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Exoantigen Studies of *Sporothrix schenckii*, *Ceratocystis minor*, and *Graphium penicillioides* Cultures

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Cultures of *Sporothrix schenckii* were serologically tested by the exoantigen immunodiffusion technique of Kaufman and Standard (L. Kaufman and P. G. Standard, J. Clin. Microbiol. **8**:42–45, 1978). This rapid and sensitive technique permitted the identification of 10 isolates of *S. schenckii* within 3 days. The production of the antigen-antiserum reference system and exoantigens with two different methods are reported. The demonstration of common antigens in *S. schenckii* and *Ceratocystis minor*, the suspected perfect state of *S. schenckii*, indicates that the two are antigenically related; however, the question as to whether *C. minor* represents the perfect form of *S. schenckii* will depend upon the induction of a sexual state in *S. schenckii*.

Sporothrix schenckii normally gains entry into the human body through traumatic incident (3). *S. schenckii* is a dimorphic fungus developing a mycelial form when grown at room temperature and a yeast form when cultivated at 37°C on rich medium. Conventional identification of this fungus is based on the recognition of the characteristics of the colony and conversion to its yeast form. Normally, identification presents few if any problems; however, it is a time-consuming process.

The fluorescent antibody technique is an alternate and rapid method of identification. However, fluorescent antibody reagents are not generally available or readily prepared (8). Recently, Standard and Kaufman (14, 15) reported using an immunodiffusion procedure for the rapid and specific detection of cell-free antigens (exoantigens) produced by cultures of the most important systemic fungi grown in brain heart infusion broth. The same authors subsequently were able to extract such specific exoantigens directly from agar slant cultures of the same fungi with an aqueous solution of merthiolate (9). The new procedure permitted the identification of suspected cultures of the pathogens 2 days after obtaining a pure mycelial culture. In our Institute we applied the exoantigen technique to the rapid and specific identification of *S. schenckii* mycelial cultures. Their differentiation from other taxonomically or morphologically related pathogenic and saprophitic *Hyphomycetes* was also studied.

MATERIALS AND METHODS

Cultures. Some cultures used in the study were from our collection (UCSC). Others were kindly furnished by Libero Ajello, Mycotic Diseases Division, Centers for Disease Control, Atlanta, Ga. (CDC), and by Robert Samson, Centraalbureau Voor Schimmelcultures, Baarn, The Netherlands (CBS). The cultures had been isolated either from clinical lesions or from natural sources. The cultures were as follows: *S. schenckii* 0 (UCSC), 2 (UCSC), B2473 (CDC), B2560a (CDC), B2688a (CDC), B2904a (CDC), B3098a (CDC), 302.73 (CBS), 340.35 (CBS), and 930.72 (CBS); *Acremonium* sp. 0 (UCSC), *A. falciforme* 0 (UCSC), and *A. strictum* 550.73 (CBS); *Aphanocladium* sp. 44 (UCSC); *Beauveria* sp. 0 (UCSC) and *B. bassiana* 102 (UCSC) and 299.27 (CBS); *Blastomyces dermatitidis* A373 (CDC); *Ceratocystis minor* B2908a (CDC) and 2909a (CDC); *Chrysosporium parvum* 0 (UCSC) and *C. tropicum* 0 (UCSC); *Graphium penicillioides* 470.71 (CBS); and *Sporotrichum* sp. 1 (UCSC).

Antiserum production. The production of the reference antiserum was carried out by immunizing rabbits. The antigen was made up of yeast cells of *S. schenckii* isolate no. B2473 grown for 1 week in 1-liter flasks containing 400 ml of brain heart infusion broth shaken in a rotary incubator (150 rpm) and maintained at 37°C. The yeast cells were centrifuged (3,000 rpm), suspended in a formalinized saline solution, and adjusted to a McFarland no. 5 optical density standard. Rabbits were injected in the marginal ear vein with 1, 2, or 2 ml of the yeast suspension for 3 consecutive days. The same inoculation procedure was repeated after a rest period of 5 days. The rabbits were bled from the heart 10 days after the last inoculation of the second series. The anti-*S. schenckii* reference serum proved to be satisfactory, exhibiting a 1:512 titer in a tube agglutination test (2). The antiserum with merthiolate (1:10,000) was lyophilized and stored at room temperature.

Reference antigen. *S. schenckii* isolate no. B2473 was cultivated in 1-liter flasks containing 400 ml of brain heart infusion broth for 1 week in a rotary shaker (150 rpm) at 37°C. Other culture flasks were kept in constant agitation with glass beads to obtain optimal production of the antigen. Merthiolate (1:5,000) was added to the flasks and then kept for 48 h at 25°C. The yeast cells and the glass beads were removed by

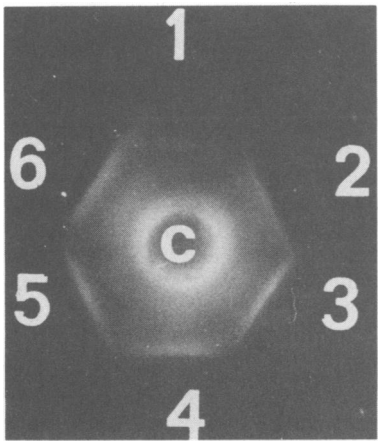

FIG. 1. Reference antigen choice for *S. schenckii* identification by immunodiffusion. Well contents: C, reference antiserum; 1 and 4, broth culture supernatant; 2 and 3, broth culture supernatant (5×); 5 and 6, broth culture (5×).

filtration after a viability control had been performed. The filtrates from cultures, simply agitated or disrupted by glass beads, were tested for their antigen content with and without 5× concentration. In addition, a filtrate was tested after centrifugation (3,000 rpm for 20 min) and concentration (5× in Amicon Minicon B 15 concentrators; Amicon Corp.). The antigen prepared from the centrifuged and concentrated filtrate gave the best results and was used as a reference (Fig. 1).

Exoantigen production. The production of cell-free antigens (exoantigens) from the *S. schenckii* isolates and other fungal cultures was carried out with two different techniques. For the first method 100-ml flasks containing 30 ml of brain heart infusion broth were inoculated with a 5-mm Sabouraud dextrose agar square of mycelial growth from slant cultures and incubated with shaking for 3 to 6 days at 25°C. The broth cultures were treated with merthiolate (1:5,000), the mycelium was removed by filtration, and the filtrate was centrifuged (3,000 rpm for 20 min). The supernatant was finally concentrated 25× in Amicon Minicon B 15 concentrators (14, 15). For the second method, cultures showing luxuriant growth on Sabouraud dextrose agar after 10, 21, 30, or 85 days were covered with 8 ml of an aqueous solution of merthiolate (1:5,000) and kept for 24 h at 25°C. After this period, 5 ml of the extract was concentrated 25× in Amicon Minicon B 15 concentrators (9).

Serological test. An agar gel double-diffusion procedure was used for serological tests. The medium and the methodology used were those recommended by the CDC for the serological diagnosis of histoplasmosis by immunodiffusion (13). The exoantigens produced with the two different techniques were tested by using the reference antigen as a control.

Adsorption of antisera. Antigens (1 ml) were placed in glass tubes and lyophilized. Reference anti-*S. schenckii* serum (1 ml) was added to the dried antigens. The antigen-antiserum mixture was agitated to dissolve the antigens completely and incubated at 37°C for 2 h in a rotary water bath. After incubation, the reagents were centrifuged for 45 min at 10,000 rpm, and the supernatant was used in the immunodiffusion test for evidence of adsorption (7).

RESULTS

All of the 10 *S. schenckii* isolates tested, independent of their source, demonstrated exoantigens identical to the specific precipitinogens of the reference antigen. There was no significant antigenic differences between preparations obtained by the two different techniques of exoantigen production. Regardless of age, the same cultures did not exhibit any significant antigenic variations. In the antigen-antiserum reference system there appeared three precipitin bands, homologous to the precipitins revealed by the reaction between the exoantigens of the *S. schenckii* isolates and the reference antiserum (Fig. 2).

The exoantigens of several *Hyphomycetes* tested did not produce any precipitin bands. The exoantigens of the *Sporotrichum* sp., however, produced heterologous precipitin bands, but these did not cause any diagnostic problems (Fig. 3).

The exoantigens of *C. minor* and *G. penicillioides* produced homologous precipitin bands when compared with the reference antiserum (Fig. 4 and 5). Adsorption of reference antiserum with the exoantigens produced from cultures of *C. minor* and *G. penicillioides* caused the disappearance of the reference precipitins.

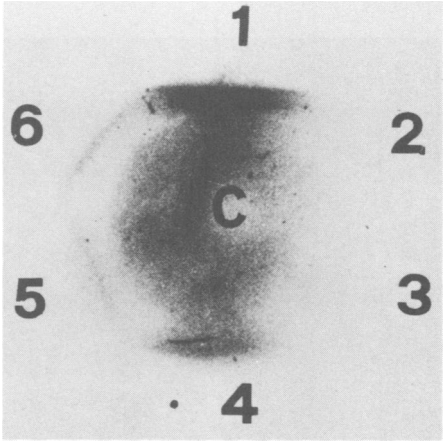

FIG. 2. Specificity of the immunodiffusion exoantigen technique for *S. schenckii*. Well contents: C, reference antiserum; 1 and 4, reference antigen; 2 and 3, *C. tropicum* exoantigens; 5 and 6, *S. schenckii* exoantigens.

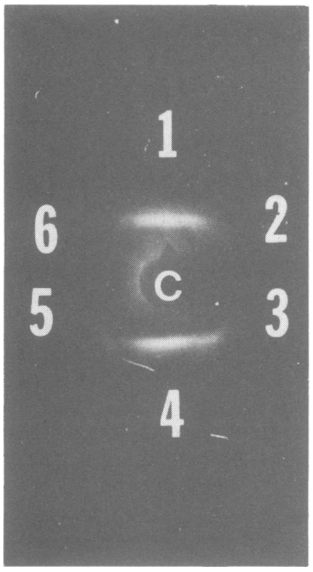

FIG. 3. Specificity of the immunodiffusion exoantigen technique for *S. schenckii*. Well contents: C, reference antiserum; 1 and 4, reference antigen; 2 and 3, *Sporotrichum* sp. exoantigens; 5 and 6, *Petriellidium boydii* exoantigens.

DISCUSSION

Sporothrix and *Ceratocystis* spp. have been extensively investigated primarily by French and Japanese investigators. Mariat (10), basing his opinions upon morphological resemblances and modalities of experimental infections in hamsters, suggested that the ascomycete *C. stenoceras* might be considered to be the perfect form of *S. schenckii*. Mariat's hypothesis is supported by comparative analysis of the lipid composition of the cell walls of *C. stenoceras* and *S. schenckii* (4).

Harada and his co-workers (6) investigated the antigenic similarity between *Ceratocystis* spp. and *S. schenckii* by an indirect immunofluorescence technique. They were able to specifically stain the isolates of *S. schenckii* from clinical specimens by adsorbing the *S. schenckii* fluorescein-conjugated antiserum with cultures of *S. schenckii* isolated from soil or cultures of other species, demonstrating, in this manner, antigenic variation in the cell wall of the strain adapted to the parasitic form.

Mariat (10) suggested that once *S. schenckii* enters a host, it loses its ability to form perithecia, thus explaining the failure to have human isolates form perithecia. The perithecia of *C. stenoceras* grown on corn meal agar do not fluoresce either with adsorbed or with Harada's unadsorbed anti-*S. schenckii* antiserum (6).

The serological differences among *S. schenckii* isolates were attributed by other authors (12) to quantitative differences in cell wall rhamnomannans which determine their antigenic properties. On the other hand, Travassos and co-workers (16), through proton and ^{13}C mag-

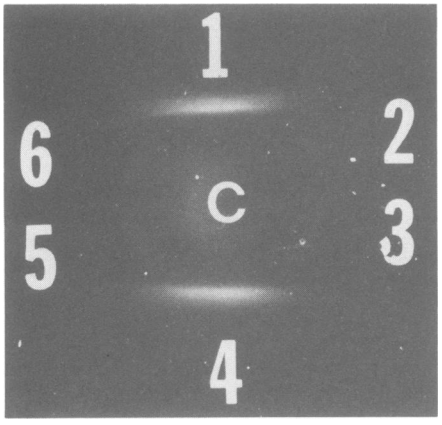

FIG. 4. Identity in the immunodiffusion exoantigen technique of the *S. schenckii* precipitin bands of *C. minor* and *S. schenckii*. Well contents: C, reference antiserum; 1 and 4, reference antigen; 2 and 3, *C. minor* exoantigens; 5 and 6, *S. schenckii* exoantigens.

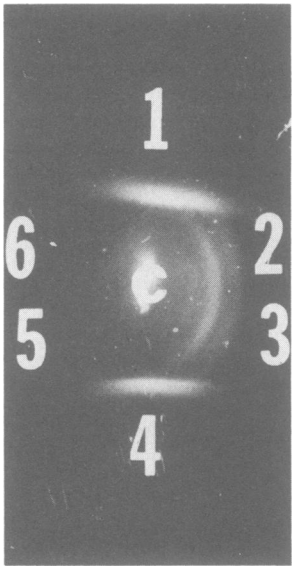

FIG. 5. Identity of the *S. schenckii* precipitin bands in the immunodiffusion exoantigen technique between *G. penicillioides* and *S. schenckii*. Well contents: C, reference antiserum; 1 and 4, reference antigen; 2 and 3, *G. penicillioides* exoantigens; 5 and 6, *S. schenckii* exoantigens.

netic resonance studies, determined that the rhamnomannans of *S. schenckii* and *C. stenoceras* were closely related, with only slight differences. Mendonca-Hagler and co-workers (11) investigated the DNA base composition of *S. schenckii* and *Ceratocystis* spp. by guanine plus cytosine content and found that only *C. minor* showed guanine plus cytosine values similar to those obtained from *S. schenckii*. Their studies would suggest that *C. minor* is more likely to be the perfect form of *S. schenckii*. Skin tests with filtrates extracted from cultures of *Ceratocystis* sp. sometimes give positive reactions in patients with cutaneous sporotrichosis (T. Iwazi, H. Kariya, H. Ishizaki, and Y. Nakamura, 530th monthly meeting of the Tokyo Branch, of the Japanese Association of Dermatologists, 1975).

A taxonomic review of the genus *Ceratocystis* was carried out by de Hoog (5). According to him the genus *Ceratocystis* may be divided as follows: (i) *Ceratocystis* sensu stricto, with *Chalara, Chalaropsis*, and *Thielaviopsis* as conidial states; and (ii) *Ophiostoma* with *Sporothrix, Verticladiella*, and *Graphium* as conidial states. In his experience it was impossible to find any constant morphological variation between *S. schenckii* and the conidial states of *Ophiostoma stenoceras* (*C. stenoceras*) and *O. tetropis*, although the average variability between these two species of *Ophiostoma* and *S. schenckii* did not correspond exactly.

During our study the only precipitin bands homologous to the reference system were found in the isolates of *C. minor* and *G. penicillioides*. This finding corroborates the close relationship between these species (1). This hypothesis was supported by the disappearance of reference precipitins after adsorption of reference antiserum with the exoantigens produced from cultures of *C. minor* and *G. penicillioides*. Adsorption of reference antiserum with reference antigen did not reveal any precipitin, confirming indirectly the specificity of the reaction.

The exoantigen technique proved to be apparently specific, sensitive, and rapid when applied to the serological identification of *S. schenckii*, allowing the identification of the cultures within 3 days regardless of the variability of the isolates. This technique is considered to be an effective tool in the investigation of complicated problems in taxonomic mycology.

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