Abstract  In vivo skin testing and in vitro lymphocyte blastogenesis were evaluated in a young adult population as methods for detecting cellular immunity to Sporotrichum schenckii. Similar procedures for Candida albicans and Coccioidoides immitis were also investigated. 5 of 143 subjects had positive skin tests and 14 had positive blastogenic responses to S. schenckii. These 14 subjects also exhibited unusually high responses to C. albicans in vitro and 11 of the 14 were female. Data demonstrated a correlation coefficient of 0.89 when comparing the blastogenic assays for S. schenckii and C. albicans, suggesting cross antigenicity.

Intact cellular immune mechanisms in combination with exposure to C. albicans may protect the host from systemic infection with S. schenckii. Although a limited number of subjects were studied, as a group, females had more vigorous cellular immune responses to C. albicans than males. The rare occurrence of sporotrichosis in females as compared to males may be the result of antigenic stimulation from commonly observed vaginal colonization with C. albicans. The present data indirectly support this hypothesis.

Introduction

Although accidental trauma with inoculation of the fungus, Sporotrichum schenckii, probably accounts for most cases of cutaneous-lymphatic sporotrichosis, systemic disease may be primarily the result of inadequate host immune defense. The overwhelming male predominance cannot be explained by outdoor exposure alone and the rarity in children, but predisposition among immunosuppressed hosts and alcoholics also suggests that endogenous host factors may be of major import (1, 2).

The immune mechanisms involved in preventing or combating systemic sporotrichosis are incompletely understood but apparently include both humoral and cellular responses since patients with defects in antibody production or cellular immunity may be predisposed. The relative importance of the cellular immune response has not been well defined, but the severe and often fatal infections of patients with cancer and those on immunosuppressive therapy, both conditions known to depress cellular immune competence, suggest that cellular immunity may provide primary host defense. Moreover, fungal infections such as systemic candidiasis have been reported in children with thymic deficiency syndromes (3, 4).

The present study was undertaken to develop an in vitro assay of cellular immunity to S. schenckii and to compare this assay to in vivo skin testing. The in vitro method employed was lymphocyte blastogenesis in response to the sporothrix antigen. The application of these assays for diagnosis of a patient with articular sporotrichosis is also presented. In addition, similar assays of cellular immunity to Candida albicans and Coccioidoides immitis were evaluated in adult males and females to delineate any cross-reactivity between these agents which might then account for the apparent protection of females from infection with S. schenckii.

Methods

Lymphocyte blastogenesis. Lymphocyte reactivity to the three fungal agents was evaluated by determining lymphocyte blastogenesis during incubation with the fungal antigens (5). Briefly, lymphocytes were separated from 4-5 ml of peripheral whole blood by a modification of the
Hypaque-Ficoll gradient technique (6). The cell suspension was washed twice in culture medium and the lymphocytes were adjusted to a concentration of \(2 \times 10^6\) ml in RPMI 1640 medium with 100 U of penicillin per ml and 100 \(\mu\)g of streptomycin per ml and 20% autologous serum. In some experiments, fetal calf serum and AB-positive serum were also employed. Using a bio- 

pipet, 0.1 ml of the lymphocyte suspension (2 \(\times 10^6\) lymphocytes) was mixed with an equal volume of fungal antigen material in one well of sterile flat-bottom microtest plates. All cultures were prepared in triplicate and three dilutions of each fungal antigen were included along with control cultures containing lymphocytes incubated with medium alone. The plates were covered with sterile plastic lids and cultures were incubated for 5 days. 24 h before harvest, 0.05 ml of RPMI 1640 medium containing 0.1 \(\mu\)Ci of [2-

\(^3\)C]thymidine was added to each well. A harvesting ap- 

paratus previously described (7) was employed for the sepa- 

ration of reacting cells on glass-fiber filters, for washing 

of these cells, and for recovery of the radioactive material 

incorporated by stimulated lymphocytes. The glass-fiber 

disks were then dried in an oven and transferred to vials 

containing 5 ml of scintillation fluid for counting in a 

Beckman Liquid Scintillation Spectrometer (Beckman In-

struments, Inc., Fullerton, Calif.). The average counts per 

minute (cpm) of triplicate samples were determined and 

results were expressed as a blastogenic index (BI)

\(\frac{\text{cpm for lymphocytes + antigen}}{\text{cpm for lymphocytes + medium}}\) 

Fungal antigens. *S. schenckii* antigen used in the in vitro 

assay of lymphocyte blastogenesis was prepared from the 

mycelial phase of the fungus which had been originally 

isolated from a patient with systemic sporotrichosis. The 

organisms were grown on Sabouraud's glucose agar at 30°C. 

The organism was harvested, washed three times with tissue 

culture medium and homogenized in a Dounce homogenizer. 

Large particles were allowed to settle, after which the 

supernate was collected and serial dilutions subsequently 

tested for antigenic reactivity as described above. Approxi-
mately 200 mg per ml of antigenic material constituted the 

stock preparation. This material was later heat inactivated 

at 56°C for 60 min after it was demonstrated that this 

process did not alter the sensitivity or specificity of the 

assay. A dilution of 1:200 was the optimal concentration 

for most positive responders.

Material for skin testing to *S. schenckii* was kindly sup- 
plied by Dr. Leo Kaufman of the Mycoses Immunology 

Unit, Center for Disease Control, Atlanta, Ga. The yeast 

phase of the organism was grown on brain-heart infusion 

broth (Difco Laboratories, Detroit, Mich.) at 37°C and 

shaken at 150–160 rpm. The preparation was treated with 

1:10,000 thimerosal, filtered to remove the whole yeast cells 

dialyzed against distilled water.

*S. albicans* antigen used both for skin testing and in vitro 

study was obtained from Hollister-Stier Laboratories, Inc., 

Yeadon, Pa., as Dermatophytin "O" undiluted, and *C. im-

mitis* antigen was obtained as a commercial lot from The 

Cutter Laboratories, Berkeley, Calif. These preparations 

were dialyzed six times against 200 ml of 0.15 M NaCl 

for 48 h to remove preservatives. Material was stored at 

\(-20°C\) until used. For skin testing, a 1:100 dilution of 

the original preparation of *C. albicans* and a 1:10 dilution 

of *C. immitis* were employed. Maximum in vitro stimulation 

of lymphocytes was obtained with a 1:2,000 dilution of *C. 

albicans* and 1:5,000 dilution of *C. immitis*.

Skin testing. Skin test material was applied in the usual 

manner by injecting 0.1 ml intradermally on the volar sur-

face of the forearm. Results were measured at 24, 48, and 

72 h with the maximum response recorded. A skin test was 

considered positive (+) if the amount of induration was 

10 mm or greater for *C. albicans* and 5 mm or greater 

for *S. schenckii* and *C. immitis*. Erythema was recorded 

but not considered of importance when the final data were 

analyzed.

Test subjects. 143 young, healthy adults, 18–39-yr-old, 

were evaluated for their skin test (in vivo) reactivity and 

lymphocyte blastogenic (in vitro) responses to the three 

fungal antigens. These subjects included 87 female and 

56 male volunteers. Most were students or hospital per-

sonnel. The average age for males and females was similar 

(27±4 yr vs. 25±3 yr) and none had histories of sporotrichosis 

infection or significant contact with gardens or nurseries.

Study case. A 58-yr-old Caucasian male with articular 

sporotrichosis was evaluated with the assays outlined. His 

diagnosis was confirmed by recovery of the organism from 

joint fluid. Serum antibody to *S. schenckii* was 1:256 by 

tube agglutination and 1:32 by latex agglutination.

He had noted the gradual onset of pain in his left knee 

2 yr before admission but was not evaluated until 1 yr 

after onset of symptoms when X rays revealed a cystic 

lesion of the left patella. The patella was removed and 

found to have granulomatous changes but no organisms 

were found by culture or by special fungal stains. 2 mo 

after operation drainage from the incision site occurred 

from which *S. schenckii* was cultured. The subject was 

treated with potassium iodide for 3 mo, but shortly there-

after swelling and pain recurred and aspiration of the left 

knee joint revealed *S. schenckii* on culture. Therapy with 

Amphotericin B was begun.

RESULTS

Data for skin testing and in vitro assays of lymphocyte 

blastogenesis are summarized in Table I. Results for 

each of the three fungal antigens were similar in that 

control subjects whose skin tests were positive also 

always had positive in vitro blastogenic reactions; 

however, occasional donors with negative skin tests had 

positive in vitro assays. More specifically, 21 control 

subjects (14.7%) had negative skin tests to *C. albicans* 

but a BI > 3 to the same antigen.

*C. albicans*. As summarized in Table I, a higher per-

centage of women than men had both positive skin 

tests, 86 vs. 73% and blastogenic indices, 99 vs. 91%. 

The mean BI was also higher in women, 15.5±8.4, as 

compared to 6.7±3.4 for male control subjects; this 

difference was statistically significant (P < 0.05).

*S. schenckii*. The mean BI to *S. schenckii* in the 

study population was 1.08±0.78. Therefore a BI of three 

or greater was considered positive. Four female and one 

male subject had positive skin tests to *S. schenckii* and 

11 females and 3 males had a BI > 3. Positive skin tests 

ranged from 6-10 mm. Fig. 1 includes all subjects with

*Abbreviations used in this paper: BI, blastogenic index; 
FBS, fetal bovine serum.*
TABLE I

Skin Test (In Vivo) and Lymphocyte Blastogenic (In Vitro) Reactions to C. albicans, S. schenckii, and C. immitis in a Young Adult Population

<table>
<thead>
<tr>
<th></th>
<th>C. albicans</th>
<th>S. schenckii</th>
<th>C. immitis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(+) Skin*</td>
<td>(+) BI</td>
<td>(+) Skin</td>
</tr>
<tr>
<td>Subjects</td>
<td>n</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Total</td>
<td>143</td>
<td>116 (81)</td>
<td>137 (96)</td>
</tr>
<tr>
<td>Female</td>
<td>87</td>
<td>75 (86)</td>
<td>86 (99)</td>
</tr>
<tr>
<td>Male</td>
<td>56</td>
<td>41 (73)</td>
<td>51 (91)</td>
</tr>
</tbody>
</table>

* A positive reaction (+) indicates induration of 10 mm or greater for C. albicans and 5 mm for S. schenckii and C. immitis.
† A blastogenic index (BI) of 3 or greater was considered positive (+).

A BI for C. albicans of > 15.0 and demonstrates that the donors with a positive BI for S. schenckii were those with unusually high blastogenic responses to C. albicans.
The data presented in Fig. 1 exhibited a correlation coefficient of 0.89 for blastogenic responses to candida as compared to sporothrix. This correlation is interpreted as being highly significant.

C. immitis. Four donors, three male and one female, had both positive skin tests and in vitro blastogenic responses to C. immitis. The average BI for these four donors was 6.1. None of these had positive results with S. schenckii but all were also positive in vivo and in vitro to C. albicans. The reactions to candida were not unusually high.
The assays were also repeated substituting fetal bovine serum (FBS) and AB-positive antibody-negative serum for autologous serum. No significant alteration was observed in the blastogenic assays. FBS frequently appeared to stimulate significantly the lymphocyte cultures, giving high background counts and thus obviating meaningful interpretation of results.

Study case. Data from the patient with articular sporotrichosis are summarized in Table II. Compared to the test group, this patient was the only one we have...
studied whose skin test was positive and whose BI to *S. schenckii* was higher than the BI to *C. albicans*. There were two test subjects with a BI > 17.1, the determination for this patient; but in both instances the response to *C. albicans* was strikingly high as seen in Fig. 1.

Other patients we have studied have included those with systemic candidiasis and systemic coccidioidomycosis. None of these patients have thus far exhibited positive responses to *S. schenckii*. Such cases, however, usually represented immune deficiency states.

**DISCUSSION**

*S. schenckii* is an uncommon cause of systemic infection in the United States as well as most parts of the world, and this rarity has certainly discouraged careful investigation into the natural history and pathogenesis of the organism. The epidemic outbreak including almost 3,000 gold mine workers in Witwatersrand, South Africa, 3 decades ago strikingly demonstrated the potential hazards of the fungus, and this mycosis does remain endemic among workers in South Africa, France, Mexico, and perhaps among nursery workers in many countries (1, 2). Moreover, the incidence of systemic infection appears to be increasing, especially in the immunologically compromised host (1). In previous reviews of systemic sporotrichosis (1, 2) one-half of reported cases were observed in patients with underlying malignant disease.

Present methods of diagnosis include primarily fungal culture and serological methods. For cutaneous-lymphatic sporotrichosis, fungal cultures offer the most direct and reliable laboratory procedure, but cultures are often negative in systemic disease since adequate specimens are difficult to obtain. For this reason, the clinician must usually rely on other methods of diagnosis. Serological assays, including slide latex agglutination, tube agglutination, complement fixation, immunodiffusion, and indirect fluorescent antibody techniques are available in a few research laboratories and, depending on the assay employed, are capable of detecting between 56 and 94% of the cases of sporotrichosis (8, 9). Reports indicate that these methods are specific and reliable. However, there is increasing evidence that development of cellular immunity precedes antibody production (10), and thus tests of cellular immunity to *S. schenckii* may detect infection earlier in the course of the disease. Early detection is, certainly, most essential when ministering to debilitated or immunologically compromised patients. In this clinical setting, immune responses may be somewhat decreased, but complete absence of reactivity in assays like those presently considered should not occur except in severe or terminal states.

This report evaluated in vivo and in vitro methods of detecting reactivity to *S. schenckii* using a skin test and an assay of lymphocyte blastogenesis. The skin test has been investigated previously and demonstrated to be a useful diagnostic tool (11, 12). In one study (12) 11% of the control population had positive skin test responses as compared to 58% in a population of nursery workers who had at least 10 yr of exposure to plants and soil. No information is available from this study as to responses to *C. albicans* skin test antigen. There were no differences with sporotrichis according to sex. In vitro assays of cellular immunity to *S. schenckii* have not been previously reported. It should be noted that blastogenic reactivity, particularly, low level responses, may represent B cell stimulation. Although there is no evidence to date that B cells respond to the antigens employed in the present report, further investigation is needed to confirm this assay as indicating only cellular immune (T cell) reactivity.

The present study confirms the sensitivity of the skin test material, as only 5 of the 143 control subjects developed a positive reaction while a patient with articulor sporotrichosis had a strongly positive response. Similarly, the in vitro blastogenic assay appeared to be a useful diagnostic procedure, as results for the study case were also positive and higher than responses to other fungal antigens employed. However, further studies on other patients with systemic sporotrichosis are certainly needed to confirm the applicability of these diagnostic tests.

Of great interest is the cross-reactivity between *C. albicans* and *S. schenckii* as demonstrated in the assays of lymphocyte blastogenesis. This is most probably on the basis of antigens common to the two mycotic agents which contribute to development of the cellular immune response. Such cross-reactivity has also been observed in previous serological studies when the whole yeast agglutination assay was employed (13, 14). Other serological methods have not demonstrated similar cross-reactivity (8, 9) suggesting that either the assays of lymphocyte blastogenesis and yeast agglutination are more sensitive or that different antigens are responsible for stimulating some antibody responses.

Most reported cases of sporotrichosis have occurred in males and the observed cross-reactivity between

**Table II**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Skin test*</th>
<th>BI</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. albicans</em></td>
<td>30</td>
<td>8.3</td>
</tr>
<tr>
<td><em>S. schenckii</em></td>
<td>15</td>
<td>17.1</td>
</tr>
<tr>
<td><em>C. immittis</em></td>
<td>Negative</td>
<td>1.4</td>
</tr>
</tbody>
</table>

* Millimeter of induration.

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C. albicans and S. schenckii may in part account for the lower incidence of infection in females. A recent report (1) indicated that only 1 of 26 patients with systemic sporotrichosis was a female. Even in children there has been a male predominance (15) for sporothrix infection further implying protection in the female host early in life.

The present laboratory data demonstrate that virtually all normal females have positive in vitro responses (i.e. a BI > 3) to C. albicans and that their responses are significantly higher than those of normal male donors. A higher percentage of females than males also have positive in vitro responses to S. schenckii but this appears most related to an increased sensitivity to C. albicans (Fig. 1).

It is well recognized that C. albicans is a frequent inhabitant of the female vaginal tract, thus providing persistent antigenic stimulation during much of life. Males certainly do not carry this mycotic agent as commonly and it is not surprising, therefore, to observe less consistent and lower cellular immune responses to C. albicans. Other groups we have evaluated who have exhibited decreased skin sensitivity and lymphocyte blastogenesis to C. albicans have included patients with malignancy and those on immunosuppressive therapy; these patients are also ones observed to have a greater predisposition to sporotrichosis infection (1, 2).

In conclusion, the present data indirectly support the hypothesis that intact cellular immune mechanisms in combination with exposure to the fungus C. albicans protect the host from systemic infection with S. schenckii; however such cross resistance remains to be proven. Many aspects of sporotrichosis infection have yet to be adequately defined, particularly the role of environmental exposure to S. schenckii and the relative importance of antibody production. There is still no direct evidence that cell-mediated immunity protects against sporotrichosis. Sporotrichosis is, of course, too uncommon for any one medical center to accumulate meaningful data, so such information can best be provided by combining the experience of many centers.

Because present therapy for systemic sporotrichosis must include the highly toxic antifungal antibiotic agent, amphotericin B, a better understanding of host defense mechanisms may direct advances in improving management. If cellular immunity is of major importance, as is suggested by our present understanding of fungal disease, then enhancement of these immune responses with agents like levamisole or with transfer factor may provide a beneficial adjunct to current therapy.

ACKNOWLEDGMENTS

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REFERENCES