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Induction of Tumor Necrosis Factor Alpha by Spherules of *Coccidioides immitis*

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The cytokine tumor necrosis factor alpha (TNF-α) functions as an immunomodulatory protein and as a mediator of cachexia. We report that viable or Formalin-killed spherules of *Coccidioides immitis* induced the secretion of TNF-α by peritoneal-exudate cells from BALB/c mice. The identification of the cytokine as TNF-α was based on its lytic activity against the TNF-α-sensitive L_S murine fibrosarcoma cell line but not the TNF-α-resistant L_R cell line, its neutralization by rabbit anti-TNF-α, and its secretion by peritoneal cells having characteristics of macrophages. The induction of TNF-α was attributable to spherules and not to contaminating lipopolysaccharide (endotoxin), as evidenced by the finding that polymyxin B, a reagent that blocks the TNF-α-inducing component of lipopolysaccharide, did not negate the production of TNF-α in response to spherules, whereas pretreatment of spherules with hyperimmune goat antiserum to spherulin neutralized the induction of TNF-α by these cells. The demonstration that *C. immitis* activates macrophages to secrete TNF-α in vitro is a new finding and warrants studies to determine whether this cytokine is produced during active coccidioidomycosis.

Tumor necrosis factor alpha (TNF-α) was identified by Carswell et al. (9) as the component present in sera from *Mycobacterium bovis* bacillus Calmette-Guérin (BCG)-infected, endotoxin-treated mice which caused hemorrhagic necrosis of transplanted tumors. In a separate line of investigation, Kawakami and Cerami (25) demonstrated that macrophages from mice sensitive to lipopolysaccharide (LPS) secreted a factor, termed cachectin, that caused lipemia and cachexia in LPS-resistant mice. Although initially studied as two separate cytokines, TNF-α and cachectin were subsequently shown to have identical functions, and when the complete primary structure of the two proteins was determined, they were found to be the same cytokine (4, 8).

There is now considerable evidence that many of the biological and physiological consequences of acute infection, immunological reactions, and tissue injury are mediated by TNF-α. In addition to its oncolytic activity and ability to induce cachexia, TNF-α can activate neutrophils, enhance the cytolytic activity of macrophages, augment natural killer cell activity, promote T- and B-cell proliferation, and modulate endothelial cell-surface antigens (reviewed in references 26 and 37). These pleiotropic immunoregulatory functions suggest that this cytokine may have a pivotal role in the induction and expression of immune response.

A variety of microbial agents have been shown to induce the secretion of TNF-α. These include bacteria (6, 20, 27, 33, 42), bacterial products (21, 23, 30, 41, 44, 45), viruses (1), parasites (2), and, in a recent study, the yeastlike fungus *Candida albicans* (16). The present investigation was undertaken to determine whether *Coccidioides immitis* might also induce the secretion of this cytokine. We report that peritoneal-exudate cells (PEC) from BALB/c mice produce significant levels of TNF-α in response to viable or Formalin-killed spherules of this pathogenic fungus. These results, together with the demonstration of TNF-α production in other infectious diseases (6, 7, 20, 27, 36), suggest that TNF-α could be a component of host response in coccidioidomycosis.

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MATERIALS AND METHODS

Mice. Female BALB/c mice 5 to 6 weeks old were purchased from Jackson Laboratory (Bar Harbor, Maine). The mice were maintained in our laboratory for 2 weeks before study.

Spherules. Spherules of *C. immitis* Silveira (ATCC 28868) were grown in modified Converse medium without Tamol as previously described (11). After incubation on a gyratory shaker (120 rpm) for 5 days at 38°C under a 20% CO_2 humidified atmosphere, the cells were pelleted by centrifugation, washed by centrifugation in pyrogen-free distilled water, and killed by the addition of Formalin (final concentration, 1%). Formalin was removed by centrifugation of the cells in pyrogen-free distilled water, and the killed spherules were then lyophilized and stored at −20°C until used.

Cell lines. The TNF-α-sensitive L_S and TNF-α-resistant L_R murine fibrosarcoma cell lines (34) were generously provided by Elizabeth Carswell Richards (Memorial Sloan-Kettering Cancer Center, Rye, N.Y.). The cell lines were grown in minimal essential medium (MEM) supplemented with nonessential amino acids (0.1 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and 8% heat-inactivated fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.). This tissue culture medium is hereafter referred to as supplemented MEM. Both cell lines were negative for mycoplasmal contamination as assessed with the Mycoplasmal T. C. II DNA hybridization assay from Gen-Probe (San Diego, Calif.).

Induction of TNF-α. PEC were elicited by injecting mice intraperitoneally with 1 ml of sterile, light-weight mineral oil (HUMCO Laboratory, Texarkana, Tex.) 72 h before assay. The mice were sacrificed by cervical dislocation, and a longitudinal abdominal incision was made through the peritoneum. The intraabdominal cavity of each mouse was lavaged three to five times with a total of 20 ml of Hanks balanced salt solution (GIBCO). The cells were washed by

centrifugation in Hanks balanced salt solution and suspended in supplemented MEM. Cell viability was consistently greater than 95% when assessed by trypan blue-dye exclusion.

The PEC were enumerated by hemacytometer counts and suspended to a concentration of 5×10^6 viable cells per ml of supplemented MEM. A sample of 100 μl was dispensed into each well of a 96-well round-bottomed microdilution plate. Lyophilized spherules and, as a positive control, LPS prepared from *Escherichia coli* serotype O55:B5 (Sigma Chemical Co., St. Louis, Mo.), were suspended in supplemented MEM at the designated concentrations and dispensed in 100-μl samples to wells in triplicate.

The cultures were incubated at 37°C in a 5% CO_2 atmosphere for 18 to 24 h, and the microdilution plates were centrifuged at 400 × *g* for 3 min. Supernatants (125 μl per well) were collected, pooled, and then centrifuged at 200 × *g* for 10 min to remove PEC or spherules.

TNF-α assay. The L_S and L_R cell lines were harvested in an exponential growth phase, washed by centrifugation in MEM, and suspended to a concentration of 10^5 cells per ml of supplemented MEM. Samples (100 μl) of the cell suspension were dispensed to wells on a 96-well flat-bottomed microdilution plate. Supernatants (100 μl) from stimulated or nonstimulated PEC were then added to triplicate wells containing the L cells.

After 48 h of incubation at 37°C under 5% CO_2, the microdilution plates were washed twice with phosphate-buffered saline, and the cells were stained for 30 min with 0.25% crystal violet. Excess crystal violet was removed by washing the plates with phosphate-buffered saline, and the cells were lysed by the addition of saponin. The A_{540} of each well was measured with a microELISA reader (Dynatech, Chantilly, Va.). Control wells consisted of L cells alone in medium and L cells in medium plus saponin, representing 0 and 100% cytotoxicity, respectively.

The percent cytotoxicity obtained with PEC supernatants was calculated as follows: [(A_{540} of the test wells − A_{540} of the saponin control wells)/(A_{540} of medium control wells − A_{540} of saponin control wells)] × 100. Recombinant murine TNF-α (rTNF-α; Genzyme, Boston, Mass.), at concentrations ranging from 8 to 250 U/ml (0.8 and 25.0 U per well, respectively), was included in each assay to confirm the sensitivity of the target cell lines.

Polymyxin B treatment. Spherules and, for comparison, LPS were suspended to a concentration of 10 μg/ml of supplemented MEM alone or MEM containing polymyxin B (20 μg/ml; Sigma). After 1 h of incubation at 37°C in a 5% CO_2 atmosphere, the polymyxin B-treated and untreated preparations were assayed for the induction of TNF-α as described above.

Fractionation of PEC. Plastic-adherent cells were isolated by incubating the PEC (5×10^6 cells per ml of MEM) in 75-cm^2 tissue culture flasks for 2 h at 37°C in a 5% CO_2 atmosphere. After removal of the nonadherent cells, adherent cells were detached with a cell scraper and suspended in supplemented MEM to the original cell concentration.

Nylon wool-nonadherent PEC were isolated by the method of Julius et al. (22). In brief, unfractionated PEC (3×10^7 cells) were suspended in 1 ml of MEM and applied to a syringe column containing 0.7 g of nylon wool. After incubation for 1 h at 37°C in a 5% CO_2 atmosphere, the nonadherent cell fraction was eluted in 30 ml of warm medium, pelleted by centrifugation, and then suspended in medium to their original volume.

Depletion of silica-sensitive macrophages was performed by suspending 2×10^7 unfractionated PEC in MEM containing silica (1 mg/ml of medium; Sigma) (24). The suspension was incubated at 37°C in a 5% CO_2 atmosphere for 2 h, washed three times in medium, and suspended in medium to the original volume. To deplete B and T lymphocytes, unfractionated PEC (1.5×10^7 cells) were treated with 150 μl of affinity-purified goat anti-mouse immunoglobulin (directed against immunoglobulins M [IgM], G, and A; Cooper Biomedical, Inc., West Chester, Pa.), or 100 μl of a 1:20 dilution of mouse monoclonal anti-Thy-1.2 (Accurate Chemical and Scientific Corp., Westbury, N.Y.), respectively. After a 30-min incubation on ice, a 1:10 dilution of rabbit Low-Tox complement (C) was added, and the cell suspensions were then incubated for an additional 30 min at 37°C under CO_2. PEC treated with C alone were included as a negative control. The treated cells were washed by centrifugation and suspended in supplemented MEM to their original volume.

Neutralization with rabbit antiserum to TNF-α. Supernatants (90 μl) from stimulated or nonstimulated PEC were added to wells in triplicate on a microdilution plate; 10 μl of rabbit anti-mouse TNF-α serum (Genzyme) or, for comparison, normal rabbit serum, each diluted 1:100 in medium, was then added to the wells. After the microdilution plates were incubated for 1 h at 37°C in a 5% CO_2 atmosphere, 100 μl each of L_S and L_R cells, at concentrations of 10^5 cells per ml, was added, and cytotoxicity was measured after 48 h of incubation.

Neutralization with antispherulin serum. Formalin-killed spherules were suspended to a concentration of 1 mg/ml of supplemented MEM and then mixed with an equal volume of undiluted goat antiserum to spherulin. This antiserum was prepared by immunizing a goat over a 1-year period with a total of 10 mg of spherulin (Berkeley Biologicals, Berkeley, Calif.) as previously described (11). Comparative assays were performed with a hyperimmune goat antiserum against *Mycobacterium tuberculosis* H37Ra (obtained from Rodney Michael, Madigan Army Medical Center, Tacoma, Wash.) and with serum from a normal (nonimmunized) goat.

After 2 h of incubation at 37°C in a 5% CO_2 atmosphere, the spherules were pelleted by centrifugation and suspended in supplemented MEM to their original concentration. The antibody-treated preparations were then assessed for their ability to induce TNF-α by peritoneal macrophages as described above.

Statistical analyses. The data were analyzed by using the Student unpaired *t* test. Probabilities of less than 0.05 were considered to be significant.

RESULTS

Induction of a cytotoxic factor(s) by spherules. Supernatants from PEC incubated with Formalin-killed spherules at concentrations of 0.1, 1.0, and 10.0 μg/ml exhibited a mean cytolytic activity against L_S cells of 13, 47, and 99%, respectively (Fig. 1). The cytotoxicity of these supernatants was not attributed to a direct toxic effect of spherule components that may have been solubilized during the incubation of the fungal cells with PEC, as evidenced by the finding that supernatants from spherules incubated in medium alone caused less than 6% lysis of the target cells. Comparisons of cytotoxin induction by spherules and LPS established that spherules were comparable to LPS when assayed at a dose of 10 μg/ml (i.e., 1 μg per well) but were significantly less stimulatory than LPS when assayed at concentrations ranging from 0.01 to 1 μg/ml ($P < 0.05$).

The kinetics of cytotoxin induction by spherules and LPS

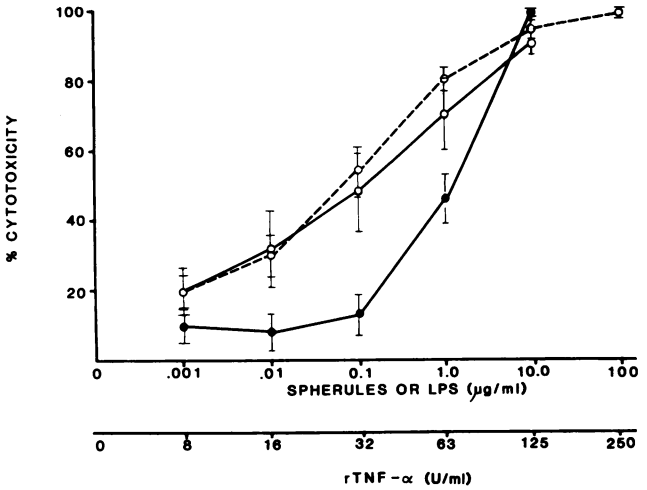

FIG. 1. Cytotoxic activity of supernatants from PEC stimulated with spherules (●——●) or LPS (○——○) when assayed against L_S cells. Also shown is the cytotoxicity obtained with murine rTNF-α (○------○) at concentrations ranging from 8 to 250 U/ml (i.e., 0.8 to 25 U per well). Results are expressed as mean cytotoxicity ± standard error.

are depicted in Fig. 2. Cytotoxin was rapidly secreted by PEC in response to 10 μg of LPS per ml; i.e., 98% of L cells were lysed by supernatants harvested after 2 h of stimulation. In contrast, maximum induction of cytotoxicity by spherules (10 μg/ml) was delayed, requiring 12 h of incubation with PEC.

Studies were also conducted to determine whether cytotoxin was induced by viable *C. immitis* spherules. Because these cells show extensive clumping when grown in liquid medium, we were unable to quantitate accurately, by hemacytometer counts or by CFU, the actual number of spherules used. As an alternative approach, we compared the cytolytic activities of serially diluted supernatants from spherule-stimulated PEC with those obtained in response to various dry weight concentrations of Formalin-killed spherules. Supernatants from PEC stimulated with freshly harvested

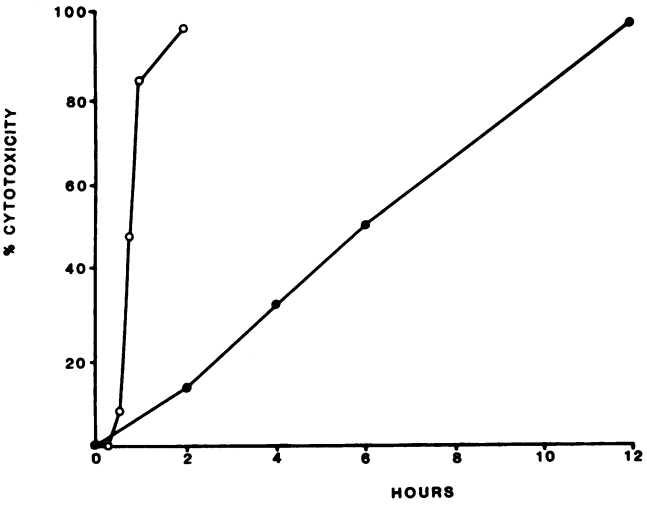

FIG. 2. Kinetics of TNF-α production by PEC after incubation with spherules (●) or LPS (○), each at a concentration of 10 μg/ml.

TABLE 1. Identification of cytotoxin as TNF-α

Assay	% Cytotoxicity (mean ± SE of 3 expts)		
	Supernatants from PEC stimulated with:		
	Spherules (10 μg/ml)	LPS (10 μg/ml)	Murine TNF-α (250 U/ml)
Target cell line			
L_S cells	98.6 ± 0.4	89.6 ± 3.3	98.2 ± 1.1
L_R cells	0	0	2.9 ± 2.9
Treatment of supernatants with:			
Normal rabbit serum	98.5 ± 0.5	82.9 ± 9.3	96.9 ± 2.0
Rabbit anti-mouse TNF-α serum	0	5.6 ± 5.6	1.3 ± 1.3

spherules induced lysis of ≥97% of the L_S cells when assayed at dilutions between 1:2 and 1:32. Dilutions of 1:64, 1:128, 1:256, and 1:512 effected cytolysis in 89, 64, 35, and 15% of the L_S cells, respectively. Extrapolation of these data indicate that the 64% cytotoxicity obtained with the 1:256 dilution of supernatants from PEC incubated with viable spherules was comparable to that induced by killed spherules at a concentration of 2 μg/ml (Fig. 1).

Identification of the cytotoxin as TNF-α. The specificity of the cytotoxin induced by spherules was assessed by using the TNF-α-sensitive L_S cell line and the TNF-α-resistant L_R cell line (34). Supernatants from spherule-stimulated PEC were selectively lytic for the L_S cell line (Table 1). Identical results were obtained with murine rTNF-α and with supernatants from PEC incubated with LPS.

Incubation of supernatants from spherule-stimulated PEC with rabbit antiserum to murine TNF-α effectively neutralized cytolytic activity against L_S cells (Table 1). Normal rabbit serum was without effect. The cytotoxin activity of supernatants from LPS-stimulated PEC and that of murine rTNF-α were also neutralized by polyclonal antiserum to TNF-α.

The preceding data are consistent with the identification of the cytotoxin as TNF-α, but do not exclude cytotoxicity by lymphotoxin (TNF-β), a lymphokine that is functionally and antigenically similar to TNF-α (18, 32, 35). Experiments were performed, therefore, to characterize the PEC population that produced cytotoxin in response to Formalin-killed spherules (Fig. 3). Cytotoxin production was associated with a cell population that was adherent to plastic, partially sensitive to silica, and removed by passage of the PEC over a nylon wool column. Depletion of T or B lymphocytes by incubation with anti-Thy-1.2 plus C or anti-mouse immunoglobulin plus C, respectively, did not diminish the induction of cytotoxin. These collective results establish that the cytotoxin induced by spherules is elaborated by a cell population having characteristics of macrophages, a finding that is consistent with macrophages being a major source of TNF-α (28).

Effect of polymyxin B on the induction of TNF-α. To establish that the induction of TNF-α was attributed to spherules and not to contamination of the preparations with LPS, Formalin-killed spherules were pretreated with polymyxin B, a reagent that neutralizes the TNF-α-inducing lipid A component of LPS (29, 30). Treatment of LPS with polymyxin B reduced cytokine production from 92.6 ± 4.2% to 31.5 ± 1.6% ($P < 0.005$). In contrast, polymyxin B was without effect on the cytokine response to killed spherules; i.e., supernatants from macrophages incubated with non-

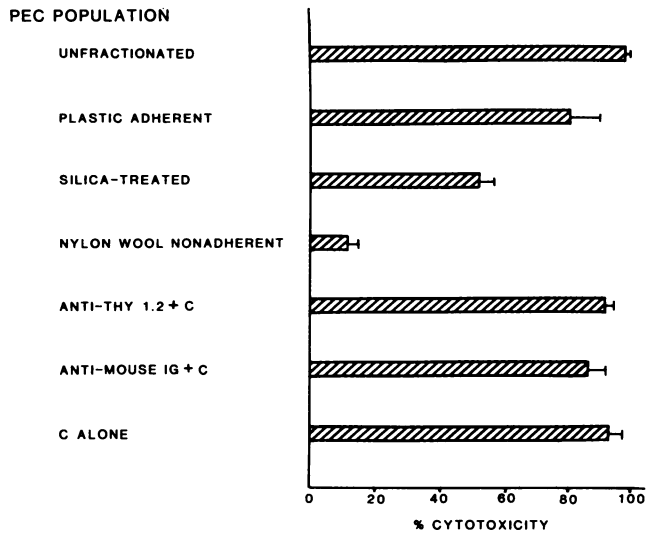

FIG. 3. Identification of the TNF-α-producing PEC as an adherent, silica-sensitive cell population that is resistant to anti-mouse Thy-1.2 plus C and anti-mouse immunoglobulin plus C. The PEC were fractionated, and the fractions assayed for the production of TNF-α in response to spherules (10 μg/ml). Results are expressed as the mean ± standard error obtained in three experiments.

treated and polymyxin B-treated spherules were cytotoxic for 99.3 ± 0.3 and 99.4 ± 0.6% of the L_S cells, respectively.

Effect of goat antispherulin serum on the induction of TNF-α. Studies were next done to assess the effect of hyperimmune goat antiserum to *C. immitis* on the induction of TNF-α by spherules. Pretreatment of spherules with goat anti-spherulin caused a significant decrease in cytotoxin induction when compared with the responses obtained after treatment of spherules with normal goat serum or serum from a goat immunized with *M. tuberculosis* H37Ra ($P < 0.005$) (Fig. 4). The induction of TNF-α by LPS was unaffected by goat antispherulin.

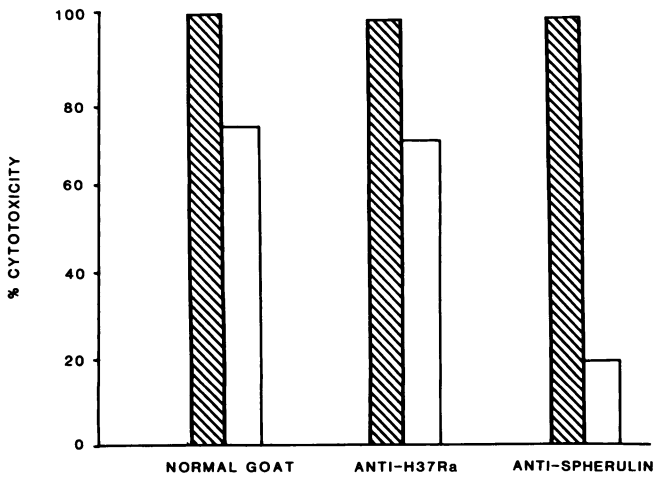

FIG. 4. Neutralization of cytotoxicity after preincubation of spherules with goat antispherulin serum. Spherules (□) or LPS (▨), each at a concentration of 1 μg/ml, were pretreated with normal goat serum, goat anti-H37Ra serum, or goat antispherulin serum before incubation with PEC.

DISCUSSION

The results of this investigation establish that viable or Formalin-killed spherules of *C. immitis* induce the secretion of TNF-α by peritoneal macrophages from BALB/c mice. The identification of the cytokine as TNF-α was established by its selective toxicity for the TNF-α-sensitive L_S cell line, its neutralization by rabbit anti-TNF-α, and its production by cells having characteristics of macrophages.

Quantitative comparisons of cytotoxin induction by spherules and LPS established that spherules were less stimulatory than LPS at concentrations of ≤1 μg/ml but were comparable to LPS when assayed at 10 μg/ml. The kinetics of TNF-α induction also differed between the two stimulants. Whereas LPS induced maximal cytokine secretion within 2 h of exposure to macrophages, cytokine response to spherules was delayed, reaching maximal levels after a 12-h incubation period. The induction of TNF-α by spherules and LPS was further distinguished in that goat antispherulin serum neutralized cytotoxin induction by spherules but not LPS, and, conversely, polymyxin B significantly decreased cytoxin induction by LPS but not spherules. These dissimilar patterns of response suggest that the TNF-α-inducing component of spherules is qualitatively distinct from the TNF-α-inducing lipid A component of LPS (29, 30) or, alternatively, that the mechanism(s) by which spherules activate macrophages to secrete TNF-α differs from that of LPS.

Although we have not established that TNF-α is induced in vivo by *C. immitis*, certain observations support this possibility. In an earlier investigation to assess antigen-induced suppression of host response in coccidioidomycosis (12), we observed that mice injected intravenously with high doses of coccidioidal antigens (i.e., ≥2.5 mg) developed a shocklike syndrome that was characterized by ruffled fur, acute diarrhea, and, in some mice, death. This pathophysiologic response is consistent with the acute shock produced in animals injected with TNF-α (39, 40) or TNF-α-inducing agents (5). Certain evidence also suggests that TNF-α may be induced during the course of naturally acquired coccidioidal disease. Primary infection with this diphasic fungus is usually asymptomatic or benign but may progress into disease involving pulmonary and extrapulmonary tissues. Fiese, in his early monograph on coccidioidomycosis (17), noted that an anorexia and wasting syndrome (cachexia) was often manifested by persons with coccidioidomycosis, including those with primary, uncomplicated infection. He further noted, "when dissemination follows, the anorexia of the primary stage may progress into profound cachexia." Cachectic responses have also been described in animals experimentally infected with *C. immitis* (31). The possibility that the cachexia is attributable to the induction of TNF-α by *C. immitis* can be evaluated by using two approaches. One would be to administer hyperimmune antiserum against the spherule-endospore phase, which in the present study was shown to significantly reduce TNF-α production in vitro. The other approach would be to neutralize the effects of TNF-α by administering antibody(s) specific for TNF-α (5).

Another potential role for TNF-α in coccidioidomycosis would be the augmentation of host response, as has been shown in other infectious diseases. Haidaris et al. (19), in 1983, reported sera from BCG-infected, LPS-treated mice were cytotoxic to *Plasmodium falciparum*-infected human erythrocytes. The antiparasitic activity was diminished after treatment of the sera with rabbit antiserum to TNF-α. This cytokine has also been shown to activate polymorphonu-

clear neutrophils to inhibit *C. albicans* (15) and *Legionella pneumophilia* (7), to augment the intracellular killing of *Mycobacterium avium* complex by human and mouse monocytes and macrophages (3), and to mediate platelet cytotoxicity against larvae of *Schistosoma mansoni* (13). A protective role for TNF-α has been further demonstrated in vivo in experimental infection with *Plasmodium* species (10, 38), *L. pneumophilia* (7), *Trypanosoma cruzi* (14, 43), and certain viruses (1). The ability of this cytokine to potentiate host defense against a large number of phytogenetically distinct pathogens merits studies to assess its role in resistance to *C. immitis*.

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