negative serologic findings in other patients has been small. These occurred in (i) two apparently immunologically normal female patients with coccidioidal inguinal adenopathy (one of these showed a faintly positive IDCF reaction with concentrated serum); (ii) one female patient with hypogammaglobulinemia; (iii) one male with coccidioidal meningitis; and (iv) one female with coccidioidal meningitis (several weeks after onset of her meningitis, antibody could be detected in her CSF but was never detected in her serum).

In a very few patients, we observed a negative CF result with serum that yielded a substantial IgG titer by QID. By adjusting the CF reagents, we found that these sera reacted positively in the CF test. Thus, the usual standardized combination of complement, hemolysin, and antigen may be appropriate for most sera but may be poised incorrectly for a few specific sera. In some instances, patients may not generate an expected CF antibody response because they have a selective deficiency of IgG1 and IgG3 subclasses, which are efficient complement binders, or they may gener-

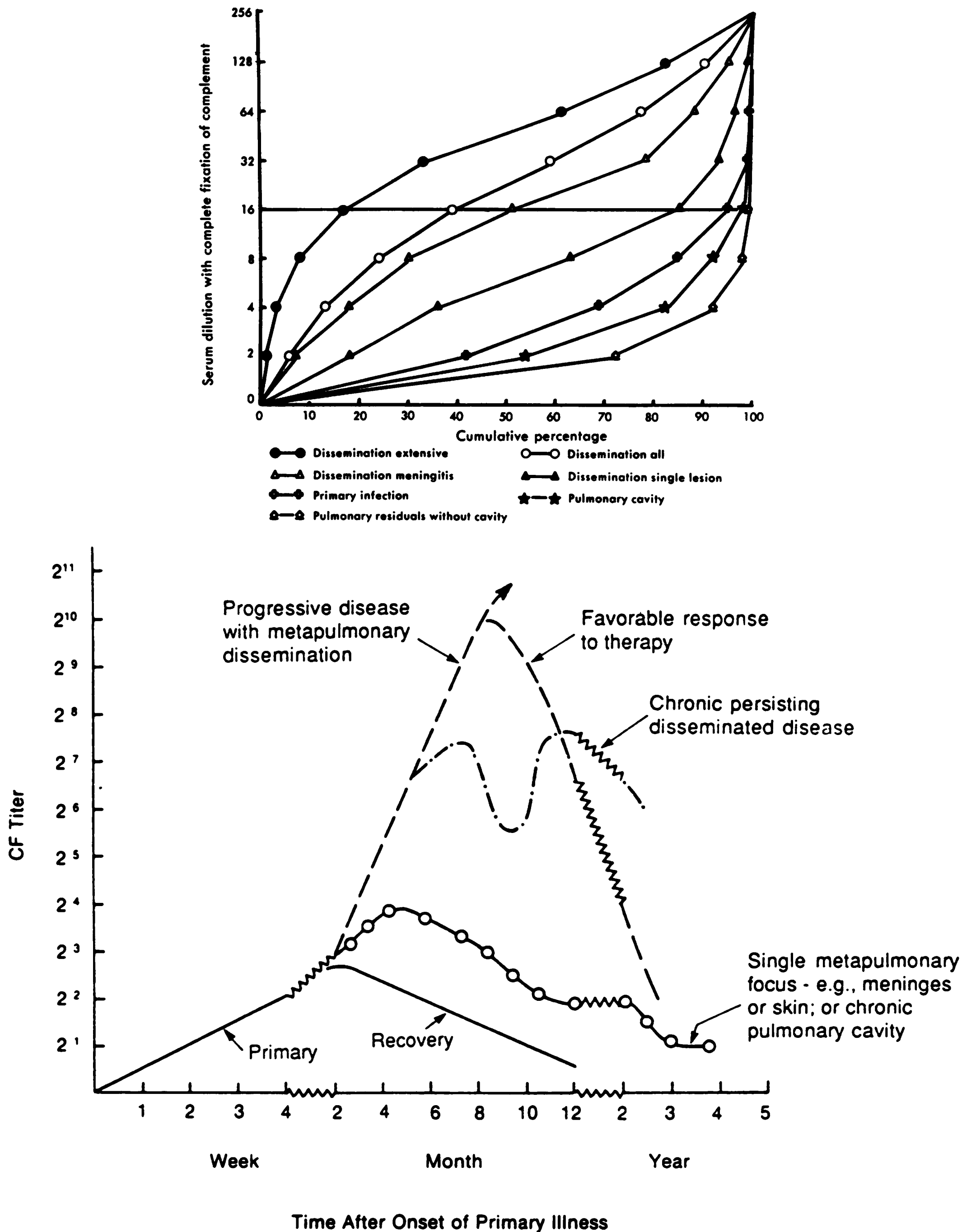

FIG. 5. (Top) Maximal titers of complement-fixing antibodies in sera of patients with various types of coccidioidal disease based on tests on 39,500 sera. Reprinted from reference 141 with permission of the publisher. Symbols: ●, dissemination extensive; △, dissemination meningitis; ♱, primary infection; ◇, pulmonary residuals without cavity; ○, dissemination all; ▲, dissemination single lesion; ☆, pulmonary cavity. (Bottom) Patterns of serum CF titers with various clinical phases of coccidioidomycosis. Reprinted from reference 101 with permission of the publisher.

ate IgG4, a nonbinder of complement. To our knowledge, we have tested sera from only a single patient with a selective deficiency (of IgG1). After concentration, her serum was positive for both IgM and IgG antibodies by ID, but was negative by CF; the QID titer was 2.

Serum concentrated by evaporation and tested by ID will yield positive findings despite negative CF results. The ID method was not available when Smith et al. (141), in the pretransplant era, reported their two serologic "misses" among 722 patients with disseminated disease, a failure rate of 0.28%. Kozub et al. (82) described negative serologic tests (CF, ID, TP, and CIE) in seven patients. Five had culture-proven *C. immitis* infection. However, neither the nature of their disease (acute, chronic, or cavitary) nor the presence of

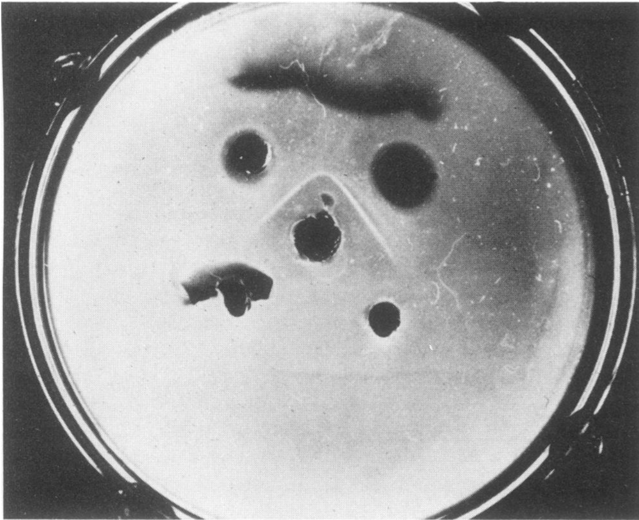

FIG. 6. Positive ID reactions between toluene-autolyzed coccidioidal mycelial antigen (top two wells) and pooled human serum in a gel consisting of bovine fibrin. The sera in the pool had CF titers between 32 and 64. This illustrates a possible setting in which an antigen-antibody precipitate can form in vivo with certain immunologic consequences such as activation of complement and chemotaxis of granulocytes.

any underlying disease was given. The other two patients were negative by CF tests but positive by one or more of the other tests.

In some cases, either very early in the course of acute coccidioidomycosis or in immunocompromised patients, tests for antibody may be negative. Tests for antigen may fulfill the serodiagnostic requirements in such cases (see section on serology).

Application of serologic tests in veterinary medicine has shown antibody responses in dogs (55, 120, 121, 129, 133, 155), horses (33), and nonhuman primates (4, 54, 88, 92, 110, 120, 134, 145) generally similar to those in humans. Serologic tests in other species (e.g., llamas) (96) have also shown that the methods are useful, but the numbers of animals tested have been too few to provide for complete evaluation of serology.

The possible protective or harmful role of coccidioidal antibodies in vivo has not been defined. The CF (IgG) antibody that produces a precipitate with its corresponding antigen in agar or gellan gum gels also produces a precipitate in a fibrin gel (Fig. 6). Such an antigen-antibody precipitate formed in fibrin in vivo could, by activation of complement, evoke an inflammatory cell response. The IgM-reactive antigen on the surface of spherules (24) may serve as an opsonizing reactant. Antibody may influence cell-mediated responsiveness.

ANTIGEN PREPARATION AND ANALYSIS

Historical Development

In 1914, Cooke was the first to use *C. immitis* derivatives for serologic testing (26). He used triturated cultured mycelium and spherules from "human lesions" in a precipitin reaction with patient sera. Davis (32) prepared a suspension of *C. immitis* for successful testing by CF. Additional crude antigens, which could be called "coccidioidins," were prepared from culture filtrates and evaluated by skin testing with little clinical application. Hirsch and d'Andrea (52, 53) prepared filtrates from mycelial *C. immitis* grown in both complex (extract of placenta) and chemically defined (ammonium lactate) media but used them primarily to show skin test reactions in humans and animals with coccidioidomycosis.

From the late 1920s to the 1940s, various other coccidioidins were prepared from culture filtrates in nonchemically defined media and used for skin testing (3, 44, 45, 68, 69, 79). A simple synthetic ammonium chloride-sodium acetate medium was used by Stewart and Kimura (144) for skin tests. However, Smith et al., who considered that these were not "sufficiently potent," tried other formulations, finally settling on modifications of the synthetic Bureau of Animal Industry medium long used for preparation of tuberculin (142).

In the 1930s, C. E. Smith and his mentor Ernest Dickson thought that it would be necessary to produce antigen from the spherule (in vivo or "parasitic" form) for a serological test and that mycelial antigen would not likely be satisfactory. However, having no method available to produce the spherule or endospore phase at that time, Smith proceeded with mycelial cultures. Smith's initiation of serologic studies, which proved to be so useful, came about as a result of his interest in the skin test reactivity of guinea pigs infected with *C. immitis*. He wanted to learn whether serum from infected guinea pigs would, when mixed with coccidioidin, "inactivate" the latter with respect to its skin test reactivity (137, 139). The mixture, allowed to sit for a week, showed a "discrete button in the bottom of the tubes." Serum from normal guinea pigs gave no precipitate. In retrospect, the observation was somewhat remarkable in that the precipitate was not bulky and was translucent and closely applied to the bottom of the tube. In further use of the precipitin test, Smith showed that a sharp flick of the tube to dislodge the button of precipitate was often needed to detect it (Fig. 2). Smith and colleagues then found that the coccidioidin and serum from patients with coccidioidomycosis would fix complement. Adaptation of the Kolmer CF test which was used for quantitation of antibody in patients with syphilis permitted similar titration of antibody in patients with coccidioidomycosis (139).

The modified Bureau of Animal Industry medium was inoculated with hyphae and arthroconidia, which led to surface growth. Cultures were incubated static for many weeks, usually 4, but for up to 3 months if the antigenic potency was considered inadequate. However, it was later shown that serologically active antigen could be detected as early as the second week of incubation in the modified Bureau of Animal Industry medium (101). Ajello et al. (2) prepared a coccidioidin that was reactive in both CF and ID tests by collecting 3-week-old culture filtrates of *C. immitis* grown in peptone-glucose-yeast extract broth. The duration of incubation resembles that of Smith's original coccidioidin preparation (142), but it was carried out on an incubator-shaker. Rowe et al. (124, 125) also produced antigen active in ID in defined synthetic and complex media in cultures incubated for 45 days on a shaker. In Smith's studies, four isolates of *C. immitis* from various geographic regions were used initially to assure antigenic coverage; later, this number was increased to 10 and then to >24 (138, 139, 142). Considerable variation in CF antigenic potency was observed by us among culture filtrates and toluene-induced autolysates of 25 strains of *C. immitis*. Huppert (57) reported that nine different strains produced variable amounts of four different antigens detectable by ID, but as yet there has been

no demonstration that strain differences pose a problem in sensitivity or specificity of serologic tests. In fact, in ID tests of >70,000 human sera, we have found that an antigen preparation from a single strain (Silveira) of *C. immitis* has been as sensitive as a multistrain antigen reagent for detecting antibody. Nevertheless, serologic antigens, including commercial preparations, are still made from many strains of *C. immitis*.

When preparing coccidioidin by the method described, Smith et al. (139) noted that despite seemingly identical inoculation and incubation the antigenic potency and anticomplementary activity varied among culture flasks. It was necessary to sample the filtrate from each flask to ascertain its suitability as serologic antigen. Numerous lots were anticomplementary and could not be used for CF tests.

The macerated undersurface of the floating mycelial mat indicated that autolysis was taking place during the prolonged incubation (105) and, depending on the strain, antigen active in the TP test was detected in 2-week-old, but not 1-week-old, cultures. In 1957, deliberate autolysis under controlled conditions was carried out to determine whether antigen could be liberated quickly and consistently (115). The method was adapted from one used to prepare yeast autolysate (118). *C. immitis* Silveira mycelial phase was cultured in 2% glucose–1% yeast extract (Difco Laboratories) for 3 days at 34 to 35°C on a shaker. The hyphae were collected by filtration on Whatman no. 1 paper, washed with sterile water, and suspended in water in a cotton-plugged flask to give a thick slurry. Toluene, 3% (vol/vol), was added to the slurry. A paper or aluminum foil cap was placed over the cotton plug and the flask was incubated at 37°C for 3 days. This led to autolysis and liberation of antigen active in skin testing and in the TP and CF tests (114, 115). CF activity (titer, >64) was evident immediately and after toluene addition, and this increased progressively throughout the 72-h autolysis period (106). The lysed cellular debris was removed by filtration on Whatman no. 1 paper, and the autolysate was retained as antigen solution. Aqueous thimerosal was added to a final concentration of 1:10,000 as a preservative.

Originally, the culture filtrate from 2% glucose–1% yeast extract medium used to produce the cells for autolysis was discarded after collection of the mycelium. However, Huppert and Bailey (59) found that the 3-day culture filtrate also possessed serologic activity. Because the Difco yeast extract used in the culture medium is itself "the water soluble portion of yeast autolysate" (36), Huppert and Bailey (60) routinely dialyzed the yeast extract against water at 80°C to avoid cross-reactive antigens. The retentate was discarded, and glucose was added to the dialysate to produce a medium similar to 2% glucose–1% yeast extract. Both autolysate and culture filtrate can be used as a source of TP, IDTP, CF, and IDCF antigens, although the autolysate is more concentrated.

In 1957, Converse (25) developed a medium later modified by Levine et al. (87) that would support in vitro growth of the spherule-endospore phase of *C. immitis*; thus, it became possible to prepare serologic antigens from this growth phase. Landay et al. (84, 85) found that thimerosal-preserved spherules induced antibody in rabbits which reacted by both CF and ID with sonicated and autoclaved spherule extracts and arthroconidial antigens. However, in ID tests, antiserum induced by arthroconidia reacted only with arthroconidial antigens, although in CF tests it reacted also with spherule antigen. Subsequently, Levine et al. (86) prepared antigen from spherules grown in vitro by allowing them to lyse in distilled water for 40 days. Initially called "spherule coccidioidin," this antigen was given the name spherulin in analogy with coccidioidin (129). Spherulin appears to have serologic reactivity at least equivalent to that of coccidioidin, and in the CF test it actually detected a higher antibody titer in human sera than did coccidioidin (130). However, spherulin showed greater cross-reactivity than coccidioidin in the CF test (64). Moreover, we have demonstrated that sera which were negative by CF with both spherulin and coccidioidin yielded positive ID tests with coccidioidin when concentrated by evaporation. Therefore, it has not been demonstrated that spherule-derived antigens should supplant mycelial coccidioidins.

The spherule-endospore-phase antigens are also active in CIE, ELISA, CF, and FA (22, 74, 84, 85, 124, 130). Endospores inoculated into modified Converse medium produce, within a few hours, antigens in the medium reactive by IDTP and IDCF (152). We have shown that toluene-induced autolysis at 35°C of mature spherules in vitro also yields antigens active in the IDTP and IDCF reactions. By exposing first-generation spherules grown in vitro to toluene at 40°C, Galgiani et al. (39) produced an antigen reactive by IDTP but lacking IDCF activity. Antigens useful in serologic tests for coccidioidomycosis are available from a number of commercial sources. Their addresses may be obtained by inquiry sent to the Division of Mycotic Diseases, Centers for Disease Control, Atlanta, GA 30333.

Subcellular Localization

The cellular location of coccidioidal antigens has been studied. Kong et al. (81) concluded that the antigen (of whole spherules) important in inducing immunity in mice and monkeys is located in the wall of spherules. The presence of antigen corresponding to that active in the IDCF and the active antigen in the TP and IDTP are released from both mature spherules and endospores produced in vitro. The release of these antigens was hastened by exogenous chitinase or lysozyme. The mechanically disrupted spherule wall fragments, when digested with chitinase, adsorbed quantitatively the antibody participating in the IDTP reaction. However, these fragments of chitinase-treated spherule walls, did not adsorb CF (or IDCF) antibody (24). This last and other observations suggest that the antigen(s) reactive in the CF or IDCF tests is not associated with the cell wall but may be cytoplasmic (157). Cultures inoculated with endospores destined to yield spherules show very early that the IDTP antigen is liberated before and persists through the formation of spherules, and IDCF antigen is found in the culture medium slightly later at a time when cleavage planes are formed (156). Cox et al. (31) also obtained an antigenic, alkali-soluble, water-soluble substance from the wall of *C. immitis* which reacted with IgM antibody.

Cole et al. (20) described the presence of a "membranous" fraction on the surface of the outer wall of spherules grown in vitro that readily sloughed and could be extracted with a detergent, *N*-octyl-β-D-glucopyranoside. This material reacted with both human IDTP-positive and IDCF-positive sera but formed a line of identity only with IDTP and not the IDCF. This suggests that the membranous surface antigen includes IDTP-reactive antigen. However, antibody to this detergent-solubilized surface antigen also reacted with cytoplasmic vesicles as well as spherule wall. The membranous layer may contain molecules that are synthesized in the cytoplasm, transported, and then incorporated into the spherule wall (22). A soluble wall fraction of

arthroconidia with a relative molecular weight of 62,000 under nonreducing conditions was found to have proteolytic activity (as a serine protease) (23). This protease was identified as "antigen 11" (Ag11) by reaction with a burro anticoccidioidin serum in two-dimensional immunoelectrophoresis, but did not react in an ELISA with serum from humans with coccidioidomycosis (the stage of disease or conventional serologic test results were not reported). This antigen showed proteolytic activity against human IgG and secretory IgA. Similar proteolytic activity was manifested by a 19-kilodalton (kDa) fraction of another soluble antigen, termed AgCS, from arthroconidial walls. AgCS, which included a 39-kDa fraction that largely lacked proteolytic activity, reacted by ELISA with sera from humans with coccidioidomycosis. The proteolytic activity of the Ag11 and AgCS against human immunoglobulins makes it likely that these antigens would digest the antibodies participating in conventional serologic tests.

Antigen Characterization

Characteristics of coccidioidal antigens have been studied by a number of workers. Hirsch and d'Andrea (52) precipitated skin-test-active antigen from *C. immitis* culture filtrate with 95% ethanol. They detected 20 to 40% reducing sugars, an osazone identified as glucosazone, and 3 to 4% nitrogen. Hassid et al. (49) also identified glucose as the major component of the alcohol-precipitated fraction of coccidioidin. They detected galacturonic and another unidentified reducing sugar. The saccharide components glucose, galacturonic acid, and unknown sugar were present in a ratio of 6:1:3, respectively. The total nitrogen content of their material was approximately 3.2%, with 0.6% as amino nitrogen. However, their tests for protein (Millon, xanthoproteic, glyoxylic acid, and biuret; and precipitation by hot trichloroacetic acid) were negative.

Subsequently, we reported that precipitation of coccidioidin with 80% ethanol yielded a serologically active material with an average molecular weight of 31,700. This material was 60 to 70% polysaccharide, based on mannose as a standard, and contained 3 to 4% nitrogen (111; D. Pappagianis, Ph.D. thesis, University of California, Berkeley, 1955). Mannose was the predominant sugar representing 90 to 98.5% of the polysaccharide, with galactose at <10%. A third sugar was detected in a very low concentration, and this migrated (by paper chromatography) similarly to rhamnose, a methyl pentose, in one solvent system and similarly to dihydroxyacetone in other solvent systems. We could demonstrate little glucose and no uronic acid. The crude polysaccharide was negative by biuret test, was not precipitated by hot trichloroacetic acid, and gave no reaction with ninhydrin, bromcresol green, or mercuric chloride-methyl orange (111). All of these tests suggested the absence of protein as had been concluded previously by Hassid et al. (49). However, when the crude polysaccharide was hydrolyzed with acid and subjected to paper chromatography, ninhydrin-reactive spots were observed which corresponded to the mobility values of six amino acids, including tyrosine and methionine (111). Furthermore, the Folin-Ciocalteau phenol reagent (93) for protein produced a reaction indicative of 15 to 20% peptide material, which approximates the expected content of protein or derivatives of protein based on the 3 to 4% nitrogen detected.

Goldschmidt and Taylor (Abstr. Annu. Meet. Soc. Am. Bacteriol. 1958, P. 98, p. 127) confirmed the presence of mannose and galactose; they, too, found an unknown sugar which they reported as monomethylmannose. Later, this was shown to be 3-*O*-methylmannose accompanied by glucose as well as mannose and galactose (119, 131). Cole et al. (21) studied the dialyzed retentate of 16-day-old mycelial culture filtrates by gas-liquid chromatography. They detected 3-*O*-methylmannose, mannose, galactose, and glucose in a ratio of approximately 3:10:1:10. Precipitation of the nitrogenous polysaccharide with varying concentrations of ethanol produced chemically varied antigens that reacted with human antiserum by TP and ID (107).

Recently, we carried out immunoaffinity isolation of antigen reactive in the TP and IDTP tests by collecting precipitate produced in the TP test, using human serum (159). The antigen was liberated from the antibody by treatment with pronase and further purified by chromatographic procedures, which yielded two serologically active fractions. These were studied by high-performance liquid chromatography and gas-liquid chromatography after acid hydrolysis. One fraction (peak I) contained mannose, 3-*O*-methylmannose, and glucose in a ratio of 8:1.2:1, and the second fraction (peak II) contained 3-*O*-methylmannose, mannose, glucose, and galactose in a ratio of 1:1:1:1. Several amino acids were present, their aggregate at a ratio of 1:5 with polysaccharide. However, despite the presence of the amino acids, or because of its size or charge, the polysaccharide(s) did not electrophoretically enter a sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and remained within the 3% polyacrylamide stacking gel. By gel filtration, peak I was estimated to have an average molecular size of 140 kDa; peak II had an average molecular size of 225 kDa (154). Some of these properties are reminiscent of those noted by Weiner (148): coccidioidal glycoprotein antigens detected by a RIA and presumed to correspond to TP antigen chromatographically were in the molecular weight range of 140,000 to 240,000 and could not be sharply resolved by PAGE with the antigen(s) remaining close to the origin. Calhoun et al. found a 100-kDa antigen separable by SDS-PAGE that was reactive with IDTP-positive sera in immunoblot analysis (10).

Kruse and Cole (83) have recently isolated two TP antigens from a concanavalin A-bound, alkali-soluble, water-soluble fraction of inner arthroconidial wall. The same antigens were also isolated from mycelial culture filtrate. One of the antigens was characterized as being a 240-kDa dimer of a 120-kDa component and the second was characterized as being a 110-kDa antigen. These polymers were electroeluted from SDS-PAGE separations.

Another approach to the study of soluble serologically active antigens of *C. immitis* entails two-dimensional immunoelectrophoresis as well as crossed- or tandem-immunoelectrophoresis (66). This study demonstrated 26 antigens in coccidioidin and 12 in spherulin. Of these, 10 appeared common to both antigen preparations, 16 were found only in coccidioidin, and 2 were unique to spherulin. Antigenic analysis of an alkali-soluble, water-soluble fraction of *C. immitis* cell walls, coccidioidin, and spherulin by immunoelectrophoresis showed IDTP activity in two peaks, the first of which has a cathodal but only a partial anodal leg and which parallels the second major polymeric peak, designated antigen 2 in this system (28, 66). A monoclonal antibody obtained by immunizing mice with spherulin recognized epitopes on the components of both of these peaks (38). Immunoblotting of coccidioidin with this monoclonal antibody revealed a molecular size range of 130 to 330 kDa, consistent with the size range observed by Kruse and Cole (83), Weiner (148), and Zimmer and Pappagianis (159).

Characterization of both spherule-endosphore-phase and

mycelial culture filtrates by SDS-PAGE and immunoblot analysis revealed electrophoretically separate antigens detected with IDCF/CF-positive sera. A 48-kDa band common to all filtrates was strongly reactive with the serum, as demonstrated by immunoblot reaction with anti-IgG (10, 157). Prior heat treatment of the antigens at 60°C for 30 min, which destroys CF and IDCF activity, altered or destroyed the component detected as the 48-kDa band (157). This component was further characterized by using size exclusion chromatography followed by ID and denaturing SDS-PAGE, as well as nondenaturing PAGE, and shown to be derived from a 110-kDa protein nonreactive with lentil lectin or concanavalin A (158). Cox et al. (30) isolated an antigen reactive with IDCF antiserum by using a combination of concanavalin A affinity and immunoaffinity chromatography. This antigen was assayed by immunoelectrophoresis and corresponded to antigen 3 in the reference system of Huppert et al. (66). Both two-dimensional immunoelectrophoresis and immunoblotting systems showed similarity between antigens derived from coccidioidin and those derived from spherulin (29). Table 1 summarizes the characteristics of coccidioidal antigens.

BODY FLUIDS TESTED

Serum should be tested in all cases of suspected coccidioidomycosis. Changes in the serologic findings with serum usually correlate with clinical changes. Nevertheless, testing CSF and pleural, peritoneal, and joint fluids (along with serum) is appropriate when their corresponding anatomical sites of origin may be affected.

Antibody can be detected in blood obtained from the umbilical cord or by venepuncture from the newborn infant (139, 141). The CF titer may be the same as or slightly lower than that of the mother's serum and will decline with time as would be expected by transplacentally transmitted maternal IgG. As described previously above, we have detected IDTP (IgM) as well as CF (IgG) antibody in the sera of two human neonates. However, neither of these two babies manifested clinical evidence of coccidioidomycosis.

Fixation of complement by CSF at 1:2 or higher dilutions usually indicates the presence of coccidioidal meningitis (139, 141). (CF by undiluted CSF may also indicate meningitis, but this has not yet been substantiated.) The significant exception is when there is juxtadural coccidioidal disease, in which case the pachymeninges may be sufficiently affected to permit CF antibody to penetrate from serum or inflammatory fluid into the CSF. Juxtadural disease can be distinguished by an increased CSF protein and normal glucose and cell count, whereas in meningitis protein and cells are increased and glucose is decreased.

CSF may be obtained from the lumbar or lateral cervical subarachnoid space, cisterna magna, or lateral cerebral ventricles. Differences in concentration of protein and glucose normally exist among these compartments. Similarly, differences in antibody concentrations may be detected in CSF from these different anatomic loci. For example, the ventricular fluid may have normal cell and chemical values and be negative for coccidioidal antibody by CF despite active basilar meningitis accompanied by chemical, cytologic, and serologic evidence of meningitis in cisternal or lumbar fluid (46). If the ventricular fluid fixes complement, it indicates either a block to the outflow of CSF or a coccidioidal ventriculitis-ependymitis.

Because of the differences in constituents of CSF from different anatomical compartments, it is important for comparisons of CF titers to be made between successive specimens from the same compartment, e.g., lumbar fluid compared with lumbar fluid, cisternal with cisternal, etc.

The initial CSF specimen may be negative by the CF test, but overall, 95% of CSF specimens are positive by CF in coccidioidal meningitis (103). Smith et al. (139) indicated that the CSF of one patient did not become positive by CF until 6 months after meningitis developed. In our study of coccid-

TABLE 1. Characteristics of antigens used in coccidioidal serologic tests

Antigen	Characteristics
TP/IDTP	1. Present in spherules, endospores, hyphae and arthroconidia in vitro; probable cell wall location.
	2. Present in both mycelial and spherule/endospore culture filtrate, and autolysates. IDTP antigen liberated before spherule maturity and persists through spherule development.
	3. Released from spherule wall by chitinase. Released from mycelial, spherule, and arthroconidial wall by alkali treatment. Chitinase-digested spherule walls adsorb TP/IDTP antibodies.
	4. Reactive with concanavalin A and lentil lectins. Heat, pronase stable. Contains mannose, glucose, galactose, and 3-*O*-methylmannose. The latter is not specific to *C. immitis*.
	5. At least two separable antigens (epitopes?) involved. Molecular weights reported as (i) 140,000 and 225,000, with different ratios of mannose and 3-*O*-methylmannose; (ii) 110,000 and a 240,000 dimer.
	6. In two-dimensional immunoelectrophoresis, activity in two peaks: one, designated "incomplete precipitating antigen 1," with a cathodal but only partial anodal leg which parallels the second major peak, designated antigen 2. The latter is not specific for *C. immitis*.
	7. Same antigen reactive in LA test?
CF/IDCF	1. Present in mature spherules and endospores, and hyphae in vitro; probable cytoplasmic location.
	2. Present in both mycelial and spherule/endospore culture filtrates only after spontaneous autolysis, or toluene induced, or spherule maturation with subsequent wall lysis and endospore release.
	3. Chitinase-digested spherule walls do not react with CF/IDCF antibodies.
	4. Nonreactive with concanavalin A and lentil lectins. Heat (60°C) and pronase sensitive.
	5. Has reported molecular weight of 110,000, but following reduction and subsequent SDS-PAGE immunoblotting, a 48-kDa band strongly reactive with IDCF/CF antiserum is detected.
	6. Immunoelectrophoretic activity in one peak, designated antigen 3.
Exoantigen (heat stable)	1. Present in mycelial culture filtrates and toluene autolysates, and spherule/endospore phase filtrates and lysates.
	2. Nonreactive with concanavalin A. Heat stable. Reaction is different and distinct from IDTP or IDCF.
	3. Immunoelectrophoretic activity reported in (i) peak designated Ag11 (?) or (ii) peak designated AgCS (?).

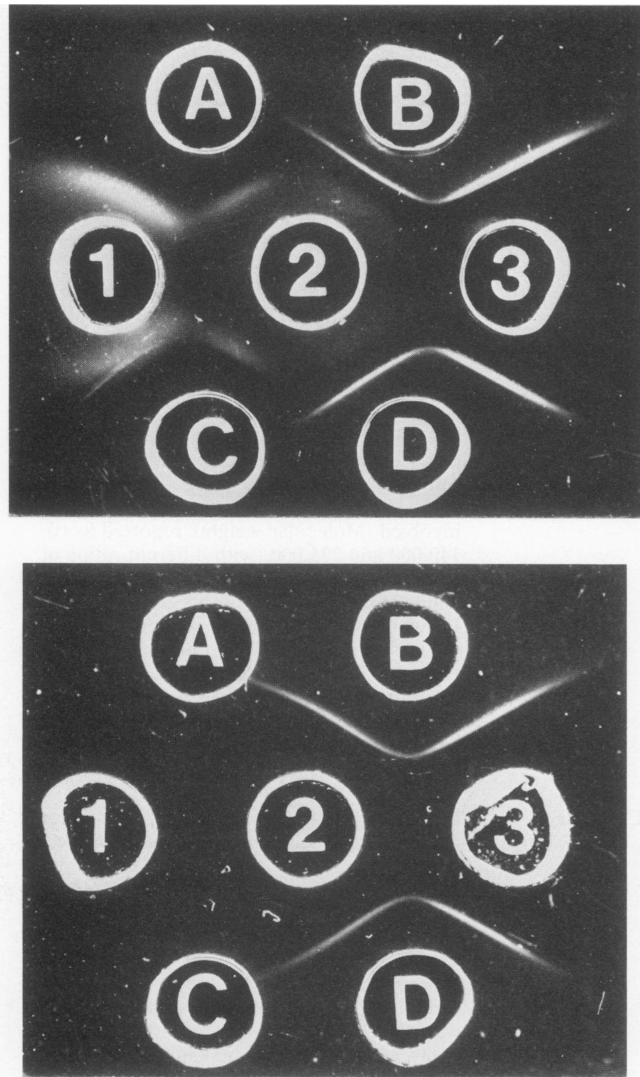

FIG. 7. ID of lumbar CSF from a patient with coccidioidal meningitis. (Top) Presence of IgG (IDCF), which is common, and IgM (IDTP), which is uncommon. (Bottom) Same patient's CSF after treatment with dithiothreitol, which dissolves the IDTP precipitate. (Wells as described for Fig. 4. CSF in center wells.)

ioidomycosis resulting from a dust storm (104, 108), the CSF became seropositive in all of 22 cases of meningitis (occurring outside usual endemic areas) by 9 weeks after onset of primary acute illness (101). In the absence of a serum specimen, a positive CSF serologic test indicates coccidioidal infection, though not necessarily meningitis. The presence of detectable IgM antibody in the CSF is infrequent; however, in our experience, its presence has always been associated with meningitis (Fig. 7). An increasing CF titer of the CSF indicates disease progression, and a decreasing titer indicates improvement. Complement-fixing antibody may persist even after return of the cellular and chemical constituents to normal, and this provides a suitable reason for continuing therapy for meningitis. The CF titer of the lumbar CSF may reach high levels (e.g., 256) accompanied by high concentrations of protein as a result of CSF loculation, e.g., as with a cervical lesion leading to a block in the circulation of the CSF. In patients with a ventriculoperitoneal or ventriculoatrial shunt, the serum as well as CSF should be tested periodically as rising serum CF titer (with or without reappearance of TP or IDTP reactivity) indicates systemic reinfection from the meninges via the shunt (101). Antibody can be detected in the CSF by ID as well as CF. However, caution is needed in the interpretation of a positive ID test, particularly when the sample has been concentrated, as the CSF may contain detectable IgG antibody even in nonmeningitic coccidioidomycosis (112). The coccidioidal LA test should not be used to test CSF because of a high rate of false-positives (107).

Pleural and pericardial effusions occur in patients with acute primary coccidioidomycosis. Both TP and IDTP antibodies may be detected in pleural fluid and the titer of CF and IDCF antibodies may be determined. Titers may be as high as in the serum, but more often they are lower (139, 141). Serologically reactive pleural fluid in patients with unreactive serum has not been reported. Smith (136) described a patient with a tuberculous pleural effusion on one side who then developed a coccidioidal pleural effusion on the opposite side. However, no information was offered as to the coccidioidal serologic activity of the fluid of the two sides.

Coccidioidal peritonitis can occur, with the peritoneal fluid showing CF or precipitin and CF antibodies. The CF titer may be the same or lower than that of the serum. Frequently, the serum CF titer is very high with coccidioidal peritonitis but the clinical prognosis is usually good.

Synovial fluid can also be used to support, by serologic reactivity, a diagnosis of coccidioidal synovitis or arthritis. It is not known, however, whether the joint fluid of patients with coccidioidal joint disease can be seronegative while their serum is seropositive.

SEROLOGIC TESTS

Detection of Early Precipitin IgM Antibody

TP. The TP test is carried out by adding 0.2 ml of serum to 0.2 ml of coccidioidin solution (139, 141). Antigen compared with a reference lot of antigen should be of such a concentration that undiluted and 1:10 dilutions of antigen will react with known TP-positive sera. Smith et al. (139, 141) originally included 1:40 and 1:100 dilutions of antigens to cover the "zone of equivalence" usually expected in a quantitative precipitin test, but these are not required. As a control, the patient's serum is added to culture medium similar to that in which coccidioidin was produced. The tubes are then shaken vigorously and placed in the 35°C incubator. The test has incorrectly been described as one in which antigen is overlaid on the serum (13, 132, 145). The tubes are incubated for 24 h and then examined for presence of a translucent gelatinous button of precipitate by flicking the tube sharply to dislodge the button from the bottom of the tube (Fig. 3). If no reaction is noted, the tubes are incubated again and read daily for the next 3 days. In 85 to 90% of instances, the TP test will show a precipitate in 24 h; only rarely will a reaction not be evident within 48 h. Although various dilutions of antigen have been used, the TP test has not been clearly standardized as a quantitative test. Presence or absence of TP reactivity is significant. The TP test is no longer recommended because of the greater sensitivity of the IDTP.

ID. In 1958, the double diffusion (Ouchterlony test) was shown to yield a band of precipitate when coccidioidin was diffused through agar toward serum from a patient with coccidioidomycosis (51). Subsequently, multiple bands of precipitation were obtained when various fractions derived

from coccidioidin were tested against sera from patients with coccidioidomycosis (111). Huppert and Bailey (59–61) developed the ID so that it could be used to detect antibody that correlated with CF (the IDCF) and TP (the IDTP). They used culture filtrate antigen solution (FAS or F) for the IDCF test and toluene lysate antigen solution (LAS or L) (110, 111) for the IDTP test, but in reality either of these antigen solutions can detect both kinds of antibody. The antigen solutions were concentrated, washed by ultrafiltration, and titrated to obtain the optimal dilution for serologic tests. However, Huppert et al. (62) indicated that ultrafiltration removed most of the TP antigen. Based on the earlier observations of Smith et al. (139) that the TP antigen is heat stable, Huppert and Bailey (61) heated the toluene or lysate antigen to 60°C for 30 min to inactivate CF (or IDCF) antigen. Their ID tests were carried out in 50-mm plastic petri dishes containing 5 ml of agar. Seven wells, 4.5 mm in diameter, were cut in the agar in a hexagonal pattern with one well in the center. The wall to wall distance between wells was 5 mm. The volume required per well was 40 to 50 μl. Other methods such as that of Kaufman and colleagues permit the testing of smaller volumes of many specimens in a single 90-mm petri dish (76, 98). The serum (or other body fluid) is placed in the appropriate well and allowed to prediffuse at room temperature for 2 h, followed by addition of the appropriate antigen dilution. This prediffusion may be unnecessary for the detection of the IDCF reaction but appears essential in order to have the IDTP reaction, which is dependent on slowly diffusing IgM, occur approximately midway between serum and antigen wells.

Huppert and Bailey (60, 61) showed that ID reactions could be read in 24 h with 69% of sera; 84% were positive at 48 h and 100% were positive at 72 h. In our experience with concentrated specimens, 85 to 90% of reactions detectable by ID are apparent in 24 h, most of the remainder becoming detectable within 48 h. Only infrequently does a reaction appear with longer incubation. Nevertheless, we recommend that final readings be made at 96 h.

Because the IDTP reaction depends on IgM, the concentration of which is normally about 120 mg/dl (10^{-3} M) of serum (that of IgG is about 10-fold higher [wt/vol], 10^{-1} M) and because the IDTP reaction was often less clear than the IDCF, we adopted a method of evaporatively concentrating serum or other body fluids prior to the ID test. Because this also effected an increase in IgG reactive with coccidioidal antigen, it improved the sensitivity for both kinds of antibody (109, 112). Our modification of the Huppert and Bailey method also includes the use of heated and unheated antigens (in different wells) to detect IDTP (IgM) and IDCF (IgG), respectively. Using control human sera positive for IDTP and IDCF in the same ID plate allows testing for both kinds of antibody in the same specimen (Fig. 4, middle). Huppert and Bailey (61) had proposed mixing optimal dilutions of IDTP and IDCF antigens to provide a single preparation to detect both types of antibody. For most sera containing both types of antibody, a single unheated antigen solution (e.g., selected at the optimal dilution to detect the IDCF reaction) can also detect IDTP antibody. However, when some sera with a low titer of CF antibody (e.g., 2 to 8) are tested, the IDCF reaction is obscured by the IDTP reaction. This can be shown by treating the bands with 0.05 M dithiothreitol (in 0.2 M Tris hydrochloride buffer, pH 8.6) which damages IgM and leads to dissolution of the IDTP band (Pappagianis et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1986). As a result, the underlying IDCF reaction is revealed because the IgG remains intact (Fig. 8).

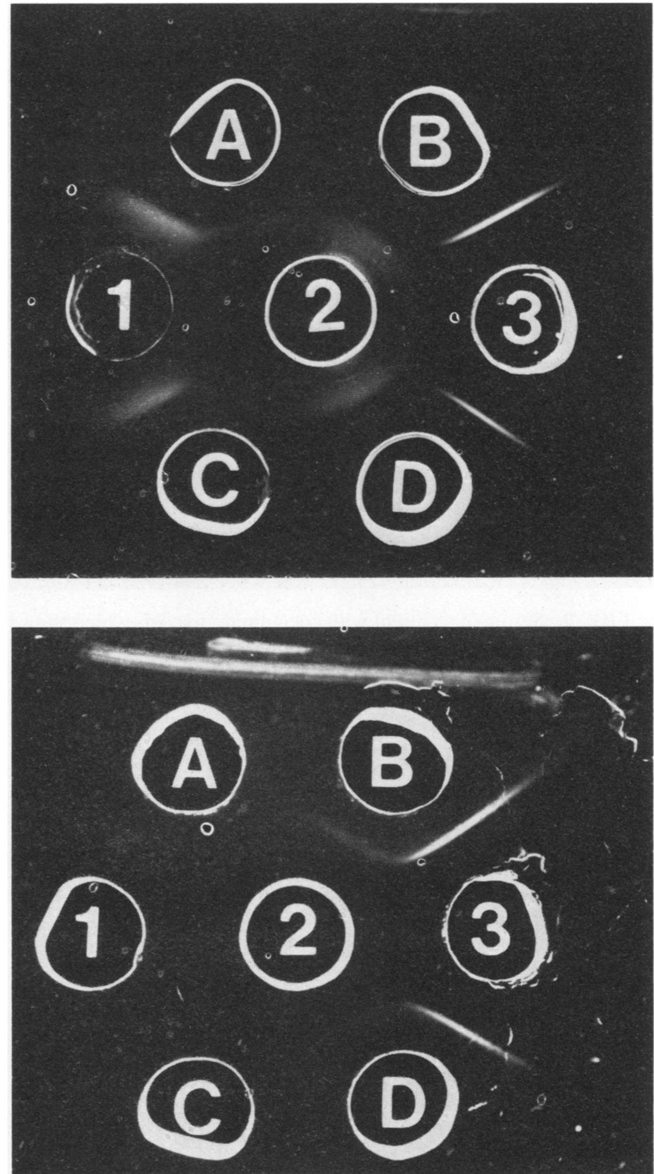

FIG. 8. ID of serum that showed both IDTP and IDCF reactivity. The latter was obscured by the IgM-antigen precipitate (top), but became evident following dissolution of the IgM-antigen precipitate with dithiothreitol (bottom). (Wells as in legend to Fig. 4.)

Occasionally, interference has been noted in the ID test by the formation of a band of precipitation resembling that produced by the IDTP reaction. The cause of the problem is unknown; however, we surmise that it may be the result of reaction between coccidioidal antigens and C-reactive protein (or related substances in serum). Such a reaction had been reported with preparations derived from *Aspergillus fumigatus* (7, 16, 91, 116). The addition of an aqueous solution (1.5%) of sodium EDTA leads to dissolution of bands that may resemble IDTP, while the authentic IDTP bands are unaffected by such treatment. An additional band of precipitation is frequently encountered with both heated and unheated antigen. It does not form a line of identity with either the IDTP or IDCF control and is not dissolved by

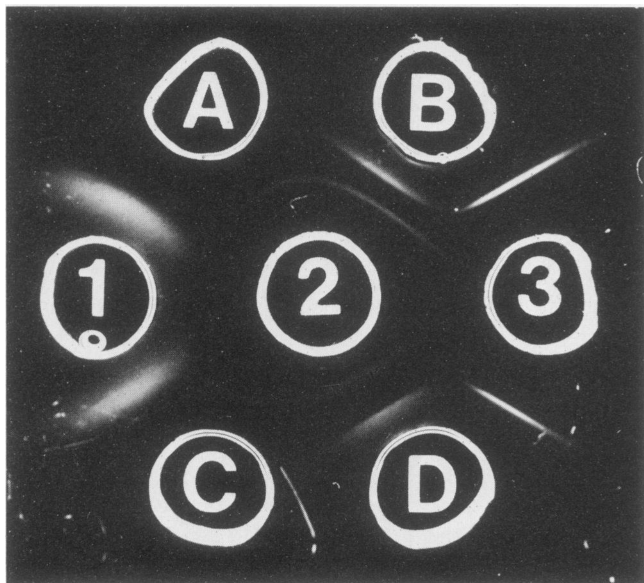

FIG. 9. Serum of a patient with coccidioidomycosis which shows presence of IgM and IgG reactions and has an additional undefined reaction lying between the IgM and IgG bands. (Wells as described in the legend to Fig. 4.)

EDTA or dithiothreitol (Fig. 9). Its identity and significance are unknown.

The substrate for ID tests can be agar, agarose, or gellan gum, a polysaccharide produced by a *Pseudomonas* species. We have used the last agent for over 3 years (approximately 20,000 specimens tested) and have found it a satisfactory substitute for agar or agarose when testing human and animal sera. (Rabbit serum, however, may not show the same reactions in gellan gum that it does in agar.)

LA. For the LA test, latex particles are coated with coccidioidal antigen obtained by toluene autolysis of mycelial *C. immitis* or from culture filtrates. Originally, polystyrene latex particles 0.15 to 0.35 μm in diameter were used (C. Mulder, S. Kiddy, and K. Lou, Abstr. Annu. Meet. Am. Soc. Microbiol. 1966, RT20, p. 141), but in more recent preparations 0.8-μm particles are used. The antigen solution is heated at 60°C to inactivate the antigenic component(s) reactive with complement. The commercial distributors also have recommended that the serum to be tested be heated at 56°C for 30 min to inactivate complement. Agglutination is read after 4 min.

The LA test is more sensitive than TP, but it has yielded at least 6% false-positive reactions (65). Results of LA tests reported to us from several different laboratories indicate that, in general use, the rate of false-positives is higher. Because of this, a confirmatory test(s), preferably ID, must follow any positive LA. Very high rates of false-positive LA occur with CSF or with sera that have been diluted (107) whether obtained from patients with coccidioidomycosis or not. Because of this, the attempted application of LA as a quantitative test (146) is likely to provide spurious information. While the LA test is designated as a test to detect antibody (IgM) corresponding to that detectable by TP, our experience shows that some LA-positive reactions are reported with sera that are positive by IDCF (IgG) but not for IgM. The LA test has been useful in diagnosis of coccidioidomycosis in the dog (155).

Detection of the CF IgG Antibody

CF. Smith et al. (139, 141) adapted the Kolmer CF test, mixing 0.25 ml of various dilutions of inactivated serum (56°C for 30 min) with 0.25 ml of antigen and 0.5 ml of complement adjusted to contain 2 U. Initially, complement binding was effected by incubation for 2 h at 37°C followed by addition of hemolysin-sensitized sheep erythrocytes and incubation for 1 h. The endpoint 4+ fixation of complement was the last tube in which there was no evidence of hemolysis to the naked eye. This method permitted an early reading; however, Smith et al. (141) recognized that complement binding carried out overnight at 4°C resulted in CF titers approximately twofold higher. Tests in several collaborating laboratories affirmed the higher titer obtained with overnight binding at 4°C (140), but because the different CF methods were used, disparities in titers were seen.

To develop a standardized procedure that could be universally adopted, comparisons of different CF methods (63) were carried out. The Smith CF macromethod, using overnight binding of complement at 4 to 5°C, gave more reproducible results than the LBCF procedure (also using complement binding at 5°C) in either its macro- or microdilution version (98). This result was unexpected because the LBCF method, although more complex, makes use of more objective methods; e.g., extent of hemolysis is interpreted with hemoglobin color standards and a 50% hemolytic endpoint is used. In the LBCF, the last tube or well showing 30% or less hemolysis is considered positive. The micro-LBCF requires approximately one-tenth the volume of serum, CSF, or other fluid required by the Kolmer or macro-LBCF, and it has been widely adopted. No direct comparison between the micro-LBCF and the original method of Smith et al. has been reported; however, binding at 4°C provides higher titers than 2-h binding at 37°C. In some instances, positive results are obtained after overnight test incubation at 4°C but not at 37°C after 2 h (135). As ordinarily described, the LBCF titration may begin with a 1:8 dilution of serum. Therefore, sera that are positive but only with titers of 2 or 4 would appear negative at the 1:8 dilution (98). Such low titers may be encountered in early coccidioidal disease, limited dissemination, pulmonary residua, or late disease when the titer has declined. The LBCF should begin with a 1:2 dilution if the IDCF is positive.

Because CF titers of 2 to 8 may represent antibody reacting with another, cross-reactive antigen (64, 139, 141), the presence of coccidioidal CF antibody should be verified in the initial specimen by the more specific ID test (76). Simultaneous testing of paired sequential sera is essential in view of possible test-to-test variation of CF and quantitative ID titers (135). Human serum from confirmed cases of coccidioidomycosis has served as a positive control for CF tests. Serum samples from horses injected intravenously with the hyphal-arthroconidial-phase *C. immitis* appeared to be satisfactory for the CF test but were never put to routine use (140). Serum samples from rabbits infected with *C. immitis* were also proposed as a reference standard (5) but not adopted. The CF test has been applied to species other than humans and appears to yield results of similar significance to those found with human sera; i.e., the titers are proportional to severity of disease (4, 54, 55, 88, 92, 110, 120, 121, 134, 145).

IDCF. The evolution and use of the ID test have been described above. In 1962, Schubert and Hampson (132) showed that CF-positive sera reacted in an ID test with coccidioidin as antigen. Huppert and Bailey (59, 60) set forth

appropriate conditions for the performance of the IDCF, which detected antibody corresponding to that detected by CF. The test relied on selection of the appropriate dilution of antigen, known to be heat labile, for the detection of IDCF. The dilution of antigen chosen, after 2-h prediffusion, yields a precipitate with antibody approximately midway between the wells. The IDCF test can be carried out without prediffusion of the serum. The higher the concentration of antibody, the closer to the antigen well will be the antigen-antibody precipitate. The location of the precipitate can even provide a rough estimate of how many dilutions of serum may be needed to obtain the endpoint titer in the CF test. While many coccidioidal sera yield a positive IDCF result without prior concentration, some specimens, e.g., from well-focalized pulmonary nodules or cavities, will not show IDCF reaction unless concentrated first. A control CF-positive human serum is used in each test.

QID. The QID test for IDCF antibody is carried out by diffusing various dilutions of serum against a fixed concentration of antigen previously determined to have appropriate reactivity. Several workers have studied the QID (48, 62, 71, 151, 153). By refilling the antigen and serum wells 24 and 48 h after the original filling and taking a final reading at 72 h, Wieden et al. (151) found that agreement between QID and CF titers (±1 two-fold dilution) was 84.7%. The QID was tested with consecutive sera from the same patients. Although it tended to yield slightly lower titers than the CF test performed by 2-h binding of complement at 37°C, over time the changes in titer were comparable. Others found similar agreement between CF and QID tests: 80% (61), 87% (I. Krasnow and M. Huppert, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, F54, p. 127), and 82% (147). The QID test requires fewer reagents and smaller volumes than the CF test. The manipulations are simpler, but no automation has been used and the results are delayed (final reading after 48 to 72 h). The QID test is practical for the laboratory that carries out few serologic determinations, but for large numbers of specimens the CF test is more practical. QID is needed when serum is anticomplementary and will not yield a readable titer by CF (frequent with canine sera) and for those unusual sera that do not show fixation of complement but yield a titer by QID (Fig. 10).

Table 2 summarizes pertinent characteristics of serologic tests for coccidioidomycosis.

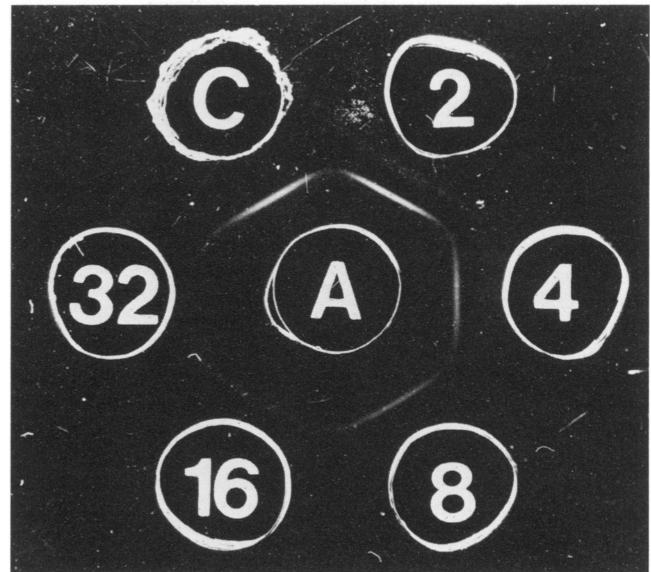

FIG. 10. QID test of human serum containing both IDTP and IDCF reactivity. The former is positive at 1:2 and 1:4 dilutions; the latter is reactive through 1:32. Well C contains control IDCF-positive serum. The antigen (Ag) is unheated coccidioidin. Wells 2 through 32 represent dilutions of patient serum 1:2 through 1:32.

Miscellaneous/Experimental Tests for Antibody

Agar gel precipitation-inhibition test. The agar gel precipitation-inhibition test is carried out by mixing serial dilutions of serum with a suitable concentration of antigen to permit binding of serum antibody to antigen. The mixture is then diffused against a serum of known titer relative to the dilution of antigen. The endpoint is taken as the highest dilution of the patient's serum that bound antigen and prevented it from reacting with the reference serum of known titer. The endpoint is read after 48 h. No advantage was apparent when compared with CF testing (120).

CIE. CIE has been used for both qualitative and quantitative antibody determination. With coccidioidin as antigen, both IDTP and IDCF antibodies were detected in sera of dogs with coccidioidomycosis (133). All canine sera that were positive by CF (titers, 2 to 128) were positive by CIE. Human sera and CSF were tested by CIE, using spherulin; the CIE provided 100% correlation with ID and 98% correlation with CF results (1). The mean titer by CIE was 1 serial dilution lower than that obtained by CF. The nature of the antigens used in two other studies is uncertain as one referred to a "toluene extract of culture filtrates" (82) and the other referred to "spherule coccidioidin" (47). The former study found that 100% of sera with CF titers of >16 were positive by CIE, but at lower CF titers CIE was proportionately less sensitive, detecting only 64% when the titer was 2. As Kozub et al. (82) pointed out, the sensitivity of the CIE would probably be improved by appropriate selection of reagents. However, if the sensitivity does not exceed that of ID, the advantage of more rapid results by CIE (1 to 2 h) may be of particular importance only in the infrequent cases of rapidly progressing coccidioidomycosis.

FA. FA methods have been used for the detection of both *C. immitis* in infected tissue (74) and antibody in human sera (75). Histologic staining was accomplished by preparing antisera in rabbits injected with viable or nonviable arthroconidia of *C. immitis*. The globulin fraction was separated from the sera and conjugated with fluorescein isothiocyanate. With this conjugate, endospores and the contents of spherules fluoresced but the walls of spherules in infected tissues did not, perhaps because antibody was already present on the surface of spherules in vivo. However, the walls of spherules produced in vitro did react with the fluorescein-labeled antibody. This reagent provided a sensitive histologic method for the detection of *C. immitis* in tissue and exudates. Its specificity was enhanced by prior adsorption with yeast cells of *Histoplasma capsulatum* or by diluting antibody globulin induced by injection of rabbits with viable (but not with killed) *C. immitis* arthroconidia (72).

The one-step FA inhibition test was used to detect the presence of coccidioidal antibody in human sera. Smears of endospores prepared from the cut surface of infected mouse lungs were exposed to a mixture (A) of fluorescein-labeled rabbit antibody plus unlabeled unknown serum. The intensity of staining was compared with that of endospores exposed to a mixture (B) of fluorescein-labeled globulin and normal serum (the negative control). In A, coccidioidal

TABLE 2. Serologic tests for coccidioidomycosis

Test	Antigen	Special considerations	Detects	Clinical correlation
TP	Coccidioidin culture filtrate/autolysate	Patient serum mixed with "undiluted" and "1:10" antigen; control patient serum plus culture medium. Now rarely used, supplanted by IDTP.	IgM	Valuable in acute disease, can reveal the only serum antibody produced in early stages; rarely positive with CSF.
IDTP	Same as TP; antigen should be heated to eliminate reactivity with IgG.	Serum or other fluid concentrated and prediffused before antigen is placed in well.	IgM	As above, but more sensitive than TP. Antibody detectable with some chronic pulmonary cavities. When positive with CSF, acute meningitis present.
LA or LPA	Latex particles coated with heat-treated toluene autolysate	Very sensitive, but false-positives occur; requires confirmatory test. Not useful with diluted serum or with CSF.	IgM (some reactions occur with sera showing only IgG by other test)	Valuable in acute disease for detection of antibody in serum.
CF	Coccidioidin culture filtrate/autolysate	Valuable for quantitation of antibody in serum and other body fluids; 2-h binding of complement at 37°C useful with sera, more sensitive 18-h binding of complement at 4°C useful with CSF or serum.	IgG	Later developing antibody but more persistent than IgM; rising titer relates to extending disease, reduction in titer relates to clinical improvement. Serum CF titer >16 often associated with dissemination. CF-positive CSF usually indicates meningitis.
IDCF, qualitative or quantitative	Coccidioidin culture filtrate/autolysate	Serum or other fluid may be tested unconcentrated and without prediffusion, but sensitivity of qualitative test enhanced by concn and prediffusion; dilutions of specimen for quantitative IDCF.	IgG	Good qualitative screening test for IgG; when serum or CSF concentrated, more sensitive than CF (may show IgG in CSF even in absence of meningitis). Can be used as a quantitative test; titers approximate those obtained by CF.

antibody in the patient serum inhibited the uptake of the fluorescent tag by endospores. The FA inhibition test showed a 93% correlation with CF-positive sera and was positive with sera from some cases of coccidioidomycosis that were negative by CF as well as some that were reactive only by TP.

FA reagent produced against killed arthroconidia was also used to visualize arthroconidia in the soil (72). This approach has not yet been used for the direct identification of the mycelial form of *C. immitis* (73) as has the exoantigen method (see below).

RIA. A solid-phase RIA method was tested by Catanzaro and Flatauer (15) as a means of detecting antibody in human sera. RIA values correlated well with the clinical status of patients with pulmonary coccidioidomycosis (type of pulmonary disease not given) and with the CF titer in disseminated infections.

ELISA. Several groups of workers have studied detection of coccidioidal antibody by ELISA (S. P. Lindsay, N. Bodie, and D. Pappagianis, Proc. Annu. Meet. Coccidioidomycosis Study Group 1981, p. 1; S. I. Freeman, C. Burke, and R. Talbot, Proc. Annu. Meet. Coccidioidomycosis Study Group 1982, p. 4; R. C. Talbot, R. C. Johnson, and J. Leonard, Proc. Annu. Meet. Coccidioidomycosis Study Group 1986, p. 3; J. Leonard and R. Talbot, Proc. Annu. Meet. Coccidioidomycosis Study Group 1987, p. 11; J. N. Galgiani, L. Lundergan, G. Grace, and K. O. Dugger, Proc. Annu. Meet. Coccidioidomycosis Study Group 1989, p. 1). A consistent shortcoming, found in about 10% of sera tested, is the inability to distinguish clearly between patient (positive) and control (negative) sera, as determined by conventional tests. Thus far, the closest correlation has been obtained between IDTP and ELISA. In a study of 792 sera, Leonard and Talbot reported an 89% agreement between ELISA and the TP test but 100% agreement between IDTP and ELISA, using anti-human globulin as the secondary antibody. In a separate study, Talbot et al. found that a large number of patients had CSF antibodies detectable by ELISA but not by CF. None of these patients ever developed coccidioidal meningitis, indicating that the greater sensitivity of the ELISA makes it unreliable for the detection of meningitis. This finding resembles that noted earlier for IDCF tests with concentrated CSF. The IDCF was overly sensitive because it detected IgG antibody in the CSF of patients with nonmeningitic coccidioidomycosis (112). Cole et al. tested antibody response to an isolated immunoreactive spherule wall antigen (20). Using anti-IgM in the ELISA they found overlapping optical density values between sera from patients and controls. When anti-IgG was the detecting antibody, there was clear differentiation between CF-positive and -negative sera; however, there was no correlation between CF titers and ELISA values, as noted also by Lindsay et al.

We recently studied various coccidioidin, spherule-endospore, and synthetic (3-*O*-methylmannose-based) antigens in the conduct of coccidioidal ELISA, using 400 sera. The use

of 20% normal goat serum as an ELISA diluent rather than traditional phosphate-buffered saline with Tween 20 provided a better distinction between IDTP-positive and -negative sera and a 97% correlation between ELISA and IDTP in serum when anti-IgM was used. Levels of anticoccidioidal IgG did not correlate with CF activity regardless of ELISA reagents (B. Zimmer, D. Pappagianis, K. VanHoosear, and M. B. Goren, Abstr. Annu. Meet. Am. Soc. Microbiol. 1990, F56, p. 418).

CROSS-REACTIVITY

The issue of serologic specificity and cross-reactivity of coccidioidal reagents has been raised in a number of reports. Smith et al. (139) obtained negative coccidioidal serologic tests from patients with tuberculosis, brucellosis, primary atypical (mycoplasmal) pneumonia, actinomycosis, influenza, syphilis, typhoid fever, tularemia, leptospirosis, pertussis, Q fever, smallpox, measles, and psittacosis. Negative serologic tests were also obtained with sera from patients with blastomycosis and cryptococcosis. However, Smith et al. as well as Campbell and Binkley (13), Salvin et al. (127), and Heiner (51) observed cross-reactions (positive coccidioidal serology, using coccidioidin) with sera from patients with histoplasmosis. Campbell and Binkley (13) studied the problem of cross-reactivity systematically and concluded that sera from patients with histoplasmosis rarely cross-reacted with coccidioidin in the CF test, and when such cross-reactions occurred, they yielded low titers compared with those produced with histoplasmal antigen. However, sera from "mild" and primary cases of coccidioidomycosis gave higher CF titers with *Histoplasma* and *Blastomyces* antigens than with *Coccidioides* antigen (11, 12). As coccidioidal CF titers increased in patients with coccidioidomycosis, heterologous CF titers decreased. When a toluene-induced autolysate of *C. immitis* hyphae was used as antigen in the CF test, no cross-reactivity was shown by a small number of sera from humans with histoplasmosis (114). Coccidioidin used in the CF test yielded cross-reactions with sera from cases of histoplasmosis and from a patient with blastomycosis and candidiasis (76). Huppert et al. (64) found that both spherulin and coccidioidin in CF tests yielded reactions with sera from several other mycoses caused by *Pseudallescheria boydii*, *Aspergillus* spp., *B. dermatitidis*, *H. capsulatum*, *Phialophora* (*Fonsecaea*) *pedrosoi*, *Candida albicans*, *Candida parapsilosis*, *Cryptococcus neoformans*, and *Torulopsis glabrata*. Recently, we noted an atypical IDTP reaction with serum from a patient with active histoplasmosis and no known coccidioidomycosis.

Sera from patients with various nonmycotic diseases were reported to fix complement in tests with coccidioidin (76, 132). These diseases included actinomycoses, tuberculosis, pneumococcal pneumonia, staphylococcal empyema, central nervous system malacia, macular degeneration, pulmonary carcinoma, and regional enteritis. Serum from patients with sarcoidosis did not react with coccidioidal antigen (17, 70).

Huppert et al. (63) reported that 20% of sera from patients with noncoccidioidal mycoses gave positive reactions by CF tests when coccidioidin was used, and 48% gave positive reactions when spherulin was used. These are inordinately high percentages considering the reported specificity of coccidioidin. Nevertheless, these and other data affirm the need for performing concomitant ID tests, at least with initial sera to confirm the presence of coccidioidal antibody (76). Based on the data of Huppert et al. (64), we should have detected a sizable proportion of sera positive by CF but negative by ID, i.e., disease not confirmed by the latter, more specific test. Indeed, among 80,000 specimens we tested over the past 8.5 years, including some 6,000 newly recognized and several thousand old cases of coccidioidomycosis, 3 were positive by CF and not by ID, a much smaller fraction of false-positives than reported by Huppert et al. (64).

The explanation for cross-reacting antibody in sera from patients with mycoses and nonmycotic diseases has not yet been provided. By ID, Heiner (51) demonstrated a reaction between coccidioidal antigen and the serum of one of several patients with histoplasmosis. He concluded that a reactive antigenic component, "c," was present also in histoplasmin. Kaplan and Clifford (74) prepared anticoccidioidal globulin from rabbits for FA tests. The globulin reacted with *H. capsulatum* and *B. dermatitidis*, but the cross-reactive component was removed by adsorption with *H. capsulatum* yeast cells. By two-dimensional immunoelectrophoresis, Huppert et al. (58) showed 9 *H. capsulatum* and 11 *B. dermatitidis* antigens in coccidioidal antigen preparations.

The 3-*O*-methylmannose present in coccidioidal antigen could explain cross-reactivity with some organisms, e.g., *Streptomyces griseus* (14), members of the family *Enterobacteriaceae* (97), mycobacteria (S. K. Maitra and C. E. Ballou, Fed. Proc. 33:1686, 1974), or the fungus *Malbranchea dendritica* (19). However, 3-*O*-methylmannose has not been demonstrated in *H. capsulatum* or *B. dermatitidis*. Infrequently, sera yield reactions in ID tests with coccidioidin but no line of identity with control sera from patients with coccidioidomycosis. Recently, we observed such a reaction with serum from a man with *Mycobacterium avium* pulmonary disease. Whether these sera contain antibody produced against one of the heterologous organisms containing 3-*O*-methylmannose or a similar sugar (e.g., 3-*O*-methylgalactose, which is the madurose from *Actinomadura madurae*) has not been determined.

ANTIGEN DETECTION

Detection of antigen would be useful in two particular situations: very early coccidioidomycosis (probably 1 week), between onset of symptoms and detectability of IgM; and in patients whose humoral responsiveness is delayed or wanting. The latter group includes those with hypogammaglobulinemia or another immunodeficient state.

Weiner (148) developed a RIA for coccidioidal antigen in human sera. Immunoreactive fractions of molecular weights between 200,000 and 230,000 (see section on antigens) were obtained by chromatographic separation, and these were conjugated to tyramine and radiolabeled with ^{125}I. The antigen appeared to be a protein-carbohydrate conjugate in which the carbohydrate represented a critical epitope because mild periodate oxidation led to loss of antigenic activity. Sera were tested after acidification and heating at 96°C for 20 min. All nine patients studied by Weiner had detectable antibody at the time the test for antigen was carried out, except a renal transplant recipient who was negative for both antigen and antibody. This patient subsequently developed detectable antibody by the IDTP test.

Galgiani et al. (40) used an inhibition ELISA to detect antigen. Human serum obtained during primary coccidioidal pneumonia was mixed with rabbit antibody to spherulin to permit the antigen in the human serum to react with lapine antibody. The concentration of rabbit antibody remaining unreacted was then measured in spherulin-coated cuvettes.

Normal human serum to which known amounts of antigen were added was used to construct a standard curve. Antigen activity was highest at 2 and 6 weeks after onset of illness and declined thereafter through 4 months and beyond.

Immunoglobulin isotype antibody levels measured in the ELISA showed changes in IgM (but not IgG or IgA) virtually parallel to antigen levels in the patient sera. This led the authors to suggest that this relationship resulted from immune complex formation. They noted, however, that normal human serum, immune human serum, or fetal calf serum (to a lesser extent) could interfere with antigen assays in that these sera reacted nonspecifically with spherulin adsorbed to the plastic cuvette (41). Of the three major isotypes, IgM exerted the greatest degree of interference. This interference could be largely overcome by acidification and heating of the serum (147, 148). All of 14 sera from 13 patients with acute pulmonary coccidioidomycosis had detectable antigen; however, no quantity was presented as the minimum level of detectable antigen. Therefore, there is uncertainty as to the significance of several readings given as "<0.1" or "<0.2" μg/ml. Three of 13 serum samples tested had antigen detectable in the absence of detectable IDTP or IDCF antibody in unconcentrated specimens. One serum sample with detectable antigen was not tested for antibody. Of the 10 serum samples positive for antibody, 3 had antigen levels of <0.2 μg/ml. The authors concluded that the assay of antigen in sera had not been sufficiently developed to use as a diagnostic method.

In part, the problem of establishing an assay for antigen may lie in the occurrence of varying immune complexes. Gamble and Ruggles (43) demonstrated the presence of a mixed cryoglobulin (IgA1–IgG3,4) associated with glomerulonephritis in a patient with a history of coccidioidomycosis some 30 years previously and a pulmonary lesion that more recently underwent cavitation. The IgG portion of the cryoglobulin reacted with coccidioidin, indicating the probable participation of immune complexes of coccidioidal antigen and IgG in the formation of IgA-IgG glomerular deposits. Furthermore, Yoshinoya et al. (154) demonstrated coccidioidal antigen-antibody complexes which, by ultracentrifugation, sedimented in the range of 6.6S to 19S and the concentration of which correlated with CF antibody titers. Subsequently, Cox et al. (27) reported that the concentration of immune complexes was proportional to IgG concentration and to CF titer only at low and intermediate titers, but less so at high titers. The acid-heat treatment of Weiner (148) and of Wack and colleagues (147) would inactivate the CF-reactive antigen and probably destroy its detectability by antibody in an ELISA or similar assay.

Antigen detection may thus best be accomplished by a method that would permit nonimmunologic detection of both heat-stable and heat-labile coccidioidal antigen or a derivative of coccidioidal antigens. Detection of the 3-*O*-methylmannose of coccidioidal antigen may obviate the problems inherent in detection of intact antigen.

EXOANTIGEN TESTING

Exoantigen serologic testing developed by Standard and Kaufman (143) has permitted identification of mycelial-phase *C. immitis* without the tedious necessity of converting it to the spherule-endospore phase in vivo or under special in vitro conditions. It has even been used to identify *C. immitis* in the presence of contaminating organisms (37). Mycelial-phase cultures produce an exoantigen that is heat stable (HS) at 56°C for 30 min and specific for this fungus (67, 143). HS exoantigen is used for rapid identification of *C. immitis* in culture by testing a filtrate of the suspected fungus by ID against a reference antiserum produced against heat-stable exoantigen (77). This IDHS reaction is different and distinct from reactions classified as IDTP (also involving a heat-stable antigen) or IDCF in human sera. The occurrence of IDHS antibody in these human sera is rare (78).

Antigen resembling HS antigen was demonstrated in (i) Formalin-treated mycelial culture filtrate, (ii) toluene autolysate and a mixture of non-Formalin-treated mycelial culture filtrate, and (iii) toluene autolysate and a mixture of non-Formalin-treated mycelial culture filtrate plus toluene autolysate (28). This antigen did not bind to concanavalin A and was considered likely to correspond to the Ag11 described by Huppert et al. (66). However, other work hinted at a relationship between HS antigen and antigen designated CS which differs from Ag11 (22).

FUTURE DIRECTIONS

Future directions in coccidioidal serology should include molecular genetics methods that can lead to purified and well-characterized reagents. Methods should be devised for the reliable detection of antigen to permit diagnosis before antibody is produced or in patients whose production of antibody is insufficient. While specific antibody for the detection of antigen, e.g., one that contains 3-*O*-methylmannose, may provide desirable specificity, there may be a need for greater breadth of antigen detection for appropriate sensitivity. ELISA and other as yet undeveloped methods may provide an approach to greater test sensitivity, but they must be able to detect antigen or antibody at various stages of coccidioidomycosis and must provide the prognostic quantitative results currently available from the venerable CF test. The rapid, specific identification of *C. immitis* in cultures as well as in sputum, exudate, or tissue should be enhanced by application of FA or enzyme-linked methods. Whatever means are adopted for serodiagnosis of coccidioidomycosis must also be consonant with environmental safety.

ACKNOWLEDGMENT

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