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Complement Activation by *Coccidioides immitis*: In Vitro and Clinical Studies

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Mycelial- or spherule-phase derivatives of *Coccidioides immitis* caused a decrease in vitro of total hemolytic complement in serum from a nonsensitized person. Activation involved both classic and alternative pathways as shown by depression of hemolytic C4 and by generation of products of activation of components C3, C4, and factor B. In addition, functional complement activity or immunoreactive levels of complement components or both were measured in 23 patients with self-limited or disseminated coccidioidomycosis. Low total hemolytic complement was found in nine, usually during the early phase of primary illness, and was transient. Hemolytic C4 was low, and the effect of inulin to decrease complement levels was blunted, suggesting both classic and alternative pathways may be deficient. However, associated depression of immunoreactive levels of components assayed (C3, C4, C5, factor B, and properdin) was not consistently found. This disparity raises the possibility of enhanced in vitro inactivation analogous to activation by immune complexes.

Coccidioidomycosis is a fungal infection which occurs in a large proportion of those living in or travelling through endemic areas such as the San Joaquin Valley and much of the southwestern United States. Persons who develop serious disseminated disease comprise only a very small percentage of those exposed. Epidemiological studies with skin test response to fungal derived antigens (14) demonstrate that usually sensitization occurs without symptoms or with mild illness. The self-limited nature of these infections is thought to reflect intact host defenses, the mechanisms of which are not well understood.

As part of studies to further this understanding, the in vitro chemotactic response of human polymorphonuclear leukocytes to substances derived from *Coccidioides immitis* was studied (5). A chemotactic response was not detected to either a filtrate of a mycelial growth or a lysate of spherule preparations alone, but activity of either substance when mixed with fresh human serum was demonstrated. Furthermore, if serum previously heated to 56°C was used, this activity was lost. These findings suggested that the chemotactic activity is mediated by complement-derived fragments. The studies presented in this report establish more directly that soluble substances interact in vitro with complement, causing generation of reaction products of complement activation. With these studies as the impetus, we also measured whole complement and complement component levels in patients with either primary self-limited or disseminated coccidioidomycosis.

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MATERIALS AND METHODS

Human serum. For most studies which examined the interaction of serum and fungal substances, serum was separated from venous blood of a person determined to be nonsensitized by the following criteria: (i) negative skin test to either coccidioidin (1:100 strength, Cutter Laboratories, Berkeley, Calif.) or spherulin (usual strength, Berkeley Biologicals, Berkeley, Calif.); (ii) serum devoid of complement-fixing antibodies for coccidioidin (measurements by Demosthenes Pappagianis, University of California, Davis); and (iii) medical history of no known illness due to *C. immitis*. Serum was either kept on ice for use that day or stored at −70°C for subsequent studies.

For some studies, serum from a patient with congenital absence of C2 was used. This serum had previously been found to have this isolated defect with normal levels of individual components C1, C3 to C9, factor B (C3PA), and C3b inactivation by radial immunodiffusion methods (tests performed by Neil R. Cooper, Scripps Clinic and Research Foundation, La Jolla, Calif.). This serum was stored at −70°C before use.

Sera from patients with coccidioidomycosis were frozen, transported on dry ice, and kept at −70°C until

used. Acute primary infection was diagnosed in persons who developed a febrile respiratory illness, demonstrated pulmonary infiltrates on radiological examination, and were found to have serological evidence of recent exposure to this fungus (precipitating antibodies and, in most, rising complement-fixing antibodies to coccidioidin). Disseminated disease was diagnosed if *C. immitis* was isolated from an extrathoracic site. Many of these patients developed symptoms in association with the epidemic conditions that occurred at Lemoore Naval Hospital and elsewhere in California during the winter of 1977 to 1978 (16).

Substances derived from *C. immitis*. Mycelial filtrate (MF), the filtrate of medium that had supported growth of the mycelial phase of *C. immitis* (lot MFS-77), was kindly supplied by D. Pappagianis at a concentration of 6.75 mg/ml. For some studies, dialyzed MF was used, prepared as described previously (3). Since the retentate increased in volume during dialysis, an appropriate dilution of the undialyzed MF was made (one part buffer to four parts MF) for comparison.

Spherulin, the hypotonic lysate of washed spherules, was generously supplied by Berkeley Biologicals free of preservative at a concentration of 1.84 mg/ml.

Assays of complement. Total hemolytic complement (CH_{50}) and hemolytic C4 (C4H) levels were determined as previously described (4, 11). CH_{50} results are expressed as activity per milliliter of serum. The normal range for human serum (two standard deviations from the mean) is 114 to 210 U/ml with a replicate variation of 3%. C4H levels are expressed as the reciprocal of the dilution of serum which yields one hemolytic site per sheep erythrocyte (11, 19). Replicate variation was less than 15%. C3, C4, C5, factor B, and properdin were measured by radial immunodiffusion (1), for which replicate variation was 5%. The normal ranges of all component assays (two standard deviations from the mean) are shown in Fig. 4.

Reaction products generated by complement activation were detected by immunoelectrophoresis with antisera to C3, C4, and factor B (6, 8, 10).

Commercially prepared antisera were used in these studies except for the properdin assay for which antisera was supplied by John T. Boyer (15). Inulin (Armar-Stone Laboratories, Inc., McGaw Park, Ill.) and aggregated gamma globulin (prepared by heating human gamma globulin to 63°C for 10 min) were used as positive controls for activation of the alternative and classic pathways, respectively. C2-deficient human serum was used in some studies to determine whether alternative pathway activation was affected when the classic pathway was blocked. This is of importance since factor B can be involved in some circumstances by classic pathway activation (9).

RESULTS

In vitro interaction of complement and fungal substances. Equal volumes of normal serum and various dilutions of MF, spherulin, or 1% gelatin-Veronal buffer were mixed and then allowed to incubate at 37°C for 1 h, after which hemolytic activity was measured. CH_{50} levels of the serum mixtures which included the fungal substances were lower than the buffer controls. Furthermore, this decrease in activity was related to the concentration of either MF or spherulin as shown in Fig. 1. C4H was also found to decrease (Table 1). Although MF contains merthiolate and low-molecular-weight medium components, dialyzed MF used in other experiments also demonstrate decreased CH_{50} values (Table 1).

Similar mixtures of normal serum plus buffer, inulin (2.5 mg/ml), aggregated gamma globulin (1.0 mg/ml), MF (3.4 mg/ml), dialyzed MF, or spherulin (0.45 mg/ml) were subjected to immunoelectrophoresis with antisera against C3, C4, or factor B. Precipitin arcs, indicating complement component activation products similar to those seen when serum was activated by inulin or aggregated gamma globulin, were demonstrated for all the fungal substances tested against each of the three antisera. Mixtures with

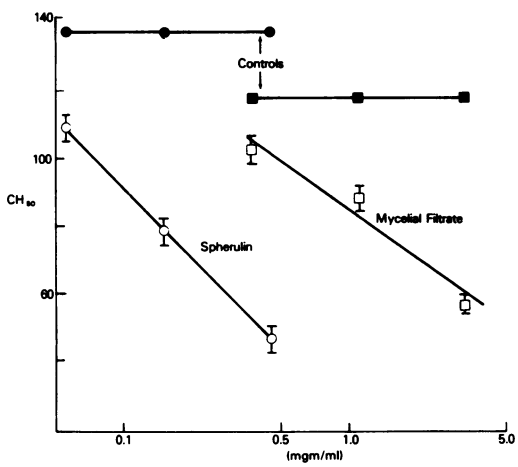

FIG. 1. *Effect of spherulin and MF at various concentrations on serum CH_{50} levels as compared with buffer controls. Symbols: ○, spherulin added; ●, buffer control for spherulin study; □, mycelial filtrate added; ■, buffer control for mycelial filtrate study.*

TABLE 1. *Effect of various coccidioidal extracts on CH_{50} and C4H after 1 h of incubation at 37°C*

Mixture	CH_{50} units/ml	C4H units/ml
Serum + buffer[a]	136	500,000
Serum + MF (2.75 mg/ml)[b]	NT[c]	258,000
Serum + MF (2.2 mg/ml)	69	NT
Serum + dialyzed MF[d]	92	NT
Serum + spherulin (0.46 mg/ml)	NT	200,000

[a] Equal parts serum + extract.
[b] Concentration of fungal substances in final volumes.
[c] NT, Not tested at this concentration.
[d] Retentate, undiluted.

C2-deficient instead of normal human serum against anti-factor B gave identical results. This indicates that alternative pathway activation is possible independent of classic pathway participation and that both pathways are being activated.

Complement measurements in infected patients. Serum was collected from 13 persons who had self-limited primary infection due to *C. immitis* and from 10 with dissemination, some with recent primary exposure, and others with a longer history of recurrent lesions. In these two groups, the CH_{50} was low in five and four persons, respectively. Although decreased hemolytic activity was found in some sera from either self-limited or disseminated patients, for both there was a temporal relationship between low values and recent onset of primary symptoms. As seen in Fig. 2, five samples collected within 9 weeks after initial symptoms were below the normal range and of these, four were very low (<10 CH_{50} units per ml). In contrast, 8 of the 11 persons tested between 9 and 18 weeks had normal levels. Furthermore, four of the five patients who were initially tested within 9 weeks after onset of symptoms were retested 10 months later, and these results are shown in Fig. 3. All rose markedly, although the patient who had disseminated disease remained below the normal range. This suggests that a congenital complement deficiency was not present in these patients.

No association was evident between the low CH_{50} levels and patient age, sex, race, signs of erythema nodosum (three persons, two with normal levels), or levels of complement-fixing antibody to coccidioidin.

To estimate alternative pathway function, the decrease in CH_{50} levels by inulin incubation was measured. Sera from seven patients with primary infection whose CH_{50}s were normal or slightly depressed (>85 CH_{50} units per ml) were incubated for 1 h with 1.0 mg of inulin per ml or an equal volume of buffer (four parts serum to one part additive), after which the CH_{50} levels were measured. For comparison, similar studies were performed with sera from 12 healthy persons. Inulin was significantly less effective in decreasing CH_{50} levels of infected patients as compared with controls (Table 2), suggesting a functional impairment of the alternative pathway.

Figure 4 shows values for several complement components assayed: hemolytic C4 activity as well as C3, C4, C5, factor B, and properdin by radial immunodiffusion assay. C4H activity paralleled CH_{50} levels in all but 1 of 15 sera tested. In contrast, components measured immunologically were generally within the normal range. One patient who had an unknown date of primary illness was found to have *C. immitis* ar-

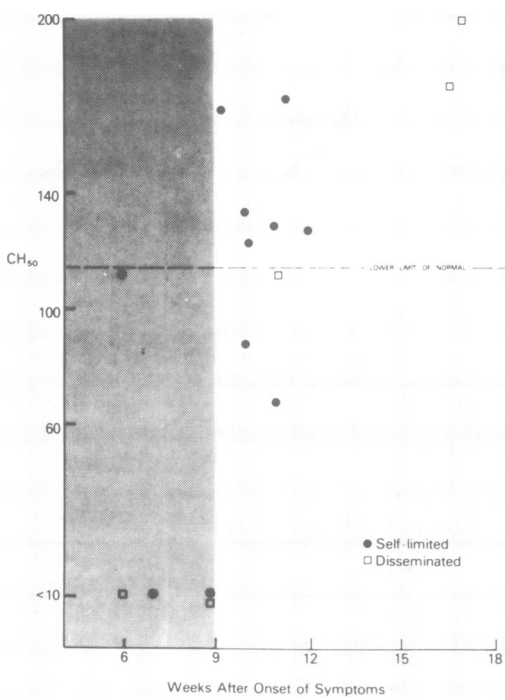

FIG. 2. *Relationship of CH_{50} levels and the time from onset of symptoms in patients with primary coccidioidol infections. Initial sera were collected from 16 persons within 18 weeks of onset of symptoms. Symbols: ●, patients with self-limited disease; □, patients with disseminated disease.*

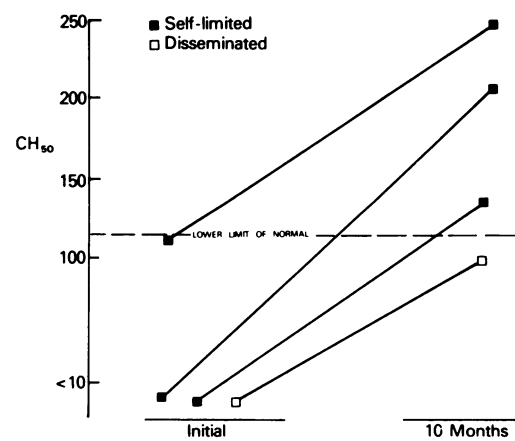

FIG. 3. *Change in CH_{50} levels in patients with primary coccidioidol infections between initial measurement and late convalescence. Symbols: ■, patients with self-limited disease; □, a patient with disseminated disease.*

thritis and then succumbed to a fulminat adult respiratory distress syndrome. Serum from this patient drawn shortly before her death yielded low CH_{50}, C3, and C4 levels with normal C5 and factor B. Two patients with primary self-limited disease had C3 levels below the normal range. In addition, three other patients, two with primary and one with chronic disseminated infection, had properdin levels below normal. In these five patients, the CH_{50} was normal.

DISCUSSION

The studies described in this report were initiated to pursue the suggestion from previous chemotaxis data that soluble substances derived from *C. immitis* could activate complement (5). Our data demonstrate that human complement activity drops in the presence of either MF or spherulin, and this effect is dependent on the incubation concentration of either fungal substance (Fig. 1). These findings are in agreement with the chemotactic response of human neutrophils, except with respect to the highest concentration of spherulin. Whereas a marked drop in chemotactic activity was observed with 0.92 mg of spherulin per ml in serum, no similar decrease in complement activation was observed in the current studies, suggesting that spherulin may have an inhibitory effect on the neutrophil function that is independent of complement interactions.

TABLE 2. *Decrease of CH_{50} in sera of coccidioidomycosis patients and controls by 1.0 mg of inulin per ml*

Patient (no. tested)	Initial CH_{50}[a] units/ml	Decrease with inulin[a]	
		Absolute (units/ml)	%[b]
Control (12)	143 ± 35[c]	66 ± 27 $P < 0.01$[d]	45 ± 13 $P < 0.04$
Infected (7)	100 ± 12	32 ± 6	32 ± 7

[a] After incubation at 37°C for 1 h.
[b] Percent = CH_{50} after inulin exposure/CH_{50} without inulin exposure × 100.
[c] Mean ± one standard deviation.
[d] P value by Student's t test (two tailed) between independent group means.

Our current findings further demonstrate that split products of components from both the classic and alternative pathways subsequently appear. The preparation of spherulin is free of preservatives and media components, and thus dialysis was unnecessary. Although our studies suggest that low-molecular-weight substances may account for some activity of MF (Table 1), dialyzed preparations decreased CH_{50} levels and qualitatively generated reaction products from all the complement components tested. Although classic pathway activation alone can involve factor B (9), our studies indicated activation by MF and spherulin of the alternative pathway in C2-deficient serum which is unable to support classic pathway activation. Complement activation by substances derived from microorganisms generally proceeds via the alternative pathway (2, 12, 18), although there is precedent for activation of both classical and alternative pathways (13). We cannot completely exclude the possibility that natural antibodies may be present in the donor's serum. However, complement-fixing antibodies to coccidioidin were not detected.

These in vitro observations prompted a search

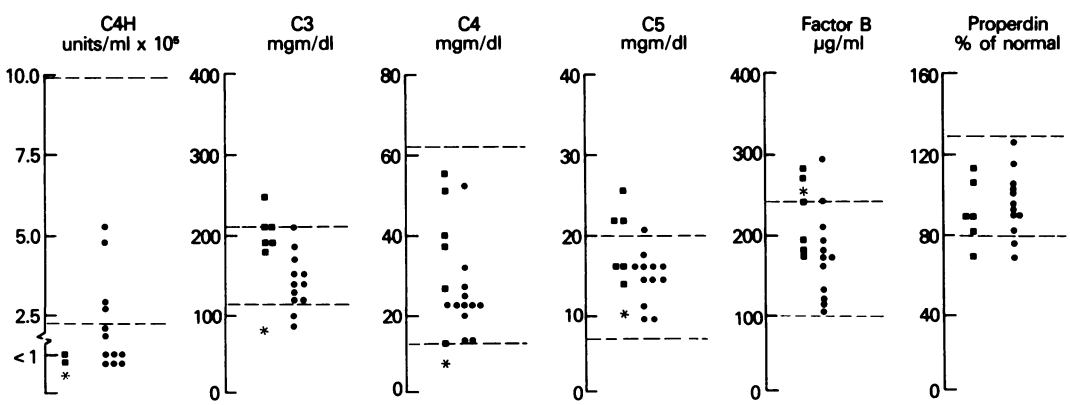

FIG. 4. *Complement component levels in serum from patients with coccidioidomycosis. Squares indicate patients with dissemination; circles indicate patients with self-limited infection; asterisk indicates patient with C. immitis arthritis described in text. Broken lines indicated the normal range (±2 standard deviations from the mean) for each assay.*

for perturbations of complement levels in patients infected with *C. immitis*. Of 23 patients, 9 showed low levels of CH_{50}, most often in the first 2 months of primary illness (Fig. 2). Convalescent sera tested in some patients demonstrated this depression to be transient. Hemolytic C4 levels paralleled the CH_{50} values in 14 of 15 sera studied. Furthermore, inulin was less effective in depleting sera taken from newly infected patients as compared to healthy persons, suggesting alternative pathway dysfunction (Table 2). From our data, a more precise statement concerning the nature of the properdin system abnormality cannot be made. It is possible that the decreased effect of inulin could be due to the somewhat lower CH_{50} values in the patient group (mean to 100 CH_{50} units per ml for those in the infected group as compared to 143 in the normals) or depletion of terminal components of complement. However, our findings are in keeping with the only study previously reporting complement levels in patients with coccidioidomycosis. Using a functional assay, Halde et al. reported decreased properdin activity in 10 of 19 patients with disseminated coccidioidomycosis (7).

Despite these abnormalities in hemolytic activities, depression of specific components of complement could not be clearly demonstrated by radial immunodiffusion techniques. Five isolated low levels of C3 or properdin were found. Additionally, in one patient who concurrently had fulminant respiratory failure, depressed CH_{50}, C3, and C4 with normal factor B indicated classic pathway activation. Generally, however, levels of components were within the normal range (Fig. 4) even when hemolytic activity was low. Since immunoassays measure hemolytically inactive reaction products of complement components as well as intact proteins, such discrepancies could arise from relatively slow clearance of the activation products from the circulation. This possibility has been suggested by Wilson et al. to account for normal immunoreactive levels of C3 in the face of low hemolytic C3 activity in patients with systemic lupus (17). However, with C4, agreement between immunological and hemolytic activities is expected in normal persons and in those with various diseases, implying that in vivo clearance of these fragments is rapid (19). Another explanation to account for these differences would be the decay of hemolytic activity in vitro between the time of venipuncture and performing the studies. Since particular attention was paid to careful handling of these specimens, such decay, if it occurred, was abnormally rapid. This occurrence would suggest the presence of activating factors in the sera such as cold-reacting immune complexes. The relevance of our findings to the possible role of complement in the pathogenesis of or host defenses against *C. immitis* infections may be clarified by further study of these possibilities.

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