Development and Evaluation of an Immunodiffusion Test for Diagnosis of Systemic Zygomycosis (Mucormycosis): Preliminary Report

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An antigen analysis with filtrate and homogenate precipitinogens of single isolates of the zygomycetes *Absidia corymbifera*, *Mucor pusillus*, *Rhizopus arrhizus*, and *Rhizopus oryzae* demonstrated the presence of common antigens among the three genera as well as antigens which permit their differentiation. Selected homogenate antigens were valuable in developing a diagnostic immunodiffusion (ID) test for systemic zygomycosis. When sera from 43 patients with various proven mycoses other than zygomycosis were tested against each of the antigens, none formed precipitin bands identical to those formed by *A. corymbifera*, *M. pusillus*, and the *Rhizopus* spp. rabbit reference antisera. Sera from 23 normal persons and 25 diabetics did not react with any of the antigens. Homogenate antigens detected antibody in 8 of the 11 sera (73%) from suspected or proven cases of zygomycosis, whereas ID tests with filtrate antigens detected antibody in only 2 of the 11 sera (18%). Of the eight sera that reacted with the homogenate antigens, five only reacted with a specific *Rhizopus* sp. antigen, two only reacted with a specific *M. pusillus* antigen, and one only reacted with a specific *A. corymbifera* antigen. Study results show the ID test with homogenate antigens to be more specific and sensitive than the ID test with filtrate antigens and indicate that the former is a promising technique for diagnosing human zygomycosis.

Systemic zygomycosis (mucormycosis) is a serious mycotic infection caused by several members of the class *Zygomycetes* (1). The zygomycetes are filamentous coenocytic fungi which produce zygospores. A few species of the many zygomycetes can cause systemic infections in debilitated or immunologically compromised persons. Because these fungi can spread rapidly in the susceptible host, the diagnosis of zygomycosis is often made at autopsy. The primary sites of infection are the paranasal sinuses, lungs, and gastrointestinal tract, but the fungi can spread to other parts of the body by direct extension or through vascular dissemination (5).

According to Landau and Newcomer (5), one of the major obstacles in treating patients for zygomycosis has been the failure to diagnose the disease in time, as indicated above. The disease can be unequivocally diagnosed when broad aseptate mycelial elements are evident by microscopic examination of tissue. Although a single isolation of the organism is of limited diagnostic value since the etiological fungi are com-

mon laboratory contaminants, repeated isolation of the fungus from clinical material indicates that it is the likely source of infection.

Few reports on the immunodiagnosis of zygomycosis appear in the literature. To date, the results of only one preliminary and inconclusive study have been published on the detection of antibody to the etiological fungi of systemic zygomycosis (3). Since early diagnosis of zygomycosis greatly increases a patient's chance of survival, a rapid diagnostic test is needed. This study was undertaken to analyze antigenically the agents of zygomycosis and to determine whether a sufficiently specific immunodiffusion (ID) test could be developed.

MATERIALS AND METHODS

Cultures. *Absidia corymbifera* (A-7637), *Mucor pusillus* (B-1199), *Rhizopus arrhizus* (A-863), and *R. oryzae* (A-885) were obtained from the Center for Disease Control (Atlanta, Ga.) stock culture collection. *R. arrhizus* and *R. oryzae* had been isolated from patients with cerebral zygomycosis; *M. pusillus* had been isolated from a patient with pulmonary zygomycosis; and *A. corymbifera* was from an unknown source.

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Preparation of antigens. Each selected fungus was inoculated into 500 ml of Sabouraud dextrose broth and grown at 25°C on a gyratory shaker set at 150 rpm. After 72 h, the cultures were killed by adding Merthiolate to a final concentration of 1:10,000.

Filtrate antigens were obtained by filtering out the mycelial growth of the broth cultures with a Whatman no. 2 filter. The filtrates were then concentrated to 0.1 their original volumes by ultrafiltration through an Amicon E-20 apparatus with a PM-10 filter (Amicon Corp., Lexington, Mass.). The protein concentration of each filtrate was adjusted to between 0.7 and 0.8 mg of protein per ml of solution by the method of Lowry et al. (6).

Homogenate antigens were prepared by washing the separated mycelium five times with phosphate-buffered saline solution (pH 7.2) containing 0.01% Merthiolate. The washed mycelium was diluted 1:4 by adding 1 part of packed mycelium to 3 parts of Merthiolate-treated phosphate-buffered saline. Suspended mycelium (30 ml) was placed in 60-ml serum bottles containing 40 g of glass beads (0.45 to 0.50 mm in diameter). The 1:4 diluted mycelium was then disrupted in a Braun MSK mechanical cell homogenizer set at 4,000 cycles per min for 3 to 5 min. The homogenate was decanted, and the beads were washed five times with Merthiolate-treated phosphate-buffered saline. The washings were pooled and added to the homogenate slurry. This slurry was then centrifuged at $16,319 \times g$ for 60 min. The supernatant was decanted and concentrated to 100 ml in an Amicon E-20 apparatus with a PM-10 filter. The protein concentration of each homogenate was standardized at between 1.0 and 2.0 mg of protein per ml of solution by the method of Lowry et al. (6).

Production of antisera. Antisera to the filtrates and homogenates were produced in rabbits by the method and schedule of Standard and Kaufman (7).

Adsorption of antisera. Antigen (1 ml) was placed in a glass tube (13 by 100 mm) and dried by bubbling air through the tube. Antiserum (1 ml) was added to the dried antigen. The antigen-antiserum mixture was agitated to dissolve the antigen completely and incubated at 37°C for 2 h in a water bath with an agitator. After incubation, the reactants were centrifuged for 45 min at $12,063 \times g$. The supernatant was decanted and tested by ID for evidence of adsorption.

ID test. For this procedure the micro-ID technique of Busey and Hinton (2) was used. Antisera were diffused for 3 h, and then antigens were added. The plates were incubated at 25°C, and results were recorded after 48 h. If the sera from the zygomycosis cases were viscous, they were incubated for an addi-

tional 24 h. Before the results were read, the plates were rinsed for 60 min with a 5% sodium citrate solution to eliminate precipitates caused by C-reactive protein. In the diagnostic test, the case serum was placed in the top central well, and rabbit reference sera were placed in the four lateral wells. Diabetic serum was placed in the bottom central well as a negative control.

RESULTS

Filtrate and homogenate antigens prepared from *A. corymbifera*, *M. pusillus*, *R. arrhizus*, and *R. oryzae* were analyzed in ID tests with homologous and heterologous rabbit antisera to determine the antigenic relationship among these fungi. To further substantiate the antigenic relationships, rabbit antisera to these zygomycetes were absorbed with the homologous and heterologous antigens and analyzed in ID tests.

The results of the filtrate analyses are summarized in Table 1. At least three of the antigens designated A, B, C, D, and E were demonstrated in each zygomycete filtrate studied. All four of the fungi had the three filtrate antigens A, C, and D. Antigen B was demonstrated in the *A. corymbifera* filtrate only, and E was present only in *R. arrhizus* and *R. oryzae*. The filtrates of the two *Rhizopus* spp. were apparently identical. No antigens specific to the *M. pusillus* filtrate were found.

The homogenates contained more genera-specific antigens than the filtrates (Table 2). In the 48-h ID test, antigens were recognized among the homogenates of the zygomycetes studied and were numbered from 1 to 12. Six antigens (1, 2, 5, 6, 7, and 8) were identified in the *M. pusillus* homogenate. Of these six, 5, 6, 7, and 8

TABLE 1. Antigenic scheme of the zygomycete filtrates

Species tested	Filtrate antigens found				
	A	B	C	D	E
<i>A. corymbifera</i>	+	+	+	+	
<i>M. pusillus</i>	+		+	+	
<i>R. arrhizus</i>	+		+	+	+
<i>R. oryzae</i>	+		+	+	+

TABLE 2. Antigenic scheme of the zygomycete homogenates

Species tested	Homogenate antigens found											
	1	2	3	4	5	6	7	8	9	10	11	12
<i>A. corymbifera</i>	+	+	+	+								
<i>M. pusillus</i>	+	+			+	+	+	+				
<i>R. arrhizus</i> ^a									+	+	+	+
<i>R. oryzae</i> ^a									+	+	+	+

^a Two additional *Rhizopus* spp. factors were recognized after 72 h of incubation (see Fig. 1).

were specific to *M. pusillus*, and 1 and 2 were also found with *A. corymbifera*. The *A. corymbifera* homogenate also contained two specific antigens, 3 and 4. The *R. arrhizus* and *R. oryzae* homogenates both contained antigens 9, 10, 11, and 12 and did not contain any of those antigens associated with the *A. corymbifera* or *M. pusillus* homogenates.

Of 102 sera tested against each of the filtrates and homogenates, 11 were from proven or suspected cases of zygomycosis, 23 were normal human sera, 25 were sera from patients with diabetes, and 43 were sera from proven human cases of aspergillosis, blastomycosis, candidiasis, coccidioidomycosis, cryptococcosis, histoplasmosis, nocardiosis, paracoccidioidomycosis, and sporotrichosis. With bands of identity as the criterion for a positive ID test, the filtrate and homogenate antigens were 100% specific. None of the normal or diabetic sera reacted with the zygomycete filtrates or homogenates. An occasional serum from a patient with candidiasis, coccidioidomycosis, or histoplasmosis formed one precipitin band. However, none of these bands was identical to those formed in the reference reactions.

The ID results for the 11 sera from proven or suspected cases of zygomycosis are shown in Table 3. The sera from proven cases were from humans or animals with histological evidence of zygomycosis. The sera from suspected cases were from patients with positive zygomycete cultures or with clinical pictures highly suggestive of zygomycosis. The etiological fungi were isolated and identified in only two cases (4 and

6) and were *R. arrhizus* and *A. corymbifera*. Of the 11 patients, only patients 9 and 10 were immunosuppressed. Case sera were considered positive if they reacted with antigens to form one or more precipitin bands identical to the bands formed in the homologous reference reaction. None of the case sera formed bands of nonidentity. Of the eight positive sera, five reacted only with antigen 11 of the *Rhizopus* spp. homogenates. Figure 1 shows the reaction of case 5 serum with antigen 11 of the *Rhizopus* spp. homogenates. As indicated earlier, *R. arrhizus* was the etiological agent in case 4, the serum of which reacted only against antigen 11 of the *R. arrhizus* and *R. oryzae* homogenates. Two of the suspected case sera (10 and 11) reacted against antigen 5 of the *M. pusillus* homogenate, and one (case 6) reacted against antigen 3 of the *A. corymbifera* homogenate. The reaction with case 10 serum is shown in Fig. 2. Two sera (6 and 11) reacted with all of the filtrates. Case 11 serum produced A, C, and D precipitin bands when reacted with the *A. corymbifera*, *M. pusillus*, and the *Rhizopus* spp. filtrates, whereas case 6 reacted only with antigen D.

DISCUSSION

A thorough review of the literature revealed only one brief and inconclusive report on the serodiagnosis of zygomycosis (3). No studies of the antigenic relationships among the etiological fungi of systemic zygomycosis were reported. ID studies with filtrates of *A. corymbifera*, *M. pusillus*, *R. arrhizus*, and *R. oryzae* showed intra-

TABLE 3. Immunodiffusion reactions of eight proven and three suspected cases of zygomycosis

Category	Case No.	Type of zygomycosis	Predisposing or concomitant disease	Immunodiffusion reactions with							
				Filtrates				Homogenates			
				A ^a	M ^b	Ra ^c	Ro ^d	A	M	Ra	Ro
Proven human	1	Cerebral	Diabetes	0 ^e	0	0	0	0	0	+ ^f	+
	2	Pulmonary	Aspergillosis	0	0	0	0	0	0	0	0
	3	Cerebral	Diabetes	0	0	0	0	0	0	0	0
	4	Cerebral	Diabetes	0	0	0	0	0	0	+	+
	5	Right kidney	Kidney stones	0	0	0	0	0	0	+	+
	6	Ethmoid sinuses	Renal disease, acidosis	+	+	+	+	+	0	0	0
Proven animal	7	Abortion	None	0	0	0	0	0	0	+	+
	8	Abortion	None	0	0	0	0	0	0	+	+
Suspected human	9	Pulmonary	Sarcoma	0	0	0	0	0	0	0	0
	10	Pulmonary	Hodgkin's	0	0	0	0	0	+	0	0
	11	Cerebral	Diabetes	+	+	+	+	0	+	0	0

^a A, *A. corymbifera* antigen.

^b M, *M. pusillus* antigen.

^c Ra, *R. arrhizus* antigen.

^d Ro, *R. oryzae* antigen.

^e 0, Negative reaction.

^f +, Positive reaction; a band of identity formed.

FIG. 1. Precipitin band formed between case 5 serum and antigen 11 of the *R. oryzae* homogenate. Wells 2, 3, 5, and 6 contained antiserum to the *R. oryzae* homogenate. Wells 1 and 4 contained case 5 and diabetic serum, respectively. Well AG contained the *R. oryzae* homogenate containing antigens 9, 10, 11, and 12. Two unclassified precipitin bands that formed after 72 h of incubation are located between band 9 and the lateral reference-serum wells.

generic cross-reactivity (Table 1). However, none of them demonstrated specific precipitin band formation with sera from 43 patients with proven nocardiosis and mycoses other than zygomycosis. It was thus apparent that a serum which formed at least one band of identity could be regarded as indicative of a zygomycotic infection.

The filtrates detected antibody in only 2 of the 11 sera from suspected or proven cases of zygomycosis. Consequently, their diagnostic value appears to be limited. Furthermore, both of the positive sera reacted with each filtrate. This indicated that the specific etiology of these cases of zygomycosis could not be determined serologically.

ID studies with the homogenates revealed that they contained more genera-specific antigens than the filtrates (Table 2). Cross-adsorption studies confirmed the greater specificity of the homogenates, and revealed 14 antigens among the fungi studied (Table 2 and Fig. 1). Twelve antigens were recognized after 48 h of incubation. Two additional antigens appeared

after 72 h of incubation. *R. arrhizus* and *R. oryzae* were antigenically identical to both filtrate and homogenate antigens. These findings are not surprising, since *R. arrhizus* and *R. oryzae* are very similar morphologically. The only morphological difference which keys cite between the two *Rhizopus* spp. is that *R. arrhizus* has sporangiophores 1 mm high and spores 5 to 7 mm long, whereas *R. oryzae* has sporangiophores 2 to 4 mm high and spores 7 to 9 mm long (4). Our antigenic analysis supports the contention that *R. arrhizus* and *R. oryzae* are probably conspecific.

The specificity of the homogenates was tested further with sera from 43 patients who had mycoses other than zygomycosis. Again, the homogenates showed good specificity. Because none of the heterologous case sera reacted with the homogenates to form bands identical to those formed in reference reactions, we concluded that sera which formed bands of identity were from patients with zygomycosis.

The homogenates appeared to be diagnosti-

FIG. 2. Precipitin band formed between case 10 serum and antigen 5 of the *M. pusillus* homogenate. Wells 2, 3, 5, and 6 contained antiserum to the *M. pusillus* homogenate. Well 1 contained serum from case 10, and well 4 contained diabetic serum. Well AG contained the *M. pusillus* homogenate containing antigens 1, 2, 5, 6, 7, and 8. Bands 1 and 2 are adjacent to the reference-serum wells.

cally useful, demonstrating a specificity of 100% and a sensitivity of 73%. Of the eight positive-case sera, five reacted only against antigen 11 of the *R. arrhizus* and *R. oryzae* homogenates (Table 3 and Fig. 1), two reacted only against antigen 5 of the *M. pusillus* homogenate (Fig. 2), and one reacted only against antigen 3 of the *A. corymbifera* homogenate. The antigen analysis had shown that antigens 11, 5, and 3 are specific to the *Rhizopus* spp., *M. pusillus*, and *A. corymbifera*, respectively. Therefore, a *Rhizopus* sp. was most likely to have been the etiological agent in cases 1, 4, 5, 7, and 8. *M. pusillus* was probably the etiological agent in cases 10 and 11 (Table 3), although one should note that the case 11 serum also reacted with the A, C, and D filtrate antigens. Unfortunately, cultures of the etiological agents were obtained in only two of the cases. *R. arrhizus* was identified as the fungus responsible for infection in case 4, and *A. corymbifera* was identified as the responsible fungus in case 6. On the basis of the results obtained, band 11 is apparently diagnostic of infection by either *R. arrhizus* or *R. oryzae*, whereas band 3 is apparently diagnostic of *A. corymbifera* infection. Although not substantiated by culture, band 5 is apparently diagnostic of infection by *M. pusillus*.

Many more sera from proven cases of zygomycosis with known etiology obviously need to be tested with the ID procedure before the diagnostic value of the homogenate and filtrate antigens can be conclusively evaluated. These

sera are difficult to obtain because zygomycosis is infrequently diagnosed. However, until such an evaluation is made, ID tests should be performed with at least the homogenate antigens when zygomycosis is suspected. Since the ID test is not entirely satisfactory, hopefully studies with other serological procedures will result in a more sensitive diagnostic test for zygomycosis.

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