Patterns of Human Antibody Reactions in Coccidioidomycosis¹

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ABSTRACT

SAWAKI, Y. (Veterans Administration Hospital, San Fernando, Calif.), M. HUPPERT, J. W. BAILEY, AND Y. YAGI. Patterns of human antibody reactions in coccidioidomycosis. *J. Bacteriol.* **91**:422-427. 1966.—At least two antigen-antibody systems in coccidioidomycosis have been demonstrated by immunodiffusion and immunoelectrophoresis, including the use of I¹³¹-coccidioidin. Sera which were positive in the conventional complement-fixation (CF) test, but negative in the tube precipitin (TP) test, yielded only one line of precipitate in the immunodiffusion test. In immunoelectrophoresis, also, there was only one precipitate arc which appeared in the γ_2 -globulin region. On the other hand, sera which were positive in both CF and TP tests yielded two different lines in the immunodiffusion test. In immunoelectrophoresis, also, there were two precipitate arcs: one in the γ_2 -globulin region and the other in the γ_1 -globulin area. Radioimmunoelectrophoresis demonstrated that the antibody activity in IG-G globulin, roughly parallels the CF titer. A similar correlation existed between the IG-M antibody activity and the TP reactivity. Antibody activity was present in the IG-A globulin but it did not reflect either CF or TP activity.

Serological studies are important diagnostic and prognostic aids in coccidioidomycosis. Currently, the tube precipitin (TP) test and the complement-fixation (CF) test are employed, and these are highly specific. Smith and his colleagues have demonstrated a relationship between the clinical stages and serological patterns (7, 8). Resolution of the various antigen-antibody systems involved in coccidioidomycosis was facilitated with the application of immunodiffusion techniques. The results suggested that this procedure is useful as a screening test for the disease (3). Huppert and Bailey (4) demonstrated the successful separation of the antigens involved in the CF and TP test for coccidioidomycosis. These two antigen-antibody systems were detected as distinctly different in the agar-gel immunodiffusion test.

The work reported here is concerned with the identification, by means of immunoelectrophore-

sis and radioimmunoelectrophoresis, of antibody components in patients' sera which are involved in these reactions.

MATERIALS AND METHODS

Human sera. Blood specimens were obtained from fasted patients with a confirmed diagnosis of coccidioidomycosis. After separation of the serum, aqueous Merthiolate was added to a final concentration of 0.01%, and the sera were stored at 5 C until used.

Antisera against human serum components. Goat or rabbit antiserum for various protein fractions of human serum was purchased from Hyland Laboratories, Los Angeles, Calif. Antiserum specific to human IG-G, IG-A, or IG-M showed only a single precipitate arc in immunoelectrophoresis when tested with normal or patients' sera (IG-G for γ -globulin, 7S γ -globulin; IG-A for γ A-globulin, γ_{1A} -globulin, β_{2A} -globulin; IG-M for γ M-globulin, γ_{1M} -globulin, β_{2M} -globulin).

CF and TP tests. CF and TP tests were performed according to the methods described by Smith et al. (7). For the CF test, the procedure of overnight fixation at 5 C was used.

Antigen from *Coccidioides immitis*. Antigens were manufactured by a modification (4) of the toluene lysis technique of Pappagianis (6). Briefly, this con-

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sisted of growing 24 strains of *C. immitis* separately in a 2% glucose and 1% yeast extract dialysate broth on a rotary shaking machine at 30 to 33 C. The fungus growth was separated by Büchner filtration. Merthiolate was added to the filtrate to a final concentration of 0.01%, and then was sterilized by filtration through a Selas 02 candle. The sterilized filtrate was concentrated by ultrafiltration behind a 6% Parlodion membrane to approximately one-sixteenth of the original volume. This concentrated antigen was standardized for optimal activity against known positive human sera.

Iodination of antigen with I^{131} . Iodination of the antigen (coccidioidin) was carried out by a modification of the method described by Hunter and Greenwood (2). A 0.2-ml amount of concentrated coccidioidin (16 times), containing 210 μ g of protein (expressed as rabbit γ -globulin by Lowry's reaction) was mixed with 0.05 ml of I^{131} (1.9 mc), 0.05 ml of 5×10^{-4} M KI, and 0.10 ml of Chloramine-T (141 μ g) at pH 8.0. After 5 min at room temperature, 0.10 ml of $NaHSO_3$ (240 μ g) was added to reduce any remaining iodine and oxidant. A 0.1-ml amount of KI (1 mg) was added after a few minutes, and the mixture (0.6 ml) was passed through a column of Sephadex G-25 (2.5 g of fine bead form, 1.1 by 11 cm). All the above solutions were made with borate buffer (pH 8.0), and the column was equilibrated with the same buffer. The portions corresponding to the peak of protein (elution volume, 5.5 to 9.5 ml) were pooled and used as I^{131} -coccidioidin. About 380 μ c of I^{131} was found in this fraction. A preliminary experiment with the same column indicated that the separation of the coccidioidin and I^{131} -iodide was almost complete, and the portion collected above contained about 57% of the applied material based on optical density at 280 $m\mu$.

Immunodiffusion. The immunodiffusion procedure was a slight modification of the method described by Huppert and Bailey (3). A 1.5% Ionagar No. 2 solution was prepared in saline containing 0.067 M Sorensen phosphate buffer and with 0.01% aqueous Merthiolate as preservative. Final pH was 8.2. Plastic petri dishes (12 by 50 mm) were charged with 5 ml of molten agar and allowed to gel. A seven-well pattern was cut out of the agar, consisting of a center well surrounded by six equally spaced peripheral wells. The dimensions were as follows: center well, 4.5 mm in diameter; peripheral wells, 3 mm in diameter; wall-to-wall distance between center and peripheral wells, 5 mm. The wells were numbered from 1 to 6 in a clockwise direction, and, routinely, a known positive control serum was placed in wells 1 and 4. The sera to be tested were placed in wells 2, 3, 5, and 6. In this manner, any reactions of the unknown sera could be compared with the control positive sera for reactions of identity, partial identity, or nonidentity.

Reverse immunoelectrophoresis. Immunoelectrophoresis was performed with an LKB 6800 A instrument. Ionagar No. 2 (1%) in Veronal buffer (pH 8.2) was used on microscope slides as a layer 2 mm thick. A central well for the sample to be tested was 1.5 mm in diameter (about 0.004 ml capacity). Electrophoresis was done at 225 v for 1 hr at room temperature, and then two parallel troughs were made at a distance of

3.1 mm from the central well on each side. One trough was charged with antiserum against a fraction of human serum, and the other contained antigen from *C. immitis*. Diffusion occurred overnight in a moist chamber at 23 C.

Radioimmuno-electrophoresis. I^{131} -coccidioidin was used either for forming precipitate arcs directly with patients' sera or for identifying the arcs of human antibody components produced by antiserum against human globulins. In both cases, the pattern differed by having a single trough down the center separating two wells to allow comparison of two serum specimens simultaneously.

In the direct precipitation procedure, the I^{131} -labeled coccidioidin, diluted in 10% normal rabbit serum (carrier), was used in the trough. The final concentration of I^{131} -coccidioidin as protein was 5 μ g/ml. After electrophoresis of the serum specimen and overnight diffusion with antigen, the slides were washed, dried, and stained with 0.5% Amidoschwartz in methanol-water-acetic acid (5:5:1). The dry stained slides were used for radioautography with Kodak Industrial Type KK X-ray film.

For identification of antibody components, a two-step procedure, similar to that used previously for ragweed-sensitive patients' sera (9), was used. After electrophoresis of the serum specimen, the trough was filled with appropriate antihuman serum. After overnight diffusion, the slides were washed for 30 hr with

FIG. 1. (upper) Typical immunodiffusion results with sera which were complement-fixation positive but tube precipitin negative. (lower) Characteristic immunoelectrophoresis result with sera which were complement-fixation positive but tube precipitin negative. Upper trough contained coccidioidin, lower trough anti-IG-G serum, and center well the patient's serum.

FIG. 2. (upper) Typical immunodiffusion results with sera which were positive by both complement-fixation and tube precipitin tests (wells 2, 3, and 5). (lower) Characteristic immunoelectrophoresis pattern with sera which were positive by both complement-fixation and tube precipitin tests. Upper trough contained coccidioidin (note two arcs of precipitate), lower trough anti-IG-M serum, and center well the patient's serum.

FIG. 3. Precipitate arcs revealed by radioautography after immunoelectrophoresis. Direct precipitation technique was used. Normal serum (negative control) is on the extreme right. The remaining sera were complement-fixation positive. The first and third specimens (from the left side) were also tube precipitin positive.

frequent changes of buffered saline (pH 8.0) to remove soluble proteins. Then, the trough was filled with a mixture of I^{131} -coccidioidin and normal goat or rabbit serum (5 μ g of antigen per ml of serum). After diffusion overnight, slides were washed, dried, stained with Amidoschwartz, and used for radioautography as above.

RESULTS

Direct precipitation of coccidioidin with patients' sera in agar layer. Coccidioidin and patients' sera were reacted in agar by Ouchterlony's immunodiffusion test and by reverse immunoelectrophoresis. Patients' sera formed specific precipitate arcs with coccidioidin in both cases. However, there was a distinct difference between the sera which differed in the TP test regardless of their CF titer. TP-negative CF-positive sera showed a single arc of precipitate in immunodiffusion. In

TABLE 1. Serological and radioimmuno-electrophoresis (direct precipitation) reactions of some representative sera from patients with coccidioidomycosis (see also Fig. 3)

Patient	Complement-fixation titer	Tube precipitin	Radioimmuno-electrophoresis	
			γ_1	γ_2
B. J. P.	1:64	+	++	+
V. S.	1:16	—	±	++
M. E.	1:256	—	—*	+++
L. J.	1:32	—	—*	+++
A. C.	1:4	—	±	±

* Long continuous arc covering both regions but with maximal intensity in the γ_2 -globulin region.

FIG. 4. Representative specimens demonstrating that antibody activity is present in all the immunoglobulins. The two-step radioimmuno-electrophoresis procedure was used, and the above reproductions were made from the radioautographs. The serum in the left well of each slide had a complement-fixation titer of 1:32 and was tube precipitin positive. The corresponding reactions for the serum in the right well of each slide were 1:64 and negative. (a) Anti-IG-G. (b) Anti-IG-A. (c) Anti-IG-M.

TABLE 2. *Clinical and immunological status of three representative cases of coccidioidomycosis**

Patient	Diagnosis	Time from onset (months)	Serological test†			Antibody component			Clinical status
			CF	TP	ID	IG-G	IG-A	IG-M	
B. J. P.	Primary pulmonary coccidioidomycosis	1	1:32	++	+	++	+	++	On admission: pneumonic infiltrate and pleural effusion. Gradual improvement.
		2	1:64	+	+	+++	+	+	
		5	1:2	—	+	+	—	—	
L. J.	Chronic pulmonary coccidioidomycosis	8	1:64	—	+	+++	+	—	History: erythema nodosum and pleural effusion for 1 month. Residual nodular lesions bilateral.
		10	1:64	—	+	+++	+	—	
		12	1:32	—	+	++	+	—	
M. E.	Disseminated coccidioidomycosis	4	1:256	—	+	++++	+	+	Treated with amphotericin B and improved. Therapy stopped and clinical condition deteriorated. Continues unstable.
		10	1:16	—	+	+++	—	—	
		12	1:16	—	+	+++	+	+	
		20	Trace	—	+	++	—	—	

* The antibody components were determined by radioimmunoelectrophoresis with the use of the two-step method.

† CF = complement-fixation titer; TP = tube precipitin test; ID = immunodiffusion test; (2) = two arcs of precipitate.

TABLE 3. *Relationship between antibody components and complement fixation (CF) test or tube precipitin (TP) test**

Determination	Relative antibody activity in each IG†								
	IG-G				IG-M			IG-A	
	—	+	++	+++	—	+	++	—	++
CF titer‡									
2-8.....	0	3	1	0	2	1	1	1	0
16-32.....	0	0	4	3	3	0	4	1	1
64-256.....	0	0	0	5	2	2	1	0	1
TP test‡									
Negative.....	0	3	0	9	7	4	1	3	0
Positive.....	0	1	5	2	2	1	5	1	1

* The number of sera belonging to each category is listed.

† Antibody activity in each immunoglobulin was estimated by comparing the radioactivity of the corresponding arc by radioautography. The activity in each component may be compared between different sera. However, the comparison of the activity between antibodies of different classes is not valid.

‡ Twenty specimens shown for TP test include 16 specimens assayed for CF titer. The titer range indicated is expressed as the reciprocal.

immunoelectrophoresis, a single arc was seen in the γ_2 -globulin region (Fig. 1). TP-positive CF-positive sera in most cases showed two precipitate arcs in immunodiffusion. In immunoelectrophoresis, two independent arcs were seen, one short arc in the γ_1 -globulin region and the other arc extending from γ_1 - to γ_2 -globulin region (Fig. 2). The presence of the two arcs indicates that two independent antigens and the corresponding antibodies are involved in these cases.

Similar results could be obtained by the use of ^{131}I -labeled coccidioidin in immunoelectrophore-

sis. Precipitate arcs were seen more clearly on the radioautographs (Fig. 3). TP-negative sera showed a long single arc with maximal intensity in the γ_2 -globulin region. TP-positive sera showed a short characteristic arc in the γ_1 -globulin region along with, or without, another arc in the γ_2 -globulin region. The data on some representative patients' sera are summarized in Table 1.

Identification of antibody components in patients' sera. Antibody active components in patients' sera were identified by radio immunoelectrophoresis. Antisera specific to each of the

known immunoglobulins, IG-G, IG-A, and IG-M, were used to form immune precipitate arcs after electrophoresis of each patient's serum. The precipitate arcs were stained with I^{131} -labeled coccidioidin (for antibody activity) and dye (for protein). The antibody activity in each component was estimated by the density of arcs revealed on the radioautographs.

The radioautographs showed that the antibody activity is present in all the immunoglobulins (Fig. 4). The data are summarized in Table 2. All the sera contained antibody of the IG-G class. The antibodies of IG-A and IG-M class were found in some of the sera. Studies with antiserum against human globulins indicated that only the arcs of immunoglobulins fix I^{131} -labeled coccidioidin. A number of other arcs seen on the stained slides failed to fix the antigen, indicating the lack of antibody activity in serum components other than immunoglobulins.

Table 3 shows the relationship between the antibody activity in each immunoglobulin and the CF test or the TP test on a larger number of sera. The results indicate that the titer roughly parallels the activity of IG-G class antibody. Furthermore, it was noted that most sera containing significant amounts of IG-M class antibody were positive in the TP test and showed a distinct direct precipitation arc in the γ_1 -globulin region in immunoelectrophoresis and two precipitate arcs in immunodiffusion (Table 2). These observations would indicate that the IG-M antibody is responsible for the positive TP test and that the IG-G antibody is responsible for the positive CF test.

DISCUSSION

Resolution of various antigen-antibody systems involved in coccidioidomycosis has been demonstrated. As in other diseases (1, 9, 10), antibody activity appears in at least three components of patients' sera: the IG-A, IG-M, and IG-G globulins. This was demonstrated clearly with the radioimmunoelectrophoresis technique.

The sera used in these studies were obtained from patients in various clinical stages of coccidioidomycosis; primary infection, chronic, and disseminated stages. Antibody of IG-G class was present in all cases as demonstrated by radioimmunoelectrophoresis. Antibody of IG-A class was found also, but the presence and activity of this component were more variable and less marked than those of the IG-G antibody. The antibody of IG-M class was found most clearly in serum specimens which yielded a positive TP test. In coccidioidomycosis, the latter test is positive only in the early stage and after exacerbation of pre-existing disease.

Immunodiffusion and immunoelectrophoresis indicated the presence of two independent systems of antigens and corresponding antibodies, as represented by two independent precipitate arcs. The antigen responsible for the TP test seems to be associated mainly with the antibody of IG-M class. Sera containing higher amounts of IG-M antibody showed positive results in the TP test. A short characteristic arc of precipitate in the γ_1 -globulin region, observed by direct precipitation reaction of coccidioidin and serum (after electrophoresis), seems to represent this antigen-antibody system. Two of the sera gave positive results in the TP test, but did not contain IG-M antibody (Table 3). IG-G antibody in these sera may have been directed against the above antigen.

On the other hand, some sera of high CF titer containing no IG-M antibody (e.g., L. J. in Tables 1 and 2) were negative in the TP test, in spite of strong antibody activity in IG-G globulin. These sera gave a single arc of direct precipitation in the γ_2 -globulin region upon reverse immunoelectrophoresis. This arc is probably due to another antigen which hardly precipitates with the antibody in the test tube. The antibody against this antigen appears to be mainly IG-G globulin. Since the CF titer roughly parallels the antibody activity in IG-G globulin, regardless of the results in the TP test, this antigen-antibody system seems to be mainly responsible for the fixation of complement. These findings are similar to those reported recently by Pappagianis et al. (5).

The successful separation and identification of the *C. immitis* antigens, corresponding to the activity in CF and TP tests, has made it possible to standardize each antigen for optimal activity and to use the antigens during the course of the disease in humans (4). Further studies on the relation of clinical status and serum antibodies are in progress. In this regard, the disseminated case of coccidioidomycosis (patient M.E.) is interesting (Table 2). The 12-month specimen from this patient yielded a positive CF test but a negative TP test. The immunodiffusion and immunoelectrophoresis tests on the same specimen produced precipitate arcs indicating the presence not only of IG-G antibody, but also of IG-M antibody, which seems to be the reactive serum component in the TP test. It has been our experience that the TP test is less sensitive than the immunodiffusion test and the immunoelectrophoresis test with I^{131} -coccidioidin. Furthermore, this patient had been started on amphotericin B therapy at the time of the 10-month specimen. He showed an excellent clinical improvement, and the therapy was stopped shortly before the 12-month specimen was obtained. Thereafter, his clinical status

deteriorated, but his CF titer continued to an essentially negative reaction in 20 months. Subsequent specimens from this patient have shown a return of the positive CF reaction to a titer of 1:16. Additional studies on patients not reported here appear to indicate that the immunodiffusion and immunoelectrophoresis results reflect the clinical condition of the patient more accurately than does the CF test, as has been illustrated in the case of patient M. E.

The recognition of at least two of the *C. immitis* antigens involved in known serological reactions of coccidioidomycosis offers the opportunity to study the changing patterns of immunologically active serum proteins as they occur during the course of the disease. Isolation and study of additional antigens may reflect even more accurately whether a patient's clinical condition will improve or deteriorate. Such information would serve as an objective guide in the clinical management of cases of coccidioidomycosis.

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