RAPID PRODUCTION OF COMPLEMENT FIXATION ANTIGENS FOR SYSTEMIC MYCOTIC DISEASES

I. COCCIDIOIDIN: INFLUENCE OF MEDIA AND MECHANICAL AGITATION ON ITS DEVELOPMENT

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The complement fixation test is of great diagnostic and prognostic value in the study of suspected cases of blastomycosis, coccidioidomycosis, and histoplasmosis. As the number of requests for these tests continues to grow along with the increasing awareness of the public health importance of these diseases, the need to produce large stocks of antigens more efficiently has arisen. Accordingly, the Mycology and Serology Units of the Communicable Disease Center are collaborating in an experimental program to develop procedures for the rapid production of satisfactory, reproducible antigens. The production of a complement fixation antigen by *Coccidioides immitis* was studied first.

Until recently, virtually the only antigen available for coccidioidomycosis serology was prepared by Dr. C. E. Smith and his co-workers (1948, 1950) at the University of California's School of Public Health in Berkeley. Although Smith's method is capable of producing reliable and effective antigens, the procedure is very time consuming and frequently yields antigens that are highly anticomplementary or extremely low in complement fixing activity.

In order to overcome these defects, Pappagianis and his co-workers (1957) investigated an alternate method of producing coccidioidin. Their antigen was prepared through the lysis of *C. immitis* mycelium.

In this paper another simple, rapid, and consistently reproducible method for preparing a *C. immitis* complement fixation antigen is described.

MATERIALS AND METHODS

Four human strains of *C. immitis* were used in this study. These strains were designated as: A 429, A 453, A 540, and A 659. Individual strains were grown at 25 C in 125-ml Erlenmeyer flasks containing 50 ml of medium. The inoculum consisted of a 1-mm square piece of mycelium cut from a 1-week-old Sabouraud glucose agar culture. The flasks were stationary or subjected to continuous agitation. The shaking apparatus was adjusted to deliver 90 two-inch reciprocating cycles per minute. For every run, each of the 4 strains was inoculated into 8 flasks of each of the 5 test media. Half of these were held stationary and half were shaken. One flask from each medium was harvested at weekly intervals over a 1-month period. Merthiolate (1:5000) was used to kill the mycelial growth before Seitz filtration. In order to broaden antigenic coverage, the supernatant from the individual flasks of each medium inoculated with a single strain of *C. immitis* was pooled before testing. Thus the antigens used in this study represented a pool of antigenic material produced by 4 different isolates of *C. immitis*. Five media were tested:

1. Smith's modified Bureau of Animal Industry Tuberculin Medium (Smith et al., 1948). L-Asparagine, 7.0 g; NH_4Cl, 7.0 g; K_2HPO_4 $3H_2O$, 1.31 g; $Na_3C_6H_5O_7 + 5\frac{1}{2}\ H_2O$, 0.90 g; $Mg\ SO_4 + 7\ H_2O$, 1.5 g; ferric citrate, 0.3 g; glucose, 10.0 g; glycerine, 25.0 g; and distilled water to make 1000 ml.

2. Glucose broth. Peptone (Difco), 10.0 g; glucose, 40.0 g; and distilled water to make 1000 ml.

3. Neopeptone dialyzate (Evans and Kessel, 1951) (NPD medium). Dialyzate,[1] 1 part; distilled water, 2 parts; and glucose, 2 per cent.

4. Peptone-glucose-yeast extract (PGY) broth (Whiffen, 1946, personal communication). Peptone (Difco), 1.0 g; yeast extract (Difco), 1.0 g; glucose, 10.0 g; and distilled water to make 1000 ml.

5. Roessler's medium (Roessler et al., 1946). Dipotassium phosphate C.P. K_2HPO_4, 2.6 g; dihy-

[1] Neopeptone (60 g per L of water) held at 70 to 80 C for 6 to 8 hr and overnight in a cold room at 4 C.

TABLE 1

Antigenic titrations of the 5 experimental antigens against a single known positive human serum

Media.....	Glucose Broth	NPD*	PGY*	Roessler's	Smith's
Titers	1:2 (1)† 1:4 (3)	1:2 (1) 1:4 (2) 1:8 (4)	1:2 (2) 1:4 (1)	0 (3) AC (1)	Undil (2) AC (4)

* NPD = neopeptone dialyzate; PGY = peptone-glucose-yeast extract.

† Number in parentheses indicates runs.

drogen phosphate C.P. KH_2PO_4, 2.0 g; magnesium sulfate C.P. $MgSO_4 + 7H_2O$, 2.0 g; ammonium acetate C.P. $NH_4C_2H_3O_2$, 6.2 g; glucose, 20.0 g; zinc sulfate C.P. $ZnSO_4 + 7 H_2O$, 0.3 g; and distilled water to make 1000 ml.

All media were sterilized at 120 C for 15 min.

The 50 per cent hemolytic end point complement fixation test as described by Schubert *et al.* (1955) was used throughout the study. All readings were made visually to 10 per cent accuracy.

Sera from culturally proved cases of coccidioidomycosis, which were positive serologically with an antigen obtained from Dr. C. E. Smith, were used in evaluating the experimental antigens.

Several lots of antigen were produced with each medium, but because of contamination and breakage the number of completed lots varied from 3 to 7 (table 1).

Typical titration results obtained with the media after 1 of the 3-week incubation periods are presented in table 2. The neopeptone dialyzate antigens were tested against 50 human sera along with the control antigen obtained from Dr. C. E. Smith. The titers obtained with the neopeptone dialyzate antigens were within a one tube variation of those obtained with the control antigen. It is evident that the two defined media were not as active as the peptone media. At the end of this 3-week period the Smith medium antigen was consistently anticomplementary at the low dilutions and developed practically no activity above the anticomplementary level. Roessler's medium never showed any significant activity. An unusual phenomenon was observed in that a small amount of activity was noted regularly at the higher dilutions. This disappeared on further dilution.

Essentially no anticomplementary activity developed in the peptone media. The complement fixation activity of the peptone-glucose-yeast extract medium (PGY) was low but the antigen could be used at a 1:2 dilution.

The anticomplementary activity noted in the undiluted glucose broth medium was not excessive. This antigen was useable at a dilution of 1:3 since at this level there was no anticomplimentary activity.

The highest reactivity was reached by the neopeptone dialyzate medium. Maximum activity occurred at the 1:8 dilution. Thus the neopeptone dialyzate antigen was as reactive as the Smith control antigen with the added advantage that it was completely free of anticomplementary activity.

DISCUSSION

Antigen production was tested at weekly intervals over growth periods of 1 month. During the first 2 weeks of development on the shaking machine, little activity developed in any of the 5 media. The peak of antigen production was reached during the 3rd week. Longer periods of agitation did not increase antigen production.

Parallel stationary cultures, prepared in an identical manner as the shake flasks, failed to produce antigens during 4 weeks of incubation. Release of antigenic material could not be detected despite vigorous growth of the inocula in the peptone media.

These studies have demonstrated that, with the use of a peptone medium and continuous shaking at 25 C, an active and consistently reproducible complement fixation antigen can be prepared from *C. immitis* in a relatively short time period.

Since completion of this investigation, several lots of neopeptone dialyzate antigen have been prepared by this method. Each of these was useable at a dilution of 1:8. In one instance, closer titration intervals were used and it was found that the optimal dilution was 1:6. This would ac-

TABLE 2

Comparative coccidioidin titers obtained with the 5 test media

Comparison of 3-week shake cultures with standard Smith coccidioidin

Medium	Medium dilution	Titration: Serum dilution 8	16	32	64	128	256	512	Hemolytic Control*: Units of complement 5	2.5	1.25
Smith standard antigen†	Undil	4‡	4	4	4	4	4	4	4	4	4
	1:2	4	4	4	4	4	3	±	±	3	4
	1:3	4	4	4	4	4	3	−	−	2+	4
	1:4	4	4	4	4	4	4	−	−	2−	3
	1:8	4	4	4	4	4	4	−	−	±	2
	1:16	4	4	4	3	2		−	−	±	2
Glucose antigen	Undil	4	4	4	4	4	4	±	±	2	4
	1:2	4	4	4	4	4	4	±	±	±	3
	1:3	4	4	4	4	4	3	±	−	±	3
	1:4	4	4	4	4	4	±	−	−	±	2
	1:8	4	4	3	2+	±	−	−	−	±	2
	1:16	4	3	±	−	−	−	−	−	±	2
NPD§ antigen	Undil	4	4	4	4	2	−	−	±	±	2
	1:2	4	4	4	4	4	±	−	−	±	2+
	1:3	4	4	4	4	4	±	−	−	±	2
	1:4	4	4	4	4	4	±	−	−	±	2+
	1:8	4	4	4	4	4	3	−	−	±	2+
	1:16	4	4	4	4−	2−	±	−	−	±	2
PGY§ antigen	Undil	4	4	4	4	4	4	2−	−	±	2−
	1:2	4	4	4	4	4	4	4	−	±	2
	1:3	4	4	4	3	2	1	±	−	±	2
	1:4	4	4	4	2	1	±	−	−	±	2
	1:8	4	2	1	−	−	−	−	−	±	2
	1:16	4	1	±	−	−	−	−	−	±	2
Roessler antigen	Undil	±	−	−	−	−	−	−	−	±	4
	1:2	±	−	−	−	−	−	−	−	±	3−
	1:3	2−	±	−	−	−	−	−	−	±	2
	1:4	2	±	−	−	−	−	−	−	±	1
	1:8	4	1	−	−	−	−	−	−	±	1
	1:16	4	3−	−	−	−	−	−	−	±	2
Smith antigen	Undil	4	4	4	4	4	4	4	4	4	4
	1:2	4	4	4	4	3	2	1	1	4	4
	1:3	4	3	2	1	±	−	−	−	±	4
	1:4	4	1	±	−	−	−	−	−	±	4
	1:8	4	2	−	−	−	−	−	−	±	4
	1:16	4	3	±	−	−	−	−	−	±	4

* Any antigen dilution which fixes more than 1.25 units of complement is considered to be anticomplementary.

† Kindly supplied by Dr. C. E. Smith, Lot 47-63.

‡ 4 = Complete fixation.

§ NPD = neopeptone dialyzate; PGY = peptone-glucose-yeast extract.

count for the occasional variation from 1:8 to 1:4 as found by our usual titration procedures.

The neopeptone dialyzate antigen was tested on four human subjects to determine if it would elicit skin test reactions on individuals known to be negative or positive reactors to the commercially available coccidioidin used for skin tests. The antigen did not elicit skin test reactions.

SUMMARY

Five different media were tested for their ability to produce complement fixation antigens from *Coccidioides immitis*. The cultures were incubated while stationary at 25 C or were continuously agitated at 25 C for 1 to 4 weeks. A neopeptone dialyzate medium with agitation produced the most satisfactory antigen, after 3 weeks of growth. It was as antigenic as the standard coccidioidin, free of anticomplementary activity, and was readily reproducible. This complement fixation antigen lacked skin test reactivity.

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