Circulating Immune Complexes in Coccidioidomycosis

DETECTION AND CHARACTERIZATION

SADAYOSHI YOSHINOYA, REBECCA A. COX, and RICHARD M. POPE, *Department of Medicine, Division of Rheumatology, University of Texas Health Science Center at San Antonio, and Audie Murphy Veterans Administration Hospital, San Antonio, Texas, 78284; Department of Research Immunology, San Antonio State Chest Hospital, San Antonio, Texas 78223*

ABSTRACT Sera of 22 patients with active and 13 with inactive coccidioidomycosis, as well as 15 healthy subjects who were skin-test positive to coccidioidin and 39 healthy subjects who were coccidioidin skin-test negative, were assayed for immune complexes. Circulating immune complexes were measured by the C1q-binding assay, the C1q-solid phase assay, the monoclonal rheumatoid factor inhibition assay, and the monoclonal rheumatoid factor solid phase assay. An increased concentration of circulating immune complexes was detected in 73% of those with active disease by at least one assay compared with 13% of the healthy controls. Significantly increased levels of immune complexes were detected in sera of patients with active coccidioidomycosis by the C1q-binding assay ($P < 0.001$), the C1q-solid phase assay ($P < 0.001$), the monoclonal rheumatoid factor inhibition assay ($P < 0.005$), and the monoclonal rheumatoid solid phase assay ($P < 0.05$) compared with the results obtained in the 54 healthy subjects. In contrast, those with inactive disease did not show significantly increased concentrations of circulating immune complexes.

Sucrose density gradient ultracentrifugation of patients' sera established that the immune complexes were of intermediate size, sedimenting between the 6.6S and 19S markers. Immune complexes were shown to contain both coccidioidin antigen and anticoccidioidin antibody. In addition, a radioimmunoassay was developed to quantitate coccidioidin antigen-containing immune complexes. The latter assay proved highly sensitive in detecting immune complexes in patients with active coccidioidomycosis.

INTRODUCTION

Coccidioidomycosis is a fungal disease acquired by inhalation of mycelial-phase arthrospores of *Coccidioides immitis*. In the infected host, the arthrospores convert to endosporulating spherules. The disease presents a diverse clinical spectrum that includes inapparent infection, primary respiratory disease usually with uncomplicated resolution, chronic pulmonary disease either stabilized or progressive, and extrapulmonary dissemination either acute, chronic, or progressive. The degree of severity varies considerably within each category.

The role of humoral and cellular immune mechanisms in host resistance to coccidioidomycosis is not fully understood. However, the immunological profile of patients with various stages of this disease suggests that cellular immunity contributes to host defense, whereas humoral immunity does not. Typically, patients with chronic or progressive disease show depressed T cell responses to coccidioidin (CDN),[1] both in vivo (skin tests) and in vitro (lymphokine production and lymphocyte transformation), but have high levels of circulating complement-fixing (CF) anti-CDN antibody (1–5). Conversely, patients with mild disease and those in clinical remission have demonstrable cell-mediated immune responses to CDN and lack or have low titers of serum CF antibody. The inverse relationship between CF antibody and cellular immune reactivity to CDN implies that antibody is either inconsequential or perhaps detrimental to host defense in coccidioidomycosis. High levels of circulating CF antibody, in the absence of demonstrable circulating *C. immitis* anti-

This work was presented in part at the 79th Annual Meeting of the American Society for Microbiology, Los Angeles, Calif., May 1979.

Received for publication 25 January 1980 and in revised form 24 April 1980.

[1] *Abbreviations used in this paper*: BA, binding assay; BSA, bovine serum albumen; CDN, coccidioidin; CF, complement fixing; INH, inhibition; mRF, monoclonal rheumatoid factor; PEG, polyethylene glycol; PBS, phosphate-buffered saline; SP, solid phase.

gen, led us to explore the possibility that sera of patients with this disease might contain immune complexes. The present report details the results of these studies, indicating that the majority of patients with active disease possess immune complexes and that these complexes are composed of *C. immitis* antigen and specific antibody to this antigen.

METHODS

Study groups. 89 persons were divided into four groups. Group I consisted of 22 patients with active culture-proven coccidioidomycosis. This group was represented equally by chronic pulmonary and disseminated disease. Group II contained 13 patients who had been in clinical remission (culture negative) for 6 mo or longer. Group III contained 15 healthy persons who were skin-test positive (≥5 mm induration at 48 h) to CDN 1:100 (Cutter Laboratories, Inc., Berkeley, Calif.). Group IV consisted of 39 healthy CDN skin-test negative persons randomly selected from the staff at the San Antonio State Chest Hospital.

Blood samples for in vitro studies were routinely taken before skin tests or several weeks thereafter. Blood was allowed to clot at room temperature for 2 h. Sera were stored at −20°C until assayed.

Because sera of some patients had been stored for up to 4 yr, sera of healthy subjects (groups III and IV) that had been stored for a similar time period were included for study. All sera were coded and assayed in a double-blind manner.

Serum antibody determinations. Serum CF antibody titers were determined by the microtiter method (6) using commercial CDN (Microbiological Associates, Walkersville, Md.). Serum IgG levels were quantitated by radial immunodiffusion using a commercially available kit (Helena Laboratories, Beaumont, Tex.).

Immune complex determinations. Immune complexes were detected by four antigen-nonspecific radioimmunoassays employing both C1q and a monoclonal rheumatoid factor (mRF) as described previously (7). C1q was isolated by the affinity chromatography method of Kolb et al. (8). The mRF was isolated from the serum of a patient with Waldenström's macroglobulinemia by gel filtration over a Sephadex G-200 column at pH 3.5. The peak excluded from the beads was neutralized before use. Both C1q and mRF were pure when examined by immunoelectrophoresis and by double diffusion in agarose with monospecific antisera and an anti-whole human serum. Both preparations were radiolabeled by the iodine monochloride (9) or the lactoperoxidase (10) methods. Specific activity ranged from 0.12 to 0.50 μCi/μg.

Normal human IgG was isolated from Cohn Fraction II by DEAE and Sephadex G-200 column chromatography (7). Antibodies specific for the Fc portion of human IgG were prepared by immunization of rabbits with IgG. Hyperimmune sera were pooled and $F(ab')_2$ anti-human Fc antibodies isolated as already described (11). Briefly, the γ-globulin fraction was pepsin-digested and eluted over an affinity column prepared with human Fc. Adherent antibodies were recovered with an acidic buffer and, after neutralization, were further purified with IgM and IgA immunoadsorbant columns. The antibodies were specific for the Fc portion of IgG when examined by radioimmunoassay (11).

The C1q-binding assay (C1q-BA) was performed by the method of Zubler et al. (12), as previously used by us (7). Serum (0.05 ml) and 0.2 M EDTA, pH 7.4 (0.1 ml), were added to polypropylene tubes. After incubation for 30 min at 37°C, tubes were transferred to an ice bath. Radiolabeled C1q (0.05 ml at 0.05 μg/ml) and 1 ml of 3% (wt/vol) polyethylene glycol (PEG, 6,000 daltons) were then added. After a 1-h incubation in the ice bath, the percent of ^{125}I-C1q precipitated was measured. The total TCA-precipitable ^{125}I-C1q served as 100% binding.

The C1q-solid phase (C1q-SP) and mRF-SP assays were performed as previously described (7, 13). Briefly, C1q (0.5 ml at 5 μg/ml) or mRF (0.5 ml at 1 μg/ml) were incubated in polystyrene tubes (12 × 75 mm, Falcon No. 2054; Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) for 16 h at 4°C. Tubes were blocked with 1% (wt/vol) bovine serum albumen (BSA) in phosphate-buffered saline (PBS, 5 mM sodium phosphate, 0.15 M sodium chloride, pH 7.4). A test sample (0.025 ml) that had been preincubated for 30 min in 0.2 M EDTA, pH 7.4 (1:2, vol/vol), was added to the coated tubes. The final volume was adjusted to 0.5 ml with PBS-BSA. After incubation (1 h at 4°C), specific ^{125}I $F(ab')_2$ anti-human Fc was added (0.5 ml at 1 μg/ml) and incubated for 1 h. After washing, radioactivity was determined and the results were calculated as nanograms of anti-Fc bound.

The mRF-inhibition (mRF-INH) assay was performed as previously described (7, 14). Briefly, human IgG was coupled to paraazobenzamidoethyl Sepharose 4B. The ability of test sample to inhibit the binding of ^{125}I-mRF to this immunoadsorbant was used to measure immune complex activity. The results were calculated as the percent inhibition of ^{125}I-mRF binding.

All assays were capable of detecting heat-aggregated IgG and preformed immune complexes composed of human IgG, rabbit antihuman IgG or BSA, rabbit anti-BSA. The C1q-BA was capable of detecting as little as 2.5 μg heat-aggregated IgG (50 μg/ml), whereas the C1q-SP detected 0.08 μg (10 μg/ml), the mRF-SP 0.08 μg (10 μg/ml), and the mRF-INH 0.025 μg (2.5 μg/ml). Monomeric-pooled normal IgG did not interfere with assays that employed C1q. At high concentrations, monomeric IgG did interfere with the assays employing mRF. Nevertheless, heat-aggregated IgG was detected over 100 times more sensitively than the monomeric IgG as previously described by us (7) and others (14).

Characterization of immune complexes. The molecular weight distribution of immune complexes was assessed after sucrose gradient ultracentrifugation. Sera (0.2 ml) were applied to 5–20% linear sucrose density gradients (12 ml total vol) prepared in 0.2 M sodium borate, 0.15 M sodium chloride, pH 8.0 (borate buffer). The gradients were centrifuged at 35,000 rpm for 16 h at 4°C in a SW 41 Ti swinging-bucket rotor in a Beckman preparative ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) Gradients were harvested into 0.35-ml fractions through holes in the bottom of the tubes. The protein content of each fraction was quantitated by the Folin-Lowry method (15). Immune complex assays were performed on each fraction (0.075 ml) after the addition of normal human serum (0.025 ml diluted 1:6 in PBS). Normal human serum was added to the fractions to standardize the concentration of C1q because differences in C1q concentration across the gradient may lead to false positive results (16).

A series of experiments were performed to establish that immune complexes were comprised, at least in part, of *C. immitis* antigen and of specific antibody to this antigen. Soluble immune complexes were precipitated from serum or plasma using PEG (6,000 daltons) at a final concentration of 3% (wt/vol), according to the method of Creighton et al. (17). Parallel experiments established that CDN prepared as a toluene-induced lysate of *C. immitis* strain Silveira (Demosthenes Pappagianis, Davis, California) did not precipitate at this concentration of PEG.

For initial experiments, PEG-precipitated immune complexes were dissociated with 4 M urea and analyzed for

IgM, IgG, C1q, and CDN antigen and antibody by double diffusion in agarose. For chromatographic characterization, the precipitates were washed three times with 3% PEG, suspended in distilled water, and dialyzed against borate buffer, pH 8.0. The small amount of precipitate not resolubilized was removed by centrifugation at 1,500 *g* for 20 min. The samples were divided and one-half subjected to gel filtration (Sepharose 6B, 90 × 2.6 cm) in borate buffer and the other half to gel filtration in borate buffer containing 4 M urea. A normal control sample and one from a patient with a CF titer of 1:16, but without demonstrable immune complexes, were processed in an identical fashion and served as the controls. The effluents were monitored for protein by ultraviolet adsorption at 280 nm. Fractions were analyzed for CDN antibody by radioimmunoassay. For these studies, CDN (diluted 1:10,000) was incubated in polystyrene tubes overnight. After blocking with BSA, the column fractions were added and incubated for 1 h. After aspiration and washing, ^{125}I F(ab')$_2$ rabbit anti-human Fc was added. The IgG anti-CDN antibodies were recorded as nanograms anti-Fc bound per tube. All samples were run in duplicate.

Quantitation of antigen-specific immune complexes containing CDN antigen was performed by a radioimmunoassay adopted after the C1q-SP. Polystyrene tubes were coated with C1q and blocked with BSA. Serum samples diluted 1:60 were incubated in the tubes for 1 h. 0.5 ml of rabbit anti-CDN antiserum was added to each tube and incubated for 1 h. This antiserum, prepared as described below, was used at a final dilution of 1:20 (vol/vol). The tubes were aspirated and washed and a radiolabeled goat anti-rabbit IgG (Fc specific), at 1 μg/ml, was added for 1 h. After aspiration and washing, radioactivity was quantitated and the results were expressed as nanograms anti-rabbit Fc bound. Controls included CDN antigen in buffer, in normal human serum, and in human serum, which contained a CF antibody titer of 1:512.

The rabbit anti-CDN antibody employed in this study was obtained after serial immunizations of three rabbits with CDN in Freund's adjuvant at multiple sites. The sera were harvested after the third immunization. No reactivity against normal human sera was apparent by double diffusion in agarose, whereas several precipitin lines developed against the immunizing antigen. For the radioimmunoassay, the antiserum was adsorbed with an immunoadsorbant column prepared with C1q. After adsorption, rabbit antiserum (1:10) was mixed with an equal volume of normal human serum (diluted 1:10). Reference anti-CDN and reference CDN were generously donated by Dr. Milton Huppert, Veterans Administration Hospital, San Antonio, Tex.

Statistical analyses. Because of the heterogeneity of the variances, the data were analyzed by nonparametric methods (18). Differences between the four study groups were analyzed by the Mann-Whitney rank-sums test. Correlation analyses were performed using Spearman's rank correlation coefficient.

RESULTS

Immunological reactivity of study groups to CDN. The skin test reactivities to CDN and the serum CF antibody titers of subjects in groups I–IV are summarized in Table I. Group I consisted of 11 patients with nondisseminated pulmonary involvement and 11 with disseminated coccidioidomycosis. Six patients with pulmonary disease had CF titers of 1:8, three had titers of 1:16, and the remaining two had titers of 1:32 and 1:64. None of the 11 patients with pulmonary disease responded to skin testing with CDN at 1:100; only 3 patients reacted to the 1:10 dilution. 2 of the 11 patients were skin-test negative to a battery of skin-test antigens (trichophytin, *Candida*, streptokinase-streptodornase, and PPD) indicating a generalized anergy.

Serum CF titers were ≥1:32 in 9 of 11 patients with disseminated disease. The remaining two patients had serum CF titers of 1:8. Four patients were skin-test reactive to CDN at 1:100 and two additional patients responded to the 1:10 dilution. All but one showed skin-test reactivity to one or more recall antigens.

TABLE I
Immunological Responses of Groups I through IV to CDN

Group	Clinical status	Mean serum CF titers	Skin-test responses to CDN*
			No. positive/No. tested
I	Active pulmonary disease	1:16 (8–64)‡	3/11 (0–32)§
	Active disseminated disease	1:256 (8–1,024)	6/11 (0–21)
II	Inactive disease	1:4 (0–16)	9/13 (0–40)
III	Healthy, CDN-reactive	<1:2 (0–8)	15/15 (10–53)
IV	Healthy, CDN-nonreactive	0	0/39

* Indicates results obtained with CDN 1:100 and/or CDN 1:10.
‡ Indicates range of reciprocal titer.
§ Indicates range of induration (millimeters diameter) responses at 48 h.

TABLE II

Mean Values for Immune Complexes for Each Patient Group Studied

Group	Clq-BA	Clq-SP	mRF-SP	mRF-INH
	% binding	*ng anti-Fc*	*ng anti-Fc*	*% inhibition*
I (22)*	5.00±0.68‡ (<0.002)§ (<0.001)‖	34.5±4.69 (<0.02) (<0.001)	41.3±3.51 (NS) (<0.05)	8.54±1.29 (NS) (<0.005)
II (13)	2.64±0.32 (NS)¶	21.3±3.63 (NS)	37.9±4.10 (NS)	5.90±1.40 (NS)
III (15)	2.16±0.13	15.1±1.86	27.31±4.09	4.67±1.43
IV (39)	2.47±0.16	17.9±1.37	34.0±1.91	4.53±0.62

* Numbers in parentheses represent the numbers of sera examined in each group.
‡ Mean value±SE.
§ *P* value obtained by statistical comparison of group I and II. NS, not significant.
‖ *P* value obtained by statistical comparison of group I vs. III and IV combined.
¶ *P* value obtained by statistical comparison of group II vs. III and IV combined.

Seven patients in clinical remission had a CF titer of 1:8; one had a titer of 1:16, and the remaining five patients were CF negative. 9 of 13 patients in remission showed skin-test reactivity to the 1:100 dilution of CDN and 4 others responded to the 1:10 dilution. With one exception, subjects in groups III (healthy, CDN reactive) and IV (healthy, CDN nonreactive) were negative for serum CF titers.

Mean IgG serum levels were: 1,512 mg/100 ml in group I, 1,170 mg/100 ml in group II, 1,016 mg/100 ml in group III, and 1,040 mg/100 ml in group IV. Serum IgG levels correlated ($P < 0.01$) with serum CF antibody titers to CDN in groups I and II.

Immune complex levels. The results obtained with the antigen-nonspecific immune complex assays for subjects in groups I–IV are summarized in Table II. The mean values for patients with active disease (group I) were significantly greater than the values obtained for healthy subjects (groups III and IV combined) by the C1q-BA and C1q-SP ($P < 0.001$), the mRF-INH ($P < 0.005$), and the mRF-SP ($P < 0.05$) assays. There were no differences in immune complexes by any assay between patients with pulmonary and disseminated disease. However, immune complex levels were significantly greater in those with active disease (group I) compared with those in clinical remission (group II) by the C1q-BA ($P < 0.002$) and the C1q-SP ($P < 0.02$). No differences were detected between patients with inactive disease and healthy subjects.

Fig. 1 depicts the individual results of the immune complex assays obtained with the sera of the patients with active pulmonary and disseminated disease. Results are expressed as an immune complex index derived by dividing each patient mean by the normal control mean (group III plus IV) for that assay (7). The boxes encompass the normal mean±2 SD for each assay. Sera from 10 of 22 (45%) patients with active disease were elevated by the C1q-BA, whereas 7 of 22 (32%) were elevated by the C1q-SP. Values ranged up to seven times the normal control mean by these assays. Assays employing mRF were less sensitive in detecting immune complexes in these patients. 4 of 22 (18%)

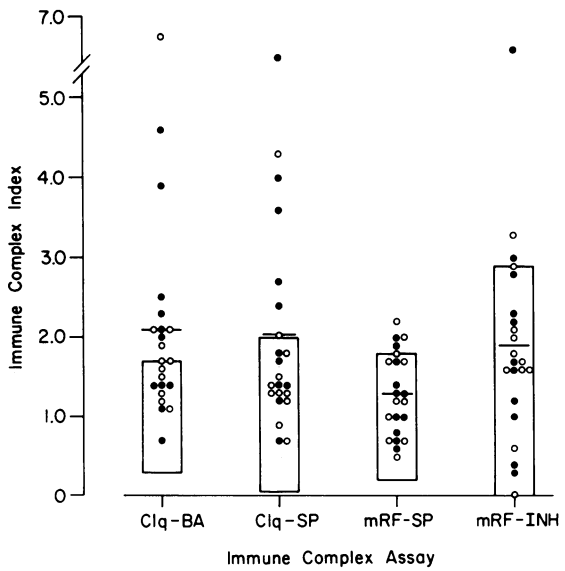

FIGURE 1 Individual results for four immune complex assays performed on sera of 22 patients with either pulmonary or disseminated coccidioidomycosis. The immune complex index was obtained by dividing each patient mean by the normal mean for that assay. ●, patients with pulmonary disease; ○, patients with disseminated disease. The normal mean ±2 SD is encompassed by the open rectangles. The patient mean for each assay is represented by the horizontal bar.

sera demonstrated elevated values for immune complexes by the mRF-SP, and 3 of 22 (14%) sera by the mRF-INH. Of the 13 patients with inactive disease, 2 (15%) were elevated by the C1q-SP, mRF-SP, and mRF-INH, whereas only 1 (8%) was elevated by the C1q-BA.

Of the 22 patients with active disease, 16 (73%) were positive for immune complexes by at least one assay, 5 (23%) by two assays, and 2 (9%) by three assays. Of the 13 patients with inactive disease, 4 (31%) were positive by at least one immune complex assay, whereas 2 (15%) were positive by two, and 1 was positive by three assays. Seven (13%) of the healthy controls were positive for immune complexes by at least one assay and two (4%) were positive by two assays. Although these two individuals were without symptomatology, the results of the immune complex assays on their sera were repeatedly positive, even with freshly obtained samples.

The correlation between immune complex values obtained by the different methods was examined. For those with active disease, the C1q-BA and C1q-SP correlated, $P < 0.01$. No correlation existed between the assays employing C1q and mRF for those with active disease alone. When comparisons were made with patients with both active and inactive disease, correlations were observed between the C1q-BA and C1q-SP (Fig. 2A, $r = 0.63$, $P < 0.001$), the C1q-BA and the mRF-INH (Fig. 2B, $r = 0.53$, $P < 0.001$), the C1q-SP and the mRF-INH ($r = 0.49$, $P < 0.01$), and the C1q-SP and the mRF-SP assays ($r = 0.46$, $P < 0.01$).

The relationships between serum immune complex levels, IgG concentrations, and CF antibody levels were examined. No correlation existed between the CF titer or IgG concentration for any immune complex assay in patients with active disease alone. However, when patients with active and inactive disease were grouped together, both the IgG and serum CF levels correlated with immune complexes detected by the C1q-BA ($r = 0.49$, $P < 0.01$ and $r = 0.62$, $P < 0.001$, respectively) and by the C1q-SP assay ($r = 0.48$, $P < 0.01$ and $r = 0.42$, $P < 0.05$). No correlations were apparent when complexes were detected by the mRF assays except between mRF-INH and IgG concentration ($r = 0.54$, $P < 0.001$).

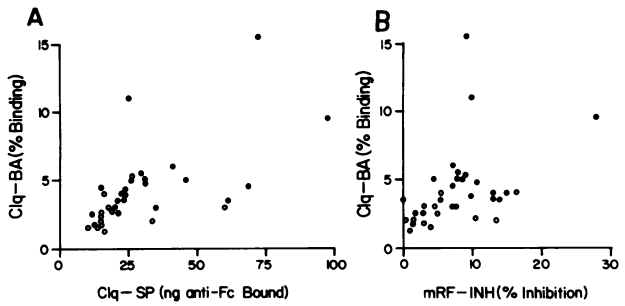

FIGURE 2 Scattergram demonstrating the relationships between the results obtained in the C1q-BA and the C1q-SP assay (Fig. 2A) and in the C1q-BA and the mRF-INH assay (Fig. 2B) in patients with active coccidioidomycosis (closed circles) and inactive coccidioidomycosis (open circles).

Characterization of immune complexes. Sucrose density gradient ultracentrifugation was employed to examine the size distribution of immune complexes detected in the sera of patients with coccidioidomycosis. The results of C1q-BA and mRF-SP assays on fractions obtained by sucrose density gradient ultracentrifugation are depicted in Figs. 3 and 4. Immune complexes migrated primarily as intermediate-sized complexes sedimenting between the 6.6S and the 19S markers. For comparison, the results obtained with a normal human serum are shown for each assay.

To more fully define the specificity of the immune complexes detected in our patients, three lines of investigation were employed to characterize their antigen and antibody composition. First, immune com-

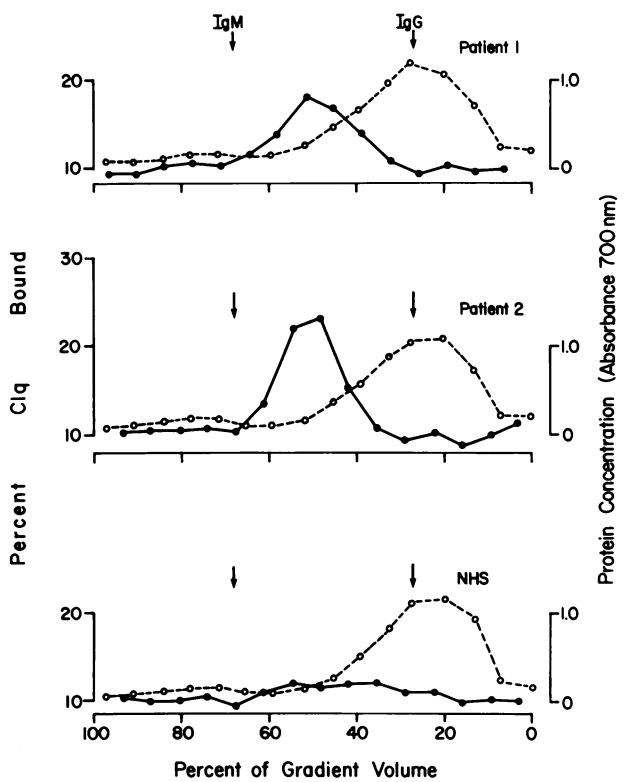

FIGURE 3 Distribution of immune complexes detected by the C1q-BA and of the total protein after preparative ultracentrifugation of sera from two patients and one healthy donor. The gradients were prepared with 5–20% sucrose. 100% of gradient volume represents the bottom and 0% the top of the gradient. The positions of 19S IgM and 6.6S IgG are indicated at the top of the figure. See Methods for further details. NHS, normal human serum.

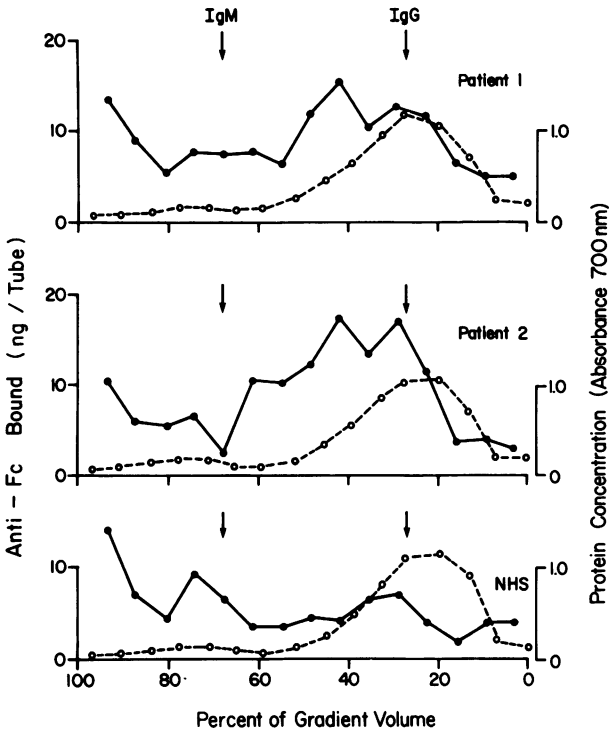

FIGURE 4 Distribution of immune complexes detected by the mRF-SP assay and of the total protein after preparative ultracentrifugation of sera from two patients and one healthy donor. See Fig. 3. NHS, normal human serum.

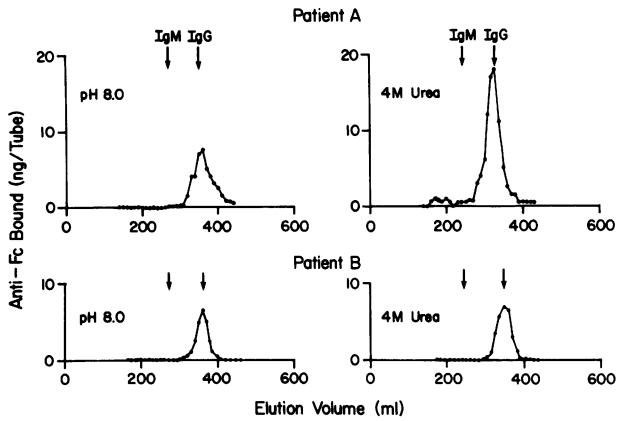

FIGURE 5 Distribution of free IgG anti-CDN after gel filtration over a Sepharose 6B column in borate buffer alone (left) and in borate buffer containing 4 M urea (right). The 4 M urea containing fractions were dialyzed against borate buffer before analysis. The elution volumes and the position of the molecular weight markers are indicated at the bottom and top of the figure. Patient A with active disease had circulating immune complexes and a CF titer to CDN. Patient B with inactive disease was negative for immune complexes but had a CF titer to CDN.

plexes were precipitated from sera using PEG. The precipitated complexes were dissociated with 4 M urea and examined for CDN antigen and antibody by double diffusion in agarose (not shown). CDN antigen was demonstrable only after precipitated complexes were dissociated with 4 M urea, indicating that antigen was bound to antibody. Anti-CDN antibody was demonstrable both before and after dissociation of complexes. IgG, IgM, and C1q were also demonstrable in the PEG precipitates before and after dissociation. Control sera, processed in an identical fashion, failed to reveal specific antigen or antibody; high concentrations of CDN antigen alone did not precipitate with PEG.

Next, studies were performed to establish that the anti-CDN antibody demonstrable in PEG precipitates before and after dissociation with 4 M urea was involved in immune complex formation. PEG precipitates from patients were subjected to Sepharose 6B column chromatography in borate buffer and in borate buffer containing 4 M urea. The fractions were examined for free anti-CDN antibody, employing a radioimmunoassay to detect IgG antibody to CDN (Fig. 5). The PEG precipitates from a patient with active disease and circulating immune complexes (patient A) demonstrated a peak of free IgG antibody that eluted in the 6.6S IgG region when run in borate buffer without 4 M urea. This was not unexpected because some IgG was precipitated from the controls by PEG under the same conditions. After elution of the PEG precipitate in borate buffer containing 4 M urea, a marked increase of free IgG antibody to CDN was observed. This indicated that the IgG antibody to CDN was involved in immune complex formation and that the complexes were dissociated in 4 M urea. The PEG precipitate of a patient with a CF titer of 1:16 to CDN, but without demonstrable circulating immune complexes (patient B), demonstrated similar-sized peaks both in borate buffer alone and in borate buffer containing 4 M urea. Thus, 4 M urea alone did not effect an increase in anti-CDN antibody in the absence of immune complexes. A normal control, run under identical conditions, showed no antibody to CDN either in borate alone or in borate buffer containing 4 M urea.

As a final proof that CDN antigen was present in immune complexes, an antigen-specific immune complex radioimmunoassay was developed using a method similar to the C1q-SP. Immune complexes were bound to a C1q-SP. Those that contained CDN antigen were detected by a specific rabbit anti-serum to CDN. Bound rabbit antibody was quantitated by a specific ^{125}I-goat anti-rabbit IgG (Fc specific). There was a significant difference ($P < 0.001$) by the antigen-specific C1q-SP between the patients with active disease and the controls (Table III). 8 of 13 patients (62%) with active coccidioidomycosis were positive for CDN-specific immune complexes. 3 of these 13 patients (23%) had circulating IgG immune complexes by the C1q-SP antigen-nonspecific assay. Of interest, 1 of the 13 healthy subjects (groups III and IV), who was positive for immune

TABLE III
Antigen-specific and Antigen-nonspecific Immune Complex Detection by the C1q-SP

	C1q-SP (antigen)	C1q-SP (IgG)
	ng anti-rabbit Fc	*ng anti-human Fc*
Group I (13)*	4.23±0.69‡ (62%)§ [$P < 0.001$]‖	26.93±2.67 (23%) [$P < 0.002$]
Group III & IV (13)	0.68±0.28 (0%)	17.54±3.22 (8%)
Control¶		
CDN in buffer	0.00	—
CDN in NHS**	0.60	—
CDN in patient serum	63.6	—

* Number of sera examined.
‡ Mean±SE.
§ Frequency of values greater than the normal mean±2 SD.
‖ *P* values obtained by statistical comparison of group I vs. III plus IV.
¶ CDN was diluted 1:100. The patient's serum had a CF titer of 1:512 to CDN.
** NHS, normal human serum.

complexes by the C1q-SP antigen-nonspecific assay, was negative by the C1q-SP CDN antigen-specific assay. CDN antigen in buffer or added to normal human serum was not detected by the antigen-specific method, whereas addition of the same quantity of CDN antigen to a patient's serum containing high levels of serum CF antibody was strongly positive (Table III).

DISCUSSION

The present study established that circulating immune complexes were present in the majority of patients with active coccidioidomycosis. A variety of antigen-nonspecific techniques were employed because no single assay is capable of detecting all types of immune complexes. For example, the assays employing C1q are capable of detecting only complement-fixing complexes composed either of IgG or IgM (C1q-BA) or IgG only (C1q-SP). The mRF assays may detect both complement-fixing and noncomplement fixing immune complexes of the IgG class (7). In the present study, both the frequency and degree of elevated immune complexes was highest with the C1q-BA and C1q-SP, followed by the mRF-SP and mRF-INH assays. Differences in sensitivity of these assays may be expected depending on antigenic valences, antibody class and subclass, antibody avidity, size of immune complexes, and antigen-antibody ratio (7, 20, 21). It has been previously documented that the immune complexes of one disorder may be more sensitively detected by one method than another. For example, assays using C1q are extremely sensitive in systemic lupus erythematosus, whereas those using mRF are not (7, 12–14, 20). A similar observation has been made in patients with acute rheumatic fever (7). On the other hand, the immune complexes present in rheumatoid arthritis are readily detected by assays using either mRF or C1q (7, 14, 20). Using four antigen-nonspecific radioimmunoassays for immune complexes, 73% of patients with active coccidioidomycosis had circulating immune complexes detected by at least one radioimmunoassay. The frequency of elevated immune complexes within the four assays ranged from 14% (mRF-INH) to 45% (C1q-BA), thus emphasizing the need for multiple assay methods.

To confirm that the antigen-nonspecific techniques were truly detecting immune complexes, sera were subjected to sucrose density gradient ultracentrifugation. The complexes detected sediment primarily in a range intermediate between 19 and 6.6S. The relatively small size of the immune complexes was not unexpected because these patients were known to possess an excess of free circulating antibody.

A serious criticism of the antigen-nonspecific assays is the inability to definitely relate the presence of the complexes to the specific disease process. The results of the analyses of the PEG precipitates by double diffusion in agarose demonstrated both *Coccidioides* antigen and antibody. The antigen detected in the PEG precipitates was not the result of precipitation of unbound antigen because it was not demonstrable until the precipitate was dissociated with 4 M urea. Although these results were useful in establishing the presence of the specific reactants, this method lacked sensitivity and was not quantitative. Therefore, an antigen-specific radioimmunoassay was developed to specifically detect and quantitate antigen involved in immune complex formation. The binding of patients' sera was most likely through the Fc portion of specific antibody bound to antigen. Neither CDN alone nor CDN in normal serum bound to the C1q-coated tubes. This assay proved superior for detection of immune complexes in patients with coccidioidomycosis. The sensitivity was increased from 23% for the antigen-nonspecific to 62% for the antigen-specific assay. None of the 13 control sera studied possessed a significant elevation of immune complexes by the antigen-specific method, including 1 control patient with a repeatedly increased value by the antigen-nonspecific method. Therefore, this method is particularly useful because it not only specifically detects antigen that has formed immune complexes but also may be readily quantitated.

A question not resolved by this study relates to the nature of the antigen(s) involved in immune complex formation. The correlation between immune complex levels and serum CF antibody titers to CDN suggests that it may be the antigen that binds the CF antibody that comprises immune complexes. Additional studies will be needed to confirm this hypothesis. In regard to

antigen-antibody ratios, the data suggest that immune complexes are formed in antibody excess. Antibody was readily detected in PEG precipitates before dissociation with 4 M urea; antigen was detected only after dissociation. Secondly, high titers of circulating antibodies to *C. immitis* are demonstrable in sera of patients with chronic or progressive coccidioidomycosis; free circulating antigen has not been detected. That antigenic determinants are available for binding in immune complexes is evidenced by the ability to detect antigen in immune complexes adsorbed onto C1q-coated tubes without prior dissociation of complexes. The antigenic determinants may be more easily detected in this assay because excess unbound antibody is removed before the addition of rabbit anti-CDN antibody. Under these conditions an equilibrium is developed that aids the binding of rabbit antibodies.

The presence of circulating immune complexes in collagen vascular diseases, cancer, and various infectious processes has been widely documented (2). However, the demonstration of immune complexes in fungal diseases has been limited. Geha (22) reported C1q precipitins in the serum of a 13-yr-old child during acute bronchopulmonary aspergillosis. This study did not establish the presence of *Aspergillus* antigen. More recently, Bullock et al. (23) reported immune complexes in the serum of a patient with disseminated histoplasmosis who had mesangiopathic glomerulonephritis, presumably secondary to histoplasmosis. Complexes were detected by C1q-SP and a Raji cell radioimmunoassay. In addition, cell-bound IgA, IgM, and C3 were demonstrable within glomeruli by direct immunofluorescence. Of particular interest, immune complexes were undetectable after the patient's clinical status improved. *Histoplasma* antigen(s) was not demonstrable in the complexes. That circulating immune complexes exist in other fungal diseases has been suggested in a report of patients with candidiasis (24). The present report is the first among those in mycotic diseases to demonstrate that immune complexes are comprised of fungal antigen and specific antibody.

The significance of immune complexes in coccidioidomycosis is not yet known. There was a strong correlation between immune complex levels detected in the C1q antigen-nonspecific assays and CF antibody titers to CDN. Previous studies have established that CF antibody is of the IgG class (25–28). The correlation between immune complex levels and serum CF antibody titers suggests that the presence of immune complexes correlates with disease severity, as does the CF antibody titer. Consistent with this, immune complexes were significantly greater in patients with active disease compared with those in clinical remission. However, there was no difference in the results of the antigen-nonspecific assays in patients with active pulmonary vs. disseminated disease, either in the frequency of detection or in the level of immune complexes. These results indicate that the presence of increased levels of immune complexes reflects disease activity, but does not distinguish pulmonary from extrapulmonary involvement.

The role, if any, of circulating *Coccidioides* antigen-antibody complexes in the pathogenesis of coccidioidomycosis is not known. Both the composition and size of circulating immune complexes affect their clearance from the circulation. Large immune complexes (>19S) are rapidly cleared from circulation by the mononuclear cell phagocytic system (29). Immune complexes of intermediate size (11–19S), such as those demonstrated in the present study, may persist in the circulation and perhaps could be deposited in blood vessels and renal glomeruli (30). This possibility has not been examined in coccidioidomycosis; however, there are no reports in the literature to suggest immune complex-mediated vasculitis or glomerulonephritis.

A more likely role of immune complexes in this disease relates to their potential immunosuppressive effects. Immune complexes have been shown to depress T cell-mediated delayed-type hypersensitivity responses (31), inhibit antibody-dependent cell-mediated cytotoxicity (32), and suppress the chemotactic response of polymorphonuclear neutrophils (33). The temporal relationship between depressed T cell function and chronic or progressive coccidioidomycosis is well documented (3–5). This, coupled with the relationship between CF antibody titers, immune complexes, and disease severity, suggests that antibody, either alone or complexed with antigen, may have an adverse effect upon the course of this disease, possibly by having a negative feedback upon T cell function.

In this regard, we have found that the lymphocyte transformation responses of patients with active coccidioidomycosis are significantly increased when their lymphocytes are cultured in serum of healthy donors as opposed to autologous serum (34). Conversely, sera of patients suppressed transformation responses of healthy CDN-reactive persons. Augmentation of patient responses in healthy donor serum and suppression of healthy donor responses in patient sera was specific for *Coccidioides* antigens because responses to mitogens and *Candida* antigen were unaffected by the source of serum. Adsorption of patients' sera with *Staphylococcus* protein A which binds the Fc portion of IgG (33) abrogated the suppressor activity. These results are consistent with, but do not distinguish between, antibody-mediated and immune complex-mediated suppression of transformation responses. Nevertheless, these data provide preliminary evidence that T cell anergy in coccidioidomycosis may be attributed, in part, to antibody and/or antigen-antibody complexes. Additional studies are in progress to elucidate the suppressive effects of antibody and immune complexes in this disease.

ACKNOWLEDGMENTS

This work was supported in part by grants AI-13573, AI-16095, and AM-25530 from the National Institutes of Health, by an Arthritis Clinical Research Center grant from the Arthritis Foundation, and by grants from the South Central Texas Chapter of the Arthritis Foundation, the Ruth and Vernon Taylor Foundation, Denver, Colo., and the Morrison Trust, San Antonio, Tex.

REFERENCES

1. Smith, C. E., R. R. Beard, H. G. Rosenberg, and E. C. Whiting. 1946. Varieties of coccidioidal infection in relation to epideminology and control of the disease. *Am. J. Public Health.* **36:** 1394–1402.
2. Smith, C. E., M. T. Saito, and S. A. Simons. 1956. Pattern of 39,500 serologic tests in coccidioidomycosis. *JAMA (J. Am. Med. Assoc.).* **160:** 546–552.
3. Smith, C. E., E. G. Whiting, E. E. Baker, H. G. Rosenberg, R. R. Beard, and M. T. Saito. 1948. The use of coccidioidin. *Am. Rev. Tuberc.* **57:** 330–360.
4. Catanzaro, A., L. Spitler, and K. M. Moser. 1975. Cellular immune response in coccidioidomycosis. *Cell. Immunol.* **15:** 360–371.
5. Cox, R. A., and J. R. Vivas. 1977. Spectrum of *in vivo* and *in vitro* cell-mediated immune responses in coccidioidomycosis. *Cell. Immunol.* **31:** 130–141.
6. Kaufman, L., E. C. Hall, M. J. Clark, and D. McLaughlin. 1970. Comparison of macrocomplement and microcomplement fixation techniques used in fungus serology. *Appl. Microbiol.* **20:** 579–582.
7. Yoshinoya, S., and R. M. Pope. 1980. Detection of immune complexes in acute rheumatic fever and their relationship to HLA-B5. *J. Clin. Invest.* **65:** 136–145.
8. Kolb, W. P., L. M. Kolb, and E. R. Podack. 1979. C1q: isolation from human serum in high yield by affinity chromatography and development of a highly sensitive hemolytic assay. *J. Immunol.* **122:** 2103–2112.
9. Nisonoff, A., F. C. Wissler, L. N. Lipman, and D. L. Woernely. 1960. Separation of univalent fragments from the bivalent rabbit antibody molecule by reduction of disulfide bonds. *Arch. Biochem. Biophys.* **89:** 230–244.
10. Heusser, C., M. Boesman, J. H. Nardin, and H. Islikes. 1973. Effect of chemical and enzymatic radioiodination on *in vitro* human C1q activities. *J. Immunol.* **110:** 820–828.
11. Pope, R. M., and S. J. McDuffy. 1979. IgG rheumatoid factor: relationship to seropositive rheumatoid arthritis and absence in seronegative disorders. *Arthritis Rheum.* **22:** 988–998.
12. Zubler, R. H., C. Lange, P. H. Lambert, and P. A. Miescher. 1976. Detection of immune complexes in unheated sera by modified ^{125}I-C1q binding test. Effect of heating on the binding of C1q by immune complexes and application of the test to systemic lupus erythematosus. *J. Immunol.* **116:** 232–235.
13. Hay, F. C., L. J. Nineham, and I. M. Roitt. 1976. Routine assay for the detection of immune complexes of known immunoglobulin class using solid phase C1q. *Clin. Exp. Immunol.* **24:** 393–400.
14. Gabriel, A., Jr., and V. Agnello. 1977. Detection of immune complexes. The use of radioimmunoassays with C1q and monoclonal rheumatoid factor. *J. Clin. Invest.* **59:** 990–1001.
15. Lowry, O. H., W. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin-phenol reagent. *J. Biol. Chem.* **193:** 265–275.
16. Soltis, R. D., M. J. Morres, D. E. Hasz, and I. D. Walson. 1979. The influence of endogenous C1q on the ^{125}I-C1q binding test. *J. Lab. Clin. Med.* **93:** 238–245.
17. Creighton, D. W., P. H. Lambert, and P. A. Miescher. 1973. Detection of antibodies and soluble antigen-antibody complexes by precipitation with polyethylene glycol. *J. Immunol.* **111:** 1219–1227.
18. Freund, J. E. 1967. *In* Modern Elementary Statistics. Prentice-Hall, Inc., Englewood Cliffs, N. J. 316–366.
19. Benveniste, J., and C. Bruneau. 1979. Detection and characterization of circulating immune complexes by ultracentrifugation. Technical aspects. *J. Immunol. Methods.* **26:** 99–112.
20. Zubler, R. H., and P. H. Lambert. 1978. Detection of immune complexes in human diseases. *Prog. Allergy.* **24:** 1–48.
21. Lurhuma, A. Z., C. L.Cambiaso, P. L. Masson, and J. R. Heremans. 1976. Detection of circulating antigen-antibody complexes by their inhibitory effect on the agglutination of IgG-coated particles by rheumatoid factor or C1q. *Clin. Exp. Immunol.* **25:** 212–226.
22. Geha, R. A. 1977. Circulating immune complexes and activation of the complement sequence in acute allergic bronchopulmonary aspergillosis. *J. Allergy Clin. Immunol.* **60:** 357–359.
23. Bullock, W. E., R. P. Artz, D. Bhathena, and K. S. K. Tung. 1979. Histoplasmosis: association with circulating immune complexes, eosinophilia, and mesangiopathic glomerulonephritis. *Arch. Intern. Med.* **139:** 700–702.
24. Weiner, M. H., and W. J. Yount. 1976. Mannan antigenemia in the diagnosis of invasive *Candida* infections. *J. Clin. Invest.* **58:** 1045–1053.
25. Sawaki, Y., M. Huppert, J. W. Bailey, and Y. Yagi. 1966. Patterns of human antibody reactions in coccidioidomycosis. *J. Bacteriol.* **91:** 422–427.
26. Pappagianis, D., N. Lindsey, C. E. Smith, and M. T. Saito. 1965. Antibodies in human coccidioidomycosis: immunoelectrophoretic properties. *Proc. Soc. Exp. Biol. Med.* **118:** 118–122.
27. Pappagianis, D., M. Saito, and K. H. V. Hoosear. 1972. Antibody in cerebrospinal fluids in non-meningitic coccidioidomycosis. *Sabouraudia.* **10:** 173–179.
28. Cox, R. A., and D. R. Arnold. 1979. Immunoglobulin E in coccidioidomycosis. *J. Immunol.* **123:** 194–200.
29. Mannik, M. W., P. Arend, A. P. Hall, and B. C. Gilliland. 1971. Studies on antigen-antibody complexes. I. Elimination of soluble complexes from rabbit circulation. *J. Exp. Med.* **133:** 713–739.
30. Haakenstad, A. O., and M. Mannik. 1977. The biology of immune complexes. *In* Autoimmunity. N. Talal, editor. Academic Press, Inc., New York. **11:** 278–360.
31. Rowley, D. A., F. W. Fitch, E. P. Stuart, H. Kohler, and H. Consenze. 1973. Specific suppression of immune responses. *Science (Wash. D. C.).* **181:** 1133–1141.
32. Pape, G. R., L. Moretta, M. Troye, and P. Perlmann. 1979. Natural cytotoxicity of human Fc-receptor-positive T lymphocytes after surface modulation with immune complexes. *Scand. J. Immunol.* **9:** 291–296.
33. Leung-Tack, J., J. Maillard, and G. A. Voisin. 1979. Chemotaxis inhibition induced in polymorphonuclear neutrophils by soluble immune complexes. *Int. Arch. Allergy Appl. Immunol.* **58:** 365–374.
34. Cox, R. A., R. Pope, and S. Yoshinoya. 1979. *American Society of Microbiology Abstracts.*
35. Kessler, S. W. 1975. Rapid isolation of antigens from cells with a *Staphylococcal* protein A antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. *J. Immunol.* **115:** 1617–1624.