Preliminary Evaluation of Whole-Blood Gamma Interferon Release for Clinical Assessment of Cellular Immunity in Patients with Active Coccidioidomycosis

Neil M. Ampel,1,3,4* Daniel K. Nelson,2 Suzette Chavez,2 Kathryn A. Naus,1 Amanda B. Herman,2 Lijin Li,2,4 Keira A. Simmons,5 and Demosthenes Pappagianis5

Assessment of the cellular immune response in coccidioidomycosis has epidemiologic and prognostic importance. Measurement of delayed-type hypersensitivity to skin testing has been used in the past to determine cellular immunity in coccidioidomycosis. However, no skin tests are currently available in the United States. Assay of gamma interferon (IFN-γ) release in whole blood in response to incubation with antigen has been used to assess cellular immunity in tuberculosis. We used a similar assay using the coccidoidal antigen preparation T27K to measure the in vitro cellular immune responses among a cohort of 69 subjects with active coccidioidomycosis. IFN-γ release was bimodal, with concentrations above and below 5 IU/ml. Using multivariate logistic regression, underlying disease and disseminated or chronic pulmonary coccidioidomycosis was significantly associated with the release of IFN-γ at a concentration of <5 IU/ml (P = 0.02 or 0.05, respectively). In addition, the release IFN-γ concentration was <5 IU/ml in all subjects with a clinical severity score of ≥6 (P = 0.02). The release IFN-γ concentration correlated with expression of CD69 on T lymphocytes in an in vitro assay using T27K as the antigen (Spearman’s ρ = 0.59; P < 0.01). These results suggest that the IFN-γ release assay with T27K as the antigen may be a useful clinical test for assessing cellular immunity in patients with active coccidioidomycosis.

Coccidioidomycosis is a fungal infection endemic in regions of the western United States, in northern Mexico, and in focal areas of Central and South America (23). Recently, the number of symptomatic cases in the United States has been increasing (7, 12). Like tuberculosis, coccidioidomycosis is a granulomatous disease, and the expression of delayed-type hypersensitivity (DTH) has both epidemiologic and prognostic importance (1). Unfortunately, the most common clinical tools to measure coccidioidal cellular immunity, the coccidioidin and spherulin skin tests, are not currently available in the United States.

We have previously demonstrated that expression of CD69 on the surfaces of T lymphocytes incubated with the coccidioidal antigen preparation T27K in whole blood is measurable in patients with various forms of coccidioidomycosis and can act as an in vitro assay of coccidioides-specific cellular immunity. This expression is inversely correlated with the severity of clinical illness in those patients with disseminated coccidioidomycosis. Moreover, the expression of CD69 is closely associated with the release of the T-helper-type cytokines gamma interferon (IFN-γ) and interleukin 2 from peripheral blood cells incubated with T27K (6).

Recently, the release of IFN-γ in whole blood incubated with mycobacterial antigens was found to correlate with results of the tuberculin skin test among patients with tuberculosis (10, 13, 16, 18, 24). In the present study, we examined the release of IFN-γ in whole blood incubated with the coccidioidal antigen preparation T27K and compared it to clinical measures of coccidioidal disease as well as to the in vitro expression of CD69 among a group of patients with clinically active coccidioidomycosis.

MATERIALS AND METHODS

Human subjects. Patients referred to the Coccidioidomycosis Clinic of the Southern Arizona Veterans Affairs Medical Center who had agreed to be part of a prospective study of coccidioidomycosis served as the subjects for this study. Whole-blood assays were performed upon entry into the clinic. All subjects were >18 years old and competent to sign a consent form. Patients with known infection with human immunodeficiency virus type 1 and those with allogeneic solid organ transplants were excluded. All work with human subjects was approved by the Human Subjects Protection Program of the University of Arizona.

Clinical data were collected during the visit, and a clinical severity score was determined. This score is based on a scale previously defined (11, 15). In this system, a point is awarded for each symptom present and two points are given for each anatomic site involved. In addition, a point is awarded if the culture for Coccidioides spp. is positive and points are given for the degree of elevation of the serum anti-coccidioidal immunoglobulin G titer, measured by the immunodiffusion complement fixation assay. Zero points were given if the titer was ≤1:2, one point was awarded if the titer was either 1:4 or 1:8, two points were awarded if the titer was either 1:16 or 1:32, and three points were awarded if the titer was ≥1:64.

Whole-blood incubation and CD69 expression. Venous blood from subjects was placed into sterile tubes containing heparin (Becton Dickinson Labware, Franklin Lakes, NJ). Aliquots of 1.0 ml were made for each sample and placed into 15-ml screw-top polystyrene centrifuge tubes (Corning, Corning, NY). For each subject, a tube received 10 μl of a 1.0-mg/ml T27K solution in RPMI 1640 (Gibco, Grand Island, NY) to yield a final concentration of 10 μg/ml; another tube received nothing and served as a control. In some samples, phytohemagglutinin L (PHA-L; Sigma Chemical Co., St. Louis, MO) was added to a final

* Corresponding author. Mailing address: Medicine and Primary Care (1-111), Southern Arizona Veterans Affairs Health Care System, 3601 S. Sixth Avenue, Tucson, AZ 85723. Phone: (520) 792-1450, ext. 6186. Fax: (520) 629-1861. E-mail: nampel@u.arizona.edu.
TABLE 1. Characteristics of 69 subjects with coccidioidomycosis who underwent testing for IFN-$\gamma$ release

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Characteristic of subjects</th>
<th>No. of subjects</th>
<th>Characteristic of subjects</th>
<th>No. of subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Male</td>
<td>61</td>
<td>Female</td>
<td>8</td>
</tr>
<tr>
<td>Age</td>
<td>$\geq$ 60 years</td>
<td>22</td>
<td>$&gt;60$ years</td>
<td>47</td>
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<tr>
<td>Race</td>
<td>African-American</td>
<td>54</td>
<td>Not African-American</td>
<td>15</td>
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<tr>
<td>Ethnicity</td>
<td>Hispanic</td>
<td>33</td>
<td>Not Hispanic</td>
<td>3</td>
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<tr>
<td>Underlying disease</td>
<td>No</td>
<td>31</td>
<td>Yes</td>
<td>38</td>
</tr>
<tr>
<td>Type of coccidioidomycosis</td>
<td>Benign pulmonary</td>
<td>36</td>
<td>Disseminated or chronic pulmonary</td>
<td>33</td>
</tr>
<tr>
<td>Antifungal therapy</td>
<td>No</td>
<td>32</td>
<td>Yes</td>
<td>37</td>
</tr>
<tr>
<td>Time from diagnosis</td>
<td>$&lt;5$ months</td>
<td>35</td>
<td>$\geq 5$ months</td>
<td>34</td>
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<tr>
<td>IDCF$^b$ titer</td>
<td>$\geq 1.4$</td>
<td>36</td>
<td>$&gt;1.4$</td>
<td>33</td>
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<tr>
<td>Clinical severity score$^c$</td>
<td>$&lt;6$</td>
<td>64</td>
<td>$\geq 6$</td>
<td>5</td>
</tr>
</tbody>
</table>

$^a$ The median time from the diagnosis of coccidioidomycosis to testing was 5.7 months (range, 0 to 207 months).

$^b$ IDCF, immunodiffusion complement fixation coccidioidal serology.

$^c$ The clinical severity score was calculated based on a method previously described (11, 15). Points were given based on the number of symptoms noted by the patient, the number of anatomic sites involved, whether a culture was positive or not for Coccidioides, and the titer of the immunodiffusion complement fixation assay.

concentration of 5 $\mu$g/ml to a third tube. Samples were incubated for 18 h at 37°C in 5% $CO_2$-95% air. To allow for adequate ventilation, the tops were left loose during incubation as previously described (6). At the end of this time, the supernatant for each tube was removed and frozen at $-70^\circ$C. The red blood cells in the rest of the sample were lysed and then analyzed by flow cytometry for the surface expression of CD69 after gating on CD3+ lymphocytes as previously described (6). Arithmetic mean fluorescent intensity (MFI) was determined for control and T27K-incubated samples. A CD69 MFI value for incubation with T27K was calculated by subtracting the control MFI from the T27K MFI result.

Measurement of IFN-$\gamma$ release. The QuantiFERON-CMI assay (Cellisist Limited, Victoria, Australia) was used to measure the IFN-$\gamma$ release for each of the frozen samples. This assay was performed according to the manufacturer’s recommendations. Samples whose values were above the highest concentration in the standard curve, 9.6 IU/ml, were not routinely diluted and reassayed. Because of this, values greater than 9.6 IU/ml were analyzed as equal to this value. For assessment of a response to PHA-L, all samples were first diluted 1:50 prior to assay to allow for measurement of concentrations up to 480 IU/ml.

Statistical analysis. Clinical and laboratory data were initially entered into a relational database (Filemaker Pro 5; Apple Computer, Cupertino, CA). Subsequently, statistical analysis was performed using Intercooled Stata 8.2 (College Station, TX). Univariate analysis was performed using logistic regression to generate the maximum likelihood estimates as odds ratios and 95% confidence intervals for categorical data. Stepwise logistic regression using backward selection with a rejected $P$ value of 0.10 and an accepted $P$ value of 0.05 was used for multivariate analysis of those factors found to be significantly associated by univariate analysis. The Spearman’s rank correlation coefficient (17) was used to assess the association between the IFN-$\gamma$ release assay and the CD69 assay, both of which measure in vitro coccidoidal cellular immune activation. The $\chi^2$ test was used to determine the association of two categories of response of the IFN-$\gamma$ release assay and other categorical factors. A $P$ value of $\leq 0.05$ was considered to be statistically significant. Regression analysis was performed to assess the relation between IFN-$\gamma$ release associated with T27K and that with PHA-L stimulation.

RESULTS

Description of the subjects. Sixty-nine patients with active coccidioidomycosis were assessed using the whole-blood release assay of IFN-$\gamma$. A clinical description of the cohort is shown in Table 1. Of the 36 patients with benign pulmonary disease, there were 23 individuals with primary pulmonary coccidioidomycosis, 9 with pulmonary nodules, and 4 with pulmonary cavities. The other 33 subjects had persistent and problematic coccidioidomycosis. Of these, 10 had chronic active noncavitary pulmonary disease and 2 had diffuse pulmonary disease. These were classified as chronic pulmonary coccidioidomycosis. Of the 21 with disseminated disease, 8 had bone or joint involvement, 6 had cutaneous disease, 5 had meningitis, and 2 had soft tissue involvement. Slightly more than half of the subjects were receiving antifungal therapy at the time of study. The data did not include the length of time each subject was on such therapy. All subjects on therapy were receivingazole antifungals, including 29 on fluconazole, 5 on itraconazole, 2 on ketoconazole, and 1 on posaconazole. No subjects were receiving amphotericin B at the time of study.

Results of the whole-blood release of IFN-$\gamma$ and correlation with clinical disease. All results are based on the initial blood sample taken in clinic. The frequency distribution of the of IFN-$\gamma$ release concentration is shown in Fig. 1. There is a bimodal distribution with results either less than or greater than 5 IU/ml. Based on this distribution, further analysis and clinical correlation were performed based on a category of a release IFN-$\gamma$ concentration of either $<5$ IU/ml or $\geq 5$ IU/ml. With 44 subjects, there was simultaneous measurement of IFN-$\gamma$ release after stimulation with 5 $\mu$g/ml of PHA-L. In these subjects, the median concentration of IFN-$\gamma$ in response to PHA-L was 108.6 IU/ml (range, 18.8 to $\geq 480$ IU/ml) and there was no association between the response to T27K and the response to PHA-L ($r^2 = 0.02; P = 0.38$). In addition, there

FIG. 1. Frequency distribution of the IFN-$\gamma$ release concentrations obtained during the initial clinic visits of the 69 subjects in the cohort.
was no significant association between the type of azole antifungal received and the concentration of IFN-γ released after stimulation with T27K (P = 0.86).

Univariate analysis revealed that three clinical conditions, underlying disease, receipt of antifungal therapy for coccidioidomycosis, and disseminated or chronic pulmonary disease, were significantly associated with a release IFN-γ concentration of <5 IU/ml (Table 2). When multivariate logistic regression was performed, only underlying disease and disseminated or chronic pulmonary disease remained significantly associated with a release IFN-γ concentration of <5 IU/ml (Table 3).

**Association between IFN-γ release and measurement of coccidioidal immunity by CD69 expression on T cells.** There was a significant association between the release IFN-γ concentration and the results of the assay of CD69 expression on the surfaces of T lymphocytes for T27K (Fig. 2) (Spearman’s rho = 0.59; P < 0.01). In addition, there was a significant association between specific categories of IFN-γ and CD69 results. Twenty-six of 35 subjects with IFN-γ concentrations of ≥5 IU/ml had CD69 values for T27K that were ≥10 above that of the control. On the other hand, 25 of 34 subjects with IFN-γ concentrations of <5 IU/ml had CD69 results for T27K that were <10 above that of the control (P < 0.01). In addition, all five subjects with clinical severity scores of >6 and IFN-γ release concentrations of <5 IU/ml also had CD69 values of <10.

**Association of the type of active coccidioidomycosis with the results of in vitro assays of coccidioidal cellular immunity.** Among the 69 subjects studied, 21 of 33 had disseminated or chronic pulmonary coccidioidomycosis and a release IFN-γ concentration of <5 IU/ml, yielding a sensitivity of 63.6%. Twenty-three of 36 subjects did not have disseminated or chronic pulmonary disease and had a release IFN-γ concentration of ≥5 IU/ml, for a specificity of 63.9%. The positive predictive value of the release IFN-γ concentration of <5 IU/ml for disseminated disease was 61.8%, and the negative predictive value was 35.3%.

Because these values are lower than those reported for latent tuberculosis (24), the combination of results for the CD69 assay and the IFN-γ release assay was analyzed. Results are displayed in Table 4 and reveal that in most cases, the results of the two tests were discordant. However, if both tests were positive, the sensitivity of predicting benign pulmonary disease was 89% [8/(8 + 1)], and if both tests were negative, the specificity was 67% [6/(6 + 3)]. Univariate analysis of the same clinical factors as displayed in Table 2 was performed among subjects for whom both assays of in vitro immunity were negative. Only an age of >60 years was significantly associated with this group (odds ratio, 5.7; 95% confidence interval, 1.3 to 25.8; P = 0.02). On the other hand, only benign pulmonary disease was significantly associated with subjects for whom both in vitro assays were positive (odds ratio, 12.2; 95% confidence interval, 1.4 to 103.9; P = 0.02).

The clinical severity score for coccidioidomycosis was originally developed to assess clinical improvement of disease in patients with various types of coccidioidomycosis undergoing

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**TABLE 2. Univariate analysis of clinical factors associated with a concentration of IFN-γ of <5 IU/ml**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Odds ratio</th>
<th>95% confidence interval</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Underlying disease</td>
<td>3.6</td>
<td>1.3–9.8</td>
<td>0.012</td>
</tr>
<tr>
<td>Subject on antifungal therapy</td>
<td>3.1</td>
<td>1.2–8.4</td>
<td>0.023</td>
</tr>
<tr>
<td>Disseminated or chronic pulmonary coccidioidomycosis</td>
<td>3.1</td>
<td>1.2–8.3</td>
<td>0.024</td>
</tr>
<tr>
<td>Diagnosis in &lt;5 months</td>
<td>0.4</td>
<td>0.2–1.1</td>
<td>0.073</td>
</tr>
<tr>
<td>Male sex</td>
<td>0.3</td>
<td>0.1–1.6</td>
<td>0.162</td>
</tr>
<tr>
<td>Age &gt;60 years</td>
<td>0.6</td>
<td>0.2–1.7</td>
<td>0.343</td>
</tr>
<tr>
<td>Not African-American race</td>
<td>1.7</td>
<td>0.5–5.6</td>
<td>0.351</td>
</tr>
</tbody>
</table>

**TABLE 3. Multivariate logistic analysis of clinical factors associated with a concentration of IFN-γ of <5 IU/ml**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Odds ratio</th>
<th>95% confidence interval</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Underlying disease</td>
<td>3.3</td>
<td>1.2–9.2</td>
<td>0.024</td>
</tr>
<tr>
<td>Disseminated or chronic pulmonary coccidioidomycosis</td>
<td>2.8</td>
<td>1.0–7.7</td>
<td>0.050</td>
</tr>
</tbody>
</table>

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**TABLE 4. Association between clinical disease and assays of in vitro coccidioidal immunity**

<table>
<thead>
<tr>
<th>Assay results</th>
<th>Benign pulmonary coccidioidomycosis</th>
<th>Disseminated coccidioidomycosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Both negative</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>CD69, ≥10, and IFN-γ release, &lt;5 IU/ml</td>
<td>8</td>
<td>17</td>
</tr>
<tr>
<td>CD69, &lt;10, and IFN-γ release, ≥5 IU/ml</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Both positive</td>
<td>8</td>
<td>1</td>
</tr>
</tbody>
</table>

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*a* The model was generated using backward stepwise selection with rejected P values of ≥0.10 and included P values of ≤0.05.
antifungal therapy (11, 14, 15). Of the 69 subjects in the study, all 35 whose samples contained ≥5 IU/ml of IFN-γ had clinical severity scores of <6. On the other hand, all five subjects with clinical severity scores of ≥6 had samples with IFN-γ concentrations of <5 IU/ml (P = 0.02).

**DISCUSSION**

These results show that the release of IFN-γ from whole blood incubated with a coccidioidal antigen preparation correlates with clinical parameters of active coccidioidomycosis. Specifically, depressed IFN-γ concentrations were found on multivariate analysis to be significantly associated with underlying disease and disseminated or chronic pulmonary coccidioidomycosis. Moreover, elevated concentrations were significantly associated with a lower clinical severity score. These results suggest that the in vitro measurement of IFN-γ release has clinical implications and utility for patients with active coccidioidomycosis. However, the sensitivity, specificity and predictive values of a low IFN-γ release concentration were not high, even when combined with the CD69 assay, suggesting that this test alone cannot predict the development of disseminated or chronic coccidioidomycosis. Because of limited follow-up data, we were unable to analyze the role of the IFN-γ release assay on predicting disease resolution or worsening over time. One important question is whether a negative test, defined in this study as <5 IU/ml, would subsequently predict the development of increasing disease severity, including dissemination. Testing of more subjects and follow-up of those already tested should further define the clinical utility of this assay.

The measurement of coccidioides-specific cellular immunity has proven in the past to be useful in determining the prognosis of coccidioidomycosis. In early studies, Smith and colleagues described the relationship between skin test reactivity and outcome (26). They noted that although dissemination of infection could occur in the presence of DTH, survival, in a time when there was no antifungal therapy, was associated with persistent reactivity to the skin test. Specifically, 75% of persons with disseminated disease survived if DTH persisted, compared to only 17% if there was no DTH response. Oldfield and coworkers, in a retrospective review of military records, found that expression of DTH after skin testing was associated with a diminished risk of relapse after antifungal therapy was discontinued (22). The results of the present study are consonant with these data.

T27K, the antigen preparation used in this study, is the soluble, aqueous supernatant derived by mechanically disrupting thimerosal-killed coccidioidal spherules and centrifuging at 27,000 × g (27). We have previously shown that using this preparation as an antigen in studies of in vitro cellular immunity distinguishes healthy immune donors with coccidioidomycosis from healthy nonimmune donors and distinguishes donors with various forms of active coccidioidomycosis (5, 6). The dose-response characteristics of this antigen preparation has been previously determined (5) with the finding that optimal reactivity is seen at 10 μg/ml, the same as that used in this study. Although T27K is prepared differently from coccidioidins and spherulin, the antigens that are used in skin tests (1), it appears to be similar to these antigens as an immunogen. T27K can be lyophilized and stored for prolonged periods and so is of potential use as a commercial antigen for in vitro assays of coccidioidal immune response. While T27K is not fully defined, this is not necessarily a drawback for its use in these assays. Currently, there are no defined coccidioidal antigens available that have been shown to be immunogenic in humans. Moreover, defined antigens, particularly those that are genetically cloned, may be less immunogenic than preparations derived directly from the organism, such as T27K, because the latter are highly glycosylated, allowing for attachment to innate receptors, such as that for mannose (8).

The QuantiFERON-TB test, similar to the assay used here, has been approved by the U.S. Food and Drug Administration for use in detecting latent tuberculosis (19). However, there remains some debate about its utility compared to that of the tuberculosis skin test (9, 16, 18, 21). Recently, Mori and colleagues demonstrated that this assay, using a mixture of mycobacterial antigens, was highly sensitive and specific (20). In our study of the coccidioidal immune response, it is important to note that we used an absolute concentration of IFN-γ, rather than a percentage of the response to a mitogen, as has been done in the tuberculosis assay (19). When we measured responses to PHA-L in a subset of subjects, the IFN-γ release concentrations were elevated in all cases and did not correlate with the response to the coccidioidal antigen T27K, suggesting that the IFN-γ concentrations in response to T27K were specific to the coccidioidal cellular immune response. We have previously found that analyzing the absolute concentration of IFN-γ in cell supernatants after incubation with coccidioidal antigen reflects the clinical cellular immune response in coccidioidomycosis (2, 3). Of note, the IFN-γ concentrations appear to be bimodal in our study, rather than continuous, as reported for tuberculosis testing (9, 21). Finally, it is important to note that this study examined subjects with clinically active disease, not simply latent infection. Hence, the sensitivity and specificity data are calculated for distinguishing benign pulmonary versus disseminated disease, not for infected versus uninfected subjects. It is understandable that these calculations might be less robust than for distinguishing infection from noninfection.

Because of the lack of availability of skin tests, there is no “gold standard” to compare the results of the IFN-γ release assay in coccidioidomycosis. Such studies should be performed prior to accepting this assay, or other in vitro assays of cellular immunity using a coccidioidal antigen, as a surrogate for the skin test. A study that compares the results of the IFN-γ release assay to induration from injection of the skin-test reagent coccidioidin among healthy donors without active coccidioidomycosis is ongoing. Results will help determine the utility of in vitro assays of coccidioidal cellular immunity among healthy donors without active coccidioidomycosis. It would be additionally useful to compare the results of skin testing with coccidioidin to the in vitro IFN-γ release in response to T27K among healthy donors living outside the region of coccidioidal endemcity to more fully assess the specificity of the in vitro assay. Such a study is currently not feasible because of the lack of coccidioidin. However, prior studies using T27K as the antigen preparation have demonstrated specificity. For example, we found that the number of CD3⁢⁺ lymphocytes producing intracellular IFN-γ was significantly higher from
samples from previously identified healthy immune donors than from nonimmune healthy donors (8) and was higher than with donors with active, disseminated coccidioidomycosis (4).

In addition, we have been able to induce a specific in vitro coccidioidal cellular immune response in lymphocytes from both nonimmune healthy donors and from nonreactive individuals with active coccidioidomycosis using mature dendritic cells incubated with T27K (25). Overall, the results from the present study demonstrate significant correlation of in vitro results with clinical parameters of disease among patients with active coccidioidomycosis. Based on this, further assessment of this test as a measure of the cellular immune response in coccidioidomycosis is warranted.

ACKNOWLEDGMENTS

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REFERENCES