A Positive Control For Coccidioidin Complement Fixation

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The use of complement fixation tests for diagnosis of coccidioidomycosis by laboratories in "fringe" and "nonendemic" areas is limited by lack of human antiserum for positive control of the coccidioidin. Lyophilized rabbit antiserum is suggested for this purpose.

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THE VALUE of the complement fixation test in suspected and proved cases of coccidioidomycosis has been adequately discussed by Smith and co-workers (*1*, *2*). Conant and associates (*3*) have also pointed out the value of complement fixation tests as an indication of the status of a coccidioidal infection. Schubert and associates (*4*) reported the results of their investigation with histoplasmin.

The continuous research on the use of complement fixation in the diagnosis of mycotic diseases emphasizes the value of this test as a diagnostic aid. As this value is recognized, the clinical laboratories will receive more requests for complement fixation tests. The serologist may properly interpret the results of any complement fixation test only if a positive control serum is used in each test.

In the endemic areas for *Coccidioides immitis* outlined by Smith (*5*), the clinical laboratories probably have little difficulty in obtaining an adequate supply of positive human serum. However, the clinical laboratories located elsewhere encounter considerable difficulty in obtaining or maintaining a supply of this control serum. These laboratories may receive only two or three requests for this complement fixation test each year. At present there appears to be no commercial source of a control serum for laboratories outside the endemic areas.

With this problem in view, a preliminary investigation was made to determine whether rabbit antiserum could be used to replace human antiserum as a source of positive control serum.

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Materials and Methods

Six young female white rabbits weighing from 5 to 7 pounds each were selected. Each of the rabbits was injected intraperitoneally with live spore mixtures of four strains of *Coccidioides immitis*. Ten days after this injection, 5 ml. of blood was removed from the ear of each rabbit, and the serum was tested for antibodies. Three of the rabbits showed high titer; all showed some titer.

As the rabbits were beginning to weaken from the intensity of the infection, they were bled approximately 50 ml. each by heart puncture on the 14th day after injection. The blood was permitted to clot, was centrifuged, and the serum separated. The serum of each rabbit was placed in a 50-ml. test tube and frozen until it could be tested. After each serum was again titered, the serums having similar titers were pooled and designated as serum pools 1 and 2. These two lots were dispensed in 10-ml. test tubes and refrozen until needed.

Autopsy of the rabbits revealed cysts, which were found to contain *C. immitis* endospores, in the peritoneal cavity.

Portions of the thawed serum pools 1 and 2 were dispensed into 2-ml. vials. The contents of the vials were frozen in a mixture of dry ice and isopropyl alcohol, desiccated, and sealed in vacuo. The vials of lyophilized serum were kept on the shelf at room temperature. When they were to be used, the serum solids

of each vial were dissolved in 2 ml. (the original serum volume) of distilled water. The reconstituted rabbit serum was heated for 30 minutes in a 60° C. water bath just before use. The heated bath was used as described by Dr. John F. Kent, department of serology, Army Medical Service Graduate School, in a private communication. He has found that the bath is necessary to destroy the nonspecific anticomplementary activity in rabbit serum. Serial dilutions of the reconstituted serum were then prepared for the titrations.

Human serum from a case of disseminated coccidioidomycosis with a high titer (reported as 1:512 by the department of mycology) was used for comparative purposes. Portions of this serum were lyophilized along with the rabbit serum and sealed in vacuo. These serum solids were dissolved in distilled water, and this reconstituted serum was heated for 30 minutes in a 56° C. water bath just before use.

Serums from normal rabbits and normal human beings were used in the investigation as negative controls.

A coccidioidin antigen for use in these complement fixation tests was supplied by Dr. Charles E. Smith of the School of Public Health, University of California.

Crude antigen preparations were also made in the laboratories of Southwest Foundation for Research and Education from cultures of the same four strains of *C. immitis* used for injection of the animals in antibody production. These four strains had been isolated from active cases of coccidioidomycosis. They were grown on the asparagine synthetic medium used by Smith and associates (*1*) and by Schubert and co-workers (*4*) in their investigations. The cultures were incubated for 6 weeks at room temperature in Roux culture bottles.

The complement fixation procedures were adapted from procedures of Kent (*6*). Titrations of all reagents used in these procedures were carried out according to the procedures outlined in the Army Technical Manual (*7*). The complement solution used in these tests was twice as concentrated as designated in the manual, but only half the recommended volume was used. Thus the same number of units of complement were used; only the volume was changed.

In the complement fixation tests the materials were added to 10 by 75-mm. test tubes and mixed thoroughly after each addition. They were added in the following order:

1. 0.25-ml. antigen dilution.
2. 0.25-ml. serum dilution.
3. 0.25-ml. complement solution (containing 2 full units).
4. All tubes incubated at 4°–7° C. for 16 to 18 hours.
5. 0.50-ml. antisheep hemolysin (containing 2 units).
6. 0.50-ml. 2-percent sheep cell suspension.
7. All test tubes incubated for 30 minutes in 37° C. water bath.
8. All tubes were compared visually with synthetic standards freshly prepared in 10-percent hemolysis steps; hemolysis was estimated and recorded to the nearest 5 percent.

The titer of a serum was recorded as the highest dilution which would permit less than 70-percent hemolysis to be exhibited in this complement fixation test. A control tube containing all components except antigen was run with each serum dilution, and a control tube containing no serum dilution was run with each antigen dilution.

Results

Preliminary titrations were performed to determine the optimum dilution for each antigen. Serial dilutions of the antigen, varying by a factor of 5, for example, 1:1, 1:5, 1:25, and 1:125, were prepared, and each of these dilutions was used for the titration of the antiserum.

The data obtained in the "crosshatch" titration of rabbit antiserum pool No. 1 with the coccidioidin furnished by Dr. Smith is shown in table 1.

The experiment showed a serum titer of 1:64 when a 1:25 dilution of this coccidioidin was used. This 1:25 dilution of Dr. Smith's antigen was used later in the titrations of rabbit antiserum. The crude antigens prepared in this laboratory also showed maximum activity at a 1:25 dilution with rabbit serum pool No. 1 if the antigens showed any activity. In contrast to these results, Dr. Smith's coccidioidin showed a maximum activity at a 1:5 dilution when used for the titration of a positive human serum in a "crosshatch" procedure identical to that outlined in table 1.

Identical titrations were performed on samples of rabbit antiserum pools Nos. 1 and 2

Table 1. "Crosshatch" titration of Smith coccidioidin and rabbit serum pool No. 1, tabulated in percent hemolysis

Antigen dilution	Serum dilution				Saline control
	1:8	1:16	1:32	1:64	
1:1	5	15	ac	—	—
1:5	0	0	25	—	—
1:25	0	0	5	50	—
1:125	5	15	20	95	—
Saline	—	—	—	—	

NOTE: Dash (—) = 100 percent hemolysis; ac = almost complete hemolysis (greater than 95 but less than 100 percent).

which had been stored in the frozen state, and samples which had been lyophilized. The results of these titrations are shown in table 2, along with results obtained when a lyophilized sample of pool No. 1 was titrated 6 months later. Table 3 shows the results of a similar titration of the one human antiserum available. The drop in titer from 1:256 to 1:16 on lyophilization could not be confirmed by repeating the experiment because no more of the human antiserum was available.

Discussion

There appears to be little difficulty in producing antiserum to *C. immitis* in rabbits. Although some rabbits develop a higher titer than others, standard immunological techniques can be used to follow the progress of the immunity reaction, enabling the worker to choose the animals showing the highest titers as a source of antiserum. As has been observed in other antiserum production, lyophilization destroys little if any of the antibody content. The lyophilized

Table 2. Titration of rabbit serum; antigen: 1:25 dilution of Smith coccidioidin, tabulated in percent hemolysis

Pool No.	Lyophilized	Serum dilution									Saline control	Titer
		1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256		
1	No	0	0	0	0	0	35	—	—	—	—	1:32
1	Yes	0	0	0	0	0	0	45	—	—	—	1:64
2	No	0	0	0	20	55	95	—	—	—	—	1:16
2	Yes	0	0	0	40	80	—	—	—	—	—	1:8
1	Yes [1]	0	0	0	0	0	0	55	—	—	—	1:64
Normal rabbit serum		—	—	—	—						—	(2)

[1] Stored for 6 months. [2] Negative.

NOTE: Dash (—) = 100 percent hemolysis.

Table 3. Titration of positive human serum; antigen: 1:5 dilution of Smith coccidioidin, tabulated in percent hemolysis

Lyophilized	Serum dilution									Saline control	Titer
	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256		
No	0	0	0	0	0	0	10	10	65	—	1:256
Yes	0	0	0	0	15	75	ac	—	—	—	1:16
Normal human serum	—	—	—	—	—	—	—	—	—	—	(1)

[1] Negative.

NOTE: Dash (—) = 100 percent hemolysis; ac = almost complete hemolysis (greater than 95 but less than 100 percent).

products appear to be stable indefinitely at room temperature, which would permit shipment to, and storage by, small laboratories until needed.

The sharp drop in titer in the one human antiserum after lyophilization is not what would be expected. No conclusions should be drawn until additional human antiserums are available for testing.

Further investigations must be carried out before a lyophilized rabbit anticoccidioidin is placed on the market as a standard reagent. The most important question to be answered is the one concerning the specificity of the antibodies produced by rabbits and humans. It is possible that the rabbit antibodies titrated in the above experiments were specific for one kind of molecules in the coccidioidin, the carbohydrates, for example, while the human antibodies were specific for an entirely different kind of molecules, possibly proteins, but not carbohydrates. Admittedly this example is an oversimplification. Nevertheless, some indirect evidence makes the possibility worthy of investigation. Martin (*8*) has shown that an alcohol-precipitated carbohydrate-like substance from *Blastomyces dermatitidis* antigens fixed guinea pig complement with rabbit antiserum but not with human antiserum although the protein portion of the *B. dermatitidis* cells did fix complement with the same human antiserum. Hassid and co-workers (*9*) have also shown that carbohydrate materials from coccidioidin failed to fix complement with human antiserum. The same human antiserum did fix complement with the original coccidioidin.

Of primary importance would be the comparison of the complement-fixing abilities of various purified fractions of coccidioidin with rabbit antiserum and with human antiserum. Additional investigations need to be accomplished; this is contemplated as soon as funds are available for this purpose. With such additional data, it appears possible lyophilized rabbit serum may become available to laboratories commercially.

Summary

Young female rabbits weighing 5 to 7 pounds were injected intraperitoneally with live spore mixtures of *Coccidioides immitis*. Fourteen days following the injection the rabbits had developed a high titer of antiserum specific for *C. immitis*. This antiserum, when lyophilized and sealed in vacuo, was shown to be stable indefinitely at room temperature.

It is suggested that, after further investigation, such an antiserum preparation might be used as a positive control serum of known titer by laboratories which have no available source of supply of fresh positive human antiserum for *C. immitis*.

Problems which remain to be investigated are discussed.

REFERENCES

(*1*) Smith, C. E., Saito, R. R., Kepp, R. McF., Clark, R. W., and Eddie, B. U.: Serological tests in the diagnosis and prognosis of coccidioidomycosis. Am. J. Hyg. 52: 1–21, July 1950.

(*2*) Smith, C. E., Whiting, G., Baker, E. E., Rosenberger, H. G., Beard, R. R., and Saito, M. T.: The use of coccidioidin. Am. Rev. Tuberc. 57: 330–360, April 1948.

(*3*) Conant, N. F., Smith, D. T., Baker, R. D., Callaway, J. L., and Martin, D. S.: Manual of Clinical Mycology. Ed. 2. Philadelphia, W. B. Saunders, 1954, pp. 113.

(*4*) Schubert, J., Ajello, L., Sanford, S., and Grant, V. Q.: Variation in complement fixation antigen production by different strains of *Histoplasma capsulatum* grown on two media. J. Lab. & Clin. Med. 41: 91–97, January 1953.

(*5*) Smith, C. E.: Diagnosis of pulmonary coccidioidal infections. California Med. 75: 385–391, December 1951.

(*6*) Kent, J. F.: The application of quantitative methods in studies of the antigen-antibody reactions. A thesis. Durham, N. C., Duke University, 1950.

(*7*) U. S. Department of the Army: Methods for medical laboratory technicians. Army Tech. Manual 8–227, AFM 160–14. Washington, D. C., U. S. Government Printing Office, 1951, pp. 562–566.

(*8*) Martin, D. S.: Serologic studies on North American blastomycosis. Studies with soluble antigens from untreated and sonic-treated yeast-phase cells of *Blastomyces dermatitidis*. J. Immunol. 71: 192–201, September 1953.

(*9*) Hassid, W. Z., Baker, E. E., and McCready, R. M.: An immunologically active polysaccharide produced by *Coccidioides immitis* Rixford and Gilchrist. J. Biol. Chem. 149: 303–311, August 1943.