In Vitro Whole-Blood Analysis of Cellular Immunity in Patients with Active Coccidioidomycosis by Using the Antigen Preparation T27K

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Measurement of cellular immunity in human coccidioidomycosis has important diagnostic and prognostic implications. The coccidioidin skin test has been the standard for the measurement of this, but it is not available in the United States. We examined the utility of measuring surface expression of CD69 on T lymphocytes in whole blood incubated with the coccidioidal antigen preparation T27K as an alternative to the skin test. Seventy donors with active coccidioidomycosis were studied. The mean fluorescent intensity (MFI) of CD69 expression on CD3 lymphocytes in response to T27K was 28.61 ± 1.77, significantly greater than the control response of 11.45 ± 0.78 (P < 0.001). The MFI CD69 response to T27K above that for the control (MFI CD69 above control) was 6.35 ± 2.18 for seven subjects with disseminated coccidioidomycosis who were studied within 5 months of diagnosis. This was significantly below the value of 20.17 ± 3.17 for 18 subjects with pulmonary coccidioidomycosis studied within 5 months of diagnosis and the value of 19.58 ± 2.91 for 27 subjects with disseminated coccidioidomycosis studied after 5 months of diagnosis (for both, P < 0.05). There was an inverse correlation between coccidioidal clinical score and MFI CD69 above control for all 34 subjects with disseminated coccidioidomycosis (r = 0.362; P = 0.036) but not for the 36 subjects with pulmonary disease (r < 0.001; P = 0.993). Among 30 subjects for whom data were available, there was a highly significant association between the MFI CD69 above control and the supernatant concentrations of gamma interferon, interleukin-2 (IL-2), and tumor necrosis factor alpha (for all, P < 0.001), but not for IL-4, IL-5, or IL-10. These data indicate that in vitro assessment of CD69 expression on T lymphocytes by using T27K may be a useful measure of cellular immune response among subjects with active coccidioidomycosis.

The association between expression of delayed-type hypersensitivity and the severity of illness in human coccidioidomycosis has been recognized for more than 50 years (26, 27). Specifically, expression of delayed-type hypersensitivity is predominantly absent often among individuals who have severe, extrathoracic dissemination. On the other hand, delayed-type hypersensitivity is usually present among those with mild, self-limited illness (13). Moreover, the failure to express coccidioidal delayed-type hypersensitivity appears to predict subsequent relapse after therapy for patients with active coccidioidomycosis is halted (24).

Immunologically, delayed-type hypersensitivity is an expression of cell-mediated immunity. While measurement of induration at the site of intradermal injection of antigen has been the traditional way to measure cell-mediated immunity, in vitro laboratory techniques also are capable of assessing cellular immunity in coccidioidomycosis (8, 12). In vitro methods are advantageous in that they do not require exposure of the subject to foreign antigen or its preservative and results are not dependent on injection technique, time of reading, and measurement of induration size. In coccidioidomycosis, the use of in vitro methods to measure cell-mediated immunity has taken on further importance because no skin-test reagent has been available in the United States for the past several years.

In the present study, we assessed in vitro expression of cellular immunity in coccidioidomycosis by measuring the T-lymphocyte surface expression of CD69, a glycoprotein that is expressed by activated lymphocytes and NK cells (25), after incubation of whole blood with the antigen preparation T27K. In the present work, we compared CD69 expression with simultaneous cytokine production among donors with various forms of active coccidioidomycosis. We have previously shown that in vitro assessment of cellular immunity by flow cytometry with the antigen T27K distinguishes immune and nonimmune healthy donors on the basis of coccidioidal skin testing when the antigen was still available (3).

MATERIALS AND METHODS

Human subjects. Seventy patients with active coccidioidomycosis were sequentially recruited and tested as they entered the Valley Fever Clinic of the Southern Arizona Veterans Affairs Health Care System. In this medical center, all those with a diagnosis of coccidioidomycosis are referred to the Valley Fever Clinic. Donors known to be infected with human immunodeficiency virus or known to have undergone allogeneic transplantation were excluded from the study. A variety of clinical data were collected at the time of study, and a clinical score of severity of coccidioidomycosis was determined. This score is based on clinical symptoms, the severity and number of clinical sites of disease, and the immune diffusion complement-fixing coccidioidal antibody titer. In this system, a higher score is associated with more severe coccidioidal illness (7, 15, 16). The study was approved by the Human Subjects Committee of the University of Arizona. In addition to these patients, 20 healthy donors with known reactivity to coccidioidomycosis were recruited and tested.
din and without active coccidioidomycosis and 11 healthy donors without known coccidioidal immunity were also studied.

Whole-blood preparation and incubation. Approximately 5 ml of blood was drawn by venipuncture from each donor and placed into tubes containing lithium heparin. Aliquots of 0.5 ml were added to 15-ml polypropylene conical centrifuge tubes (Corning, Corning, N.Y.) to which 10 or 20 μg of T27K per ml was added. Previous studies did not reveal differences between the two concentrations. Nothing was added to control tubes. T27K was prepared as a solution as described previously (3, 28). Samples were incubated for 18 to 24 h at 37°C in 5% air–5% CO2, with the caps of the tubes kept loose and without rocking. At the end of this time, 50 μl of 20 mM EDTA (GIBCO, Grand Island, N.Y.) was added to each sample for 15 min at room temperature. Subsequently, 5.0 ml of FAC-Stain (Becton Dickinson Immuno-cytometry Systems, [BDIS], San Jose, Calif.) was added to each tube to lyse red blood cells. The tubes were incubated for 10 min at room temperature, and then the contents were mixed. Following this, the samples were centrifuged, resuspended in 5.0 ml of wash buffer (phosphate-buffered saline with 1% bovine serum albumin and 0.1% NaN3), and recentrifuged; and the pellet was resuspended in 1.0 ml of wash buffer. To 200 μl of each sample was added 20 μl of phycoerythrin (PE)-labeled anti-CD69 and 20 μl of peridinin chlorophyll protein-labeled anti-CD3 (BDIS), and the mixture was incubated for 30 min at room temperature in the dark. Then, 2.0 ml of wash buffer was added to each sample, the samples were centrifuged again, and the pellet was resuspended in 500 μl of phosphate-buffered saline. After this step, the samples were immediately used for flow cytometry.

Flow cytometry. Cells were analyzed with a single argon-ion laser flow cytometer (488 nm; FACScan; Becton Dickinson, Mountain View, Calif.) with Cell Quest software (Becton Dickinson). Prior to each assay, general calibration and compensation were established by using two-color CaliBRITE beads (Becton Dickinson), prepared according to the directions of the manufacturer, with the results analyzed by using FACSComp software (version 4.1; Becton Dickinson). For every printout, a runout was generated detailing the photomultiplier tube (PMT) voltages, laser current, and power. Over the 6-month period encompassing this study, the FL1 PMT voltage varied by 14 V (620 to 634 V), the FL2 PMT voltage varied by 26 V (664 to 680 V), and the FL3 PMT voltage varied by 32 V (704 to 736 V). In addition, the laser current varied between 5.18 and 5.22 A, and the laser power varied between 15.00 and 15.05 mW.

In addition to these general steps, a sample was prepared from T27K-stimulated cells for each experiment containing fluorescein isothiocyanate-labeled immunoglobulin G2a, PE-labeled immunoglobulin G1, and PerCP-labeled anti-CD3. From this, a CD3-lymphocyte gate was established and the PMT voltage for each channel was set. Compensation was then optimized by analyzing samples containing fluorescein isothiocyanate-, PE-, and PerCP-labeled anti-CD3 within the CD3-lymphocyte gate. Compensation was considered optimized when the positive populations were aligned over the negative populations. The surface expression of CD69 was then assessed among CD3 lymphocytes after counting of 10,000 of these cells for each sample and was recorded as the mean fluorescent intensity (MFI).

Cytokine bead assay. The plasma supernatant was drawn off some of the samples prior to preparation for flow cytometry and was frozen at −70°C. Subsequently, these samples were thawed and the concentrations of the cytokines gamma interferon (IFN-γ), interleukin-2 (IL-2), tumor necrosis factor alpha (TNF-α), IL-4, IL-5, and IL-10 were measured by a flow cytometric bead assay (BDIS). Samples with levels >5,000 pg/ml, the upper limit of the assay, were not rerun at dilution but were instead listed as 5,000 pg/ml.

Statistics. Statistical analysis of continuous variables between two groups was performed by the Student t test, and the paired t test was used for comparison of data within subjects. Linear regression was used to determine the association of continuous variables. A contingency table was used to assess differences in data within subjects. Linear regression was used to determine the association of continuous variables. A contingency table was used to assess differences in data within subjects.

RESULTS

Description of patients. The 70 patients consisted of 34 individuals with disseminated coccidioidomycosis and 36 patients with pulmonary disease. Reflecting the population of veterans from which the study population was derived, 67 of the subjects in the study were male, with a median age of 66.9 years (age range, 26.8 to 85.4 years). In addition, 21 were African American, 67 were non-Hispanic, and 5 were active cigarette smokers. The median time since the diagnosis of coccidioidomycosis upon entry into the study was 8.9 months (range, 0.2 to 289.4 months). Forty-seven patients were receiving some type of antifungal therapy at the time of the study, while 23 were not. Thirty-four of the patients had underlying diseases. Of these, diabetes was the most common and occurred in 16 patients.

Expression of CD69 in response to T27K. Among all 70 patients, the mean ± standard error of the mean MFI CD69 in response to incubation with T27K was 28.61 ± 1.77, significantly greater than the response of the control, whose samples were not incubated with T27K, of 11.45 ± 0.78 (P < 0.001). In order to account for the low but persistent expression of CD69 by CD3 lymphocytes without antigen stimulation, subsequent data are expressed as the difference, for each donor, in MFI CD69 between the sample incubated with T27K and the control sample not incubated with T27K (MFI CD69 above control). Hence, the results for each donor were normalized by subtracting the results for the sample containing T27K from the results for the control sample that did not contain T27K.

The mean ± standard error of the mean MFI CD69 above control in response to T27K for all 70 patients was 17.16 ± 1.66 and ranged from −1.45 to 53.7. The MFI CD69 above control for the 34 patients with disseminated coccidioidomycosis was 16.86 ± 2.52 and was not significantly different from value for the 36 patients with pulmonary disease, 17.44 ± 2.22 (P = 0.861). The results for the patients with active coccidioidomycosis were not significantly different from the value of 15.21 ± 1.58 obtained for the 20 healthy immune donors with known coccidioidal immunity (P = 0.549). On the other hand, the MFI CD69 above control for 11 healthy nonimmune donors was only 1.58 ± 0.69, significantly less than the values for the patients with active coccidioidomyraxis and the healthy, immune donors (for all, P < 0.001). There were no differences in the MFI CD69 above control response to T27K of CD3 cells on the basis of underlying disease, including diabetes, age of 60 years or more, or race (for all, P > 0.050) (data not shown).

Because antifungal therapy may have changed the initial coccidioidal cellular immune response over time, particularly among those with disseminated disease, data were next analyzed by comparing subjects who were studied within 5 months of diagnosis to those who were studied after 5 months of diagnosis. These data are displayed in Fig. 1. Among the seven subjects with disseminated coccidioidomycosis studied within 5 months of diagnosis, the MFI CD69 above control was 6.35 ± 2.18, significantly less than the value of 20.17 ± 3.17 for the 18 subjects with pulmonary coccidioidiomycosis studied within 5 months of diagnosis (P = 0.016) and the value of 19.58 ± 2.91 for the 27 patients with disseminated coccidioidomycosis studied more than 5 months after diagnosis (P = 0.031). This difference was not due to a difference in background expression of CD69. The expression of CD69 in control, unstimulated samples from those with a diagnosis of pulmonary coccidioidomycosis of less than 5 months’ duration was 8.45 ± 1.38, not different from the value of 10.83 ± 3.30 for those with disseminated disease of less than 5 months’ duration (P = 0.434).

Association of CD69 expression in response to T27K and clinical coccidioidomycosis score. As shown in Fig. 2, among the 34 subjects with disseminated coccidioidomycosis, the MFI CD69 above control in response to T27K was found to be significantly and inversely associated with the clinical coccidioidomycosis score.
iodomycosis score ($r = 0.362; P = 0.036$). On the other hand, among the 36 donors with pulmonary coccidioidomycosis, there was no correlation between MFI CD69 above control in response to T27K and the clinical coccidioidomycosis score ($r = 0.001; P = 0.993$).

**Association of CD69 response with cytokine production.** For 30 subjects, the plasma supernatant was saved and subsequently tested to determine the relationship between CD69 expression and cytokine production after incubation with 10 μg of T27K per ml. Fifteen of these donors had disseminated coccidioidomycosis and 15 had pulmonary disease. As depicted in Fig. 3, there was a highly significant and positive association between MFI CD69 above control and the concentrations of IFN-γ, IL-2, and TNF-α in the plasma supernatant (for all three, $P < 0.001$). The levels of IL-4, IL-5, and IL-10 were low and not significantly associated with MFI CD69 above control. The relation between CD69 expression and cytokine production was not different among the donors with disseminated or pulmonary coccidioidomycosis (data not shown).

**DISCUSSION**

Measurement of delayed-type hypersensitivity by skin testing has been the standard technique by which cell-mediated immunity has been determined among individuals with coccidioidomycosis (27). This test is not available in the United States, and alternatives are required to assess coccidioidal cellular immunity. Assessment of cellular immunity in coccidioidomycosis serves several purposes. First, it may be used to establish the prevalence of coccidioidal infection in a population within a geographic region (14, 20). Second, it may be used to determine new infection, if prior assessment of immunity has been performed and has been found to be lacking. Finally, expression of cell-mediated immunity appears to reflect a protective host response and may auger an improved clinical outcome among individuals with symptomatic coccidioidomycosis (24, 26).

In the present study, the surface expression of CD69 on CD3 lymphocytes after incubation with the coccidioidal antigen preparation T27K was used to ascertain the cellular immune status of donors with active coccidioidomycosis. The response to T27K of samples from all donors was significantly above that for unstimulated control samples and above the values obtained for a group of healthy, nonimmune donors. When the MFI CD69 above control in response to T27K was examined, there was no overall difference in response between donors with disseminated coccidioidomycosis and those with pulmonary disease, nor were there differences in response on the basis of underlying disease, age, or race. The average age of the patients with active coccidioidomycosis was nearly 67 years. While this appears old, recent data indicate that symptomatic coccidioidomycosis is far more prevalent among those aged 60 years and over than among younger subjects (4, 21). Because all patients with active coccidioidomycosis at the medical center were referred to the coccidioidomycosis clinic and were eligible for study, the older age most likely reflects the predisposition for symptomatic illness among an older age group rather than any other factor. However, there were significant differences on the basis of the length of time between diagnosis and study. When studied early in the course of their illness, those with disseminated disease had a decreased response to T27K compared to the response of either those studied later or...
those with pulmonary disease. These data suggest that antifungal therapy may ameliorate the depression in cellular immune response that occurs in disseminated coccidioidomycosis that has been observed previously (1, 2, 8, 10, 11, 26). Barbee and Hicks (5) have demonstrated such a return in cellular immune response with therapy in a small cohort of patients using an in vitro lymphocyte transformation assay. We are prospectively examining the cellular immune response over time in patients with various forms of coccidioidomycosis on antifungal therapy to more fully explore this question.

The coccidioidal antigen preparation T27K used in these experiments is the soluble, aqueous supernatant obtained after mechanically disrupted thimerosal-preserved spherules are centrifuged at 27,000 x g. Previous work with formalin as the preservative has shown that it protects mice from experimental coccidioidal infection when combined with alum (28). To date, the components of T27K have not been fully defined, but several coccidioidal antigens have been identified in the preparation, including chitinase, chitobiase, aspartyl protease, Ag2/proline-rich antigen, alkaline phosphatase, serine protease, and tube precipitin. Some of these antigens have been identified in both coccidioidin and spherulin, but neither of the latter two preparations has ever been fully characterized.

The use of T27K in an in vitro assay is in contrast to earlier studies, in which either coccidioidin or spherulin was used as the skin-test reagent. While it would be of great interest to compare these older antigen preparations to T27K, they are, unfortunately, no longer available. Because of the laboratory and bioterrorism risk, preparation of either requires a licensed biosafety level 3 facility. Moreover, significant lot-to-lot variability occurs with these preparations, making any comparisons difficult. In previous work, we have found that the in vitro response to T27K among healthy donors corresponds closely with skin-test positivity to spherulin (3).

The clinical score is a measure of coccidioidal severity and is increased in patients with severe disease and declines as disease improves with antifungal therapy. There was a significant inverse association between the score of coccidioidal illness among donors with disseminated coccidioidomycosis and the expression of CD69. However, no such association was seen among those with active pulmonary disease. This observation is consistent with observations first made by Smith and Beard (26) with the use of coccidioidin skin testing and with the findings of other investigators who used both skin testing and in vitro assays of cell-mediated immunity (8, 11, 12).

CD69 expression on CD3 lymphocytes in response to T27K among donors with coccidioidomycosis was directly and positively associated with production of the cytokines IFN-γ and IL-2 but not with the production of the T-helper type 2 cytokines IL-4, IL-5, and IL-10. This suggests that CD69 expression is associated with a T-helper type 1 cellular immune response in patients with coccidioidomycosis. These data and our previous work demonstrating clear distinctions between healthy immune and nonimmune donors (3) suggest that T27K is a biologically useful antigen in determining the cellular immune response to coccidioidomycosis. The close association between the production of T-helper type 1 cytokines, particularly IFN-γ, and CD69 expression in response to T27K suggests that assessment of these cytokines alone or in association with CD69 expression might be useful in measuring the coccidioidal cellular immune response. At this time, we do not have enough data to determine whether this is the case. Studies to examine this are planned for the future.

Expression of CD69 on lymphocytes has been shown to be
associated with cellular immune function in patients with a variety of other conditions (6, 17, 22, 25). In addition, other rapid assays have been found to be useful in determining the cellular immune response in patients with other granulomatous conditions. In particular, the measurement of IFN-γ in whole blood incubated with tuberculin antigen has been found to compare favorably with purified protein derivative skin testing in patients with a variety of forms of tuberculosis (9, 18, 19, 23). In the present study, expression of CD69 on CD3 lymphocytes in response to the coccidioidal antigen preparation T27K was a rapid, easily performed assay that required less blood than many standard clinical laboratory tests and provided useful information regarding coccidioidomycosis-specific cellular immunity. Therefore, even if the reagents for performing coccidioidal skin testing were still available, there would be several advantages to using an in vitro system, such as measurement of CD69 expression. The present study, while demonstrating clinical associations with the in vitro assay, did not clearly show a direct relationship between the assay result and clinical disease or outcome. It is imperative that further studies examining the utility of in vitro measurement of coccidioidal cellular immunity be performed to further assess this possibility.

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