Clinical Usefulness of Lymphocyte Transformation in Patients with Coccidioidomycosis*

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The development of an appropriate host defense in coccidioidomycosis is predicated on the presence of a positive delayed skin test reaction to coccidioidin. In severe and/or disseminated disease, coccidioidin reactions are routinely negative. By employing serial in vitro spherulin-induced lymphocyte blast transformation (LT) studies in a group of eight severely-ill coccidioidomycosis patients, prognostic clinical data were provided which could not have been obtained from their skin test status alone. Four of the eight demonstrated positive LT responses early in the course of their disease, quickly converted their skin tests to positive, and were cured of their disease. Two patients had negative LT responses until their skin test converted after several months of therapy. The final two have continued to demonstrate negative LT values despite several years of therapy and have experienced exacerbations of their disease when treatment was discontinued. The use of LT data in such patients can be very helpful in guiding therapeutic decisions in this difficult clinical problem.

Host defense against systemic fungal infections is primarily dependent upon the development of an appropriate cell-mediated immune (CMI) response. Clinically, such a response is identified by the presence of a delayed skin reaction 36 to 48 hours after inoculation with a preparation of the fungal antigen. Among the three primary systemic fungal infections, coccidioidomyocosis, histoplasmosis, and blastomycosis, the presence or absence of a skin reaction has significant clinical value only in coccidioidomycosis. More than 40 years ago, Smith¹ demonstrated that over 90 percent of individuals exposed to the fungus Coccidioides immitis became skin test positive to coccidioiden antigen within 14 days. Such positive reactions continue to be present for many years and are associated with the presence of cell-mediated immunity to the offending organism. Conversely, the failure to develop a positive reaction following fungal exposure, or the reversion of a positive to a negative reaction in the face of clinical infection, implies an inadequate cell-mediated host immune response.

In recent years, the high sensitivity of the coccidioidin skin test reaction noted by Smith has been questioned.²⁻³ In one acute outbreak of coccidioidomyocosis among college students, Werner et al⁴ found the reaction rate to be only 65 percent. Also, the initial reports of an increased sensitivity with spherulin antigen⁵⁻⁶ have not been confirmed.⁷ At the same time, there is an increasing number of patients in the endemic coccidioidomycosis area with significantly impaired immune defenses. Recent reports indicate that a large percentage of those with disseminated, frequently fatal disease are those whose immune system is compromised secondary to immunosuppressive therapy for organ transplantation or oncologic chemotherapy.⁸⁻¹³ Bronnimann et al¹⁴ have shown a high percentage of patients in the endemic area with the acquired immune deficiency syndrome have coccidioidomycosis during their disease and at autopsy.

Because of the apparent decrease in the sensitivity of the coccidioidin skin test in immune competent individuals, and the increasing number of patients with severe and/or disseminated disease in which the skin test is uniformly negative, we have developed a specific in vitro lymphocyte transformation test to more accurately assess cellular immune competence in patients with coccidioidomyocosis. Using coccidioidal spherule antigen (spherulin), and expressing lymphocyte blast transformation (LT) values in absolute counts per minute (CPM), this test has provided excellent discrimination between immune and nonimmune subjects.¹³ When compared with skin test reactivity to spherulin and coccidioidin, the LT assay was more sensitive than the skin test. Subjects with mild self-limited disease, as well as those with positive skin tests only, were readily distinguished from nonimmune skin test negatives.¹⁴

The purpose of this study was to determine the usefulness of the spherulin LT assay in predicting clinical outcome in a group of patients with severe and/or disseminated disease, all of whom were coccidioidin skin test negative at the time of presentation.

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METHODS

Patient Population

All subjects were seen in the hospital or chest clinic at the University of Arizona Medical Center. A diagnosis of coccidioidomycosis was initially made on clinical grounds and confirmed by culture of Coccidioides immitis from disease sites. Skin tests with coccidioidin (1:100) and spherulin (standard dose) were applied and read by experienced nurses. At the time of initial evaluation, these were uniformly negative with positive reactions to either Candida or triphophyton control antigens. All patients were treated with either amphotericin, ketoconazole, or both in sequence. Informed consent was obtained from each subject in accordance with the institutional Human Subjects Committee.

Lymphocyte Function Studies

Details of the laboratory methods employed have been described previously. They may be summarized as follows: Lymphocytes were separated from heparinized blood samples by density gradient centrifugation, using Ficoll-Hypaque or lymphocyte separating media. Cells were then aspirated and washed in phosphate buffered saline solution. Viability counts ranged from 98 to 100 percent, with a mean recovery rate of 65 percent.

Lymphocyte blast transformation (LT) was carried out by a microculture technique. Transformation was induced by preservative-free spherulin and phytohemagglutinin (PHA). For the latter, the concentration employed was that which from previous studies produced the maximum transformation response. All PHA and control determinations were run in triplicate using \(1 \times 10^5\) lymphocytes in a total volume of 0.2 ml. Spherulin antigen was titered to determine the dilution that gave the maximum transformation response with cells diluted in growth media containing 20 percent autologous plasma from a healthy spherulin skin test-positive control subject. Spherulin antigen was used in 10, 20, and 30 \(\mu\)l volumes in triplicate to determine the dose response curve. Lymphocytes in a concentration of 10\(^4\)/ml were added in 0.2 ml volumes (2 \(\times 10^9\) well) to each concentration of spherulin. Unstimulated control samples were run to assess spontaneous transformation.

All microtiter plates were incubated at 37°C in a humidified incubator containing 5 percent carbon dioxide. The PHA-stimulated cells were pulsed on day 3, and spherulin stimulated cells pulsed on day 5 with 50 \(\mu\)l (1.0 mc) of tritiated thymidine. From 18 to 24 hours after pulsing, the cells were harvested onto fiberglass filters which were dried overnight. The filters were then placed in scintillation vials and counted in a scintillation counter. For each dose, antigen-induced blast transformation was expressed as the mean of the triplicate counts per minute (CPM). The highest mean value in response to the three different doses of antigen was used for each patient. On the basis of our previously reported data in a large number of immune (skin test positive [STP]) and nonimmune (skin test negative [STN]) subjects, all lymphocyte transformations were reported as CPM. Reporting in this fashion avoids the potential influence of the autologous mixed lymphocyte reaction, which influences the "spontaneous" control counts and could affect the stimulation index. Spherulin-induced LT responses of 10,000 CPM or greater to spherulin antigen have been routinely found in healthy immune control subjects and those with self-limited disease.

RESULTS

The patient characteristics and the clinical nature of their disease are shown on Table 1. A majority (six of eight) were men and dark skinned, with an age range from 18 to 71 at the time of presentation. Except for patient 8, all were seen within five months of the onset of their disease. Extrathoracic involvement uniformly involved the lymph nodes or skin or both. The latter varied from relatively innocuous-appearing erythematous pustules to widespread abscesses. In the two older patients (4 and 6), involvement was limited to the lung, but in both, the disease was bilateral and diffuse, with associated hypoxemia. C. immitis was also grown from the blood of patient 6. Coccidioidin complement fixation titers were quite variable, and often did not peak until several weeks following diagnosis. Patient 5, for example, presented with a titer of only 1:16, which reached a maximum of 1:512 three months later, while being treated with amphotericin B. In keeping with the common clinical observation in patients with severe coccidioidomycosis, all patients had negative delayed skin reactions to coccidioidin.

The LT values for the eight patients are listed in Table 2. Despite their negative coccidioidin skin tests, a wide range of LT values was evident. The lymphocytes of four patients (1 through 4) exhibited positive (CPM \(\geq\)10,000) in vitro responses to spherulin-in-
Table 2—Immunologic Characteristics of the Patients During Study Period

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Initial LT CPM</th>
<th>CF Titer</th>
<th>Treatment Status at Presentation, Mo</th>
<th>Total No. LT Studies</th>
<th>Mean LT CPM (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23,543</td>
<td>1:256</td>
<td>0</td>
<td>5</td>
<td>19349 (13,491-27,412)</td>
</tr>
<tr>
<td>2</td>
<td>10,327</td>
<td>1:8</td>
<td>0</td>
<td>4</td>
<td>9238 (4,502-14,013)</td>
</tr>
<tr>
<td>3</td>
<td>15,338</td>
<td>1:8</td>
<td>4</td>
<td>3</td>
<td>12464 (9,380-15,338)</td>
</tr>
<tr>
<td>4</td>
<td>16,121</td>
<td>1:128</td>
<td>7</td>
<td>5</td>
<td>18511 (5,609-42,549)</td>
</tr>
<tr>
<td>5</td>
<td>2,465</td>
<td>1:128</td>
<td>0</td>
<td>5</td>
<td>9916 (2,465-31,345)</td>
</tr>
<tr>
<td>6</td>
<td>3,011</td>
<td>1:64</td>
<td>0</td>
<td>5</td>
<td>7603 (1,154-21,474)</td>
</tr>
<tr>
<td>7</td>
<td>1,464</td>
<td>1:16</td>
<td>0</td>
<td>8</td>
<td>1702 (587-3,925)</td>
</tr>
<tr>
<td>8</td>
<td>3,213</td>
<td>1:16</td>
<td>8</td>
<td>6</td>
<td>3268 (2,047-5,471)</td>
</tr>
</tbody>
</table>

duced LT. This, despite the fact that they had active, severe disease, were STN, and had complement fixation titers which varied from 1:8 to 1:256. Two of the four were untreated, and the other two had received therapy with either amphotericin B or ketoconazole for four and seven months, respectively. Repeat LT determinations were somewhat variable prior to their skin test conversion, but continued to be near or above the 10,000 CPM figure. In each of these cases (1 through 4), a positive coccidioidin skin test reaction was present within months of presentation and was maintained following cessation of treatment. Clinically, their disease resolved, and they were considered cured.

Patients 5 and 6 initially showed severely decreased spherulin-induced LT (2465 and 2011 CPM, respectively). Both had received no treatment when initially tested. However, they responded well clinically, and became skin test-positive within months. The values for patient 5 fell somewhat (6,479 CPM) following the end of therapy, but he has remained STP with a negative complement fixation titer and no evidence of disease. Patient 6 displayed a normal LT response only after he became STP. He also has remained LT and ST positive while receiving no further therapy.

Despite long-term treatment with apparent clinical stability, neither patient 7 nor 8 has been able to achieve a positive LT response. Transiently, after 25 months of ketoconazole therapy, patient 8’s skin test converted to positive while her coccidioidal skin lesions were in remission and stable. Therapy was therefore discontinued. Within weeks, her lesions showed renewed evidence of activity. Subsequently, her skin test reverted again to negative, and a skin biopsy was positive for C. immitis. Ketoconazole therapy was restarted, again with good results, but she remains ST and LT negative. Except for the fact that she has been consistently STN, patient 7 has had a similar course. Her skin lesions also flared-up within weeks after discontinuing ketoconazole. Both patients have received continued treatment with excellent control of their disease. Although the data are not shown, all patients, including the latter two, had normal LT responses to the mitogen phytohemagglutinin (PHA).

DISCUSSION

Since Smith’s pioneering work over 40 years ago, it has been evident that the majority of individuals exposed to the fungus C. immitis experience either subclinical infection or a mild self-limiting disease. The hallmark of such exposure, and the evidence for an appropriate cell-mediated immune response has been a positive coccidioidin skin test result. Either because of overwhelming fungal exposure, or for other reasons that have never been completely clear, a small percentage of otherwise healthy individuals, especially those of dark skinned ethnic origin, are unable to mount a satisfactory host defense. For them, the infection disseminates to one or more extrapulmonary sites. In recent years, such impaired host defense has also been linked to underlying immunodeficiency states or immune suppressive therapeutic agents. These two factors, in addition to the major influx of immigrants to the endemic disease area, have significantly increased the population at risk for disseminated coccidioidomycosis.

In contrast to other systemic mycoses, the coccidioidin skin test has been a valuable clinical tool in the assessment of such patients. Dissemination is strongly suspected when the coccidioidin complement fixation titer rises, especially over 1:16, in the presence of a negative skin test result, or when the skin test result reverts from positive to negative in the face of
active disease. Unfortunately, the apparent decrease in the sensitivity of the skin test from over 90 percent, as reported by Smith,\(^1\) to approximately 65 percent in more recent reports\(^4\), has diminished its clinical usefulness. Also, within any group of patients with disseminated disease, a variety of responses to therapy and clinical outcomes are seen. For some, rapid reconvalescence of the skin test to positive is accompanied by cure. In others, the best that occurs is disease control, with recurrent exacerbations when therapy is discontinued. Recurrence rates as high as 25 percent have been reported in some series.\(^5,22\)

At the time of presentation, the patients in the present series represented a rather typical spectrum of severe and/or disseminated disease. A majority were either black or Mexican-American, but in none was there evidence for an underlying immune deficiency state. Complement fixation titers were all 1:16 or higher at some time in their course. In six patients, the extrapulmonary sites of involvement were lymph nodes or skin. Two patients (subjects 4 and 6), both over age 60, had severe bilateral pulmonary disease, with acute respiratory failure, but no clinically evident extrapulmonary involvement.

Despite their uniformly negative skin tests and severe disease status, LT studies revealed three categories of antigen-specific host immune response. The first category included four patients (1 through 4) who showed positive lymphocyte blast transformation to spherulin antigen initially, responded well to therapy, and rather quickly converted their skin tests to positive. Both the clinical response and the positive skin reactivity continued when therapy was discontinued. The second category included two patients (5 and 6) who converted both LT and skin test reactivity from negative to positive following more prolonged treatment, with persistent clinical stability off treatment. Subsequent skin tests and LT responses have continued to be positive. The third category includes the final two patients (7 and 8) who have consistently failed during several years of follow-up to demonstrate a positive LT response (although patient 8 had a transient positive ST) and developed recurrent disease within weeks when therapy was stopped.

A number of reports have documented the correlation between delayed skin test reactions and the in vitro LT response to specific fungal antigen.\(^14,23-26\) In most instances, the LT was found to be more sensitive than the skin test.\(^14,16,20\) Despite this advantage, the clinical usefulness of LT studies has been limited, primarily by a lack of methodologic standardization. However, for coccidioidal antigens particularly, many of these methodologic problems have recently been resolved.\(^13\) Using spherulin-derived antigen and autologous plasma, it has been possible to produce a clear separation between STP and STN subjects in an area endemic for coccidioidomycosis. All normal STP control subjects and patients with self-limited disease produced LT response of 10,000 counts per minute or greater.

The use of serial spherulin-induced LT determinations in patients with severe disease provided valuable clinical information that could not have been obtained from skin test data alone. All patients were initially STN, and could not, on that basis, be separated in terms of their anticipated response to therapy or long-term prognosis. Despite the negative skin tests, four of the eight had initial spherulin-induced LT values which indicated an active immune response. Opel and Scheer\(^4\) reported similar findings in three of six STN patients with severe disease. All four of our positive LT patients responded well to therapy and became STP within months. They continued to do well and maintained an active immune response when therapeutic support was discontinued. In each instance, a positive clinical result could have been predicted from the LT data obtained early in their course, when their disease was active and their skin tests results were negative. In patients 5 and 6, LT values also provided valuable information. Both had negative skin tests results and LTs during their early course, and developed positive LT and skin test responses which predicted a good clinical outcome following therapy. Patients 7 and 8 have remained LT negative and have experienced disease exacerbations off treatment. At best, their disease is controlled while receiving treatment, but cure has thus far not been possible. In case 8, the LT was more predictive than the skin test (which became transiently positive) of a poor clinical response off treatment. Most authors have not reported a cellular immune defect in patients with disseminated disease. However, in patients 7 and 8, such a defect appeared to be the primary immune abnormality. At no time during almost three years was there any significant stimulation response of the patients' lymphocytes to spherulin. That this cellular defect was selective was suggested by the consistently positive PHA stimulation.

Patients with active progressive coccidioidomycosis with negative coccidioidin skin tests provide a major management dilemma. They obviously require treatment, but the therapeutic endpoint is unknown and usually cannot be predicted on the basis of a predetermined drug dose or duration. Successful clinical outcome is dependent upon the presence of a normal CMI response. Traditionally, this has been measured by the return of a positive skin reaction. Even then, the maintenance of such a reaction after therapy is discontinued is uncertain, and clinical relapse is common, with skin test reversion to negative. With standardization of the methodologic variables of the spherulin-induced LT, we have demonstrated the prognostic...
usefulness of the LT assay in predicting which patients will respond to treatment and have an eventual good clinical outcome in the presence of ambiguous skin test status.

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REFERENCES