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Roles of Gamma Interferon and Interleukin-4 in Genetically Determined Resistance to *Coccidioides immitis*

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The profiles of gamma interferon (IFN-γ) and interleukin-4 (IL-4) production were evaluated during the course of coccidioidomycosis in two inbred mouse strains which differ in their susceptibility to *Coccidioides immitis*. Cytokine responses, measured at the molecular and protein levels, showed increased levels of IFN-γ in lung extracts from mice of the resistant DBA/2 strain after a pulmonary challenge, whereas the susceptible BALB/c strain manifested a predominant IL-4 response. The importance of these cytokines in host defense against *C. immitis* was established by treating the mice with recombinant cytokines or neutralizing anticytokine monoclonal antibodies. Treatment of the susceptible BALB/c mice with recombinant murine IFN-γ significantly protected mice against systemic challenge, and in the reciprocal experiment, the administration of an anti-IFN-γ monoclonal antibody to the resistant DBA/2 mice significantly decreased their capacity to control disease. Although the treatment of DBA/2 mice with recombinant IL-4 did not alter the disease, neutralization of endogenous IL-4 in infected BALB/c mice by administration of a neutralizing anti-IL-4 antibody led to a significant reduction in the fungal load in their tissues. These results, taken together, establish that IFN-γ plays a pivotal role in resistance to *C. immitis*, whereas IL-4 down-regulates protective immunity against *C. immitis*.

Coccidioidomycosis is a mycotic disease endemic to the southwestern United States and parts of Central and South America. The etiologic agent *Coccidioides immitis* propagates in the soil in a saprobic mycelial phase which gives rise to enterothallic arthroconidia. Primary infection is acquired by inhalation of the arthroconidia, which enter the alveoli and undergo a morphologic conversion into endosporulating spherules. The disease has protean manifestations, ranging from an inapparent or benign pulmonary infection to a progressive and often lethal disseminated form that most commonly involves the central nervous system, skin, and bones (3, 15, 16).

The profiles of cell-mediated immune (CMI) and humoral immune responses have consistently shown that a CMI response directly correlates with resistance to *C. immitis*, whereas antibody production inversely correlates with resistance (3, 7, 9, 10, 13, 15). Thus, persons with asymptomatic or benign disease typically manifest strong skin test reactivity and in vitro T-cell responses to *C. immitis* antigens and produce low or nondemonstrable levels of anti-*Coccidioides* antibodies. The development of progressive, disseminated coccidioidomycosis, in contrast, is associated with T-cell anergy and the production of exaggerated levels of anti-*Coccidioides* immunoglobulin G (IgG) and IgE antibodies.

The mechanisms that govern the induction and expression of CMI responses in coccidioidomycosis have not been elucidated but appear to be under genetic control. Cumulative studies have established that persons of Asian, black, or Hispanic ancestry are at high risk for developing disseminated coccidioidomycosis (3, 32). It has been hypothesized, but not proved, that this increased susceptibility is attributable to a defect in the ability of these persons to mount or maintain CMI responses to the fungus (7). A focus of our work has been to examine this hypothesis by comparing the induction and expression of T-cell responses in two inbred mouse strains which differ in their susceptibility to *C. immitis* (12, 23). In a previous report (12), the highly susceptible BALB/c mouse strain was shown to mount a delayed-type footpad hypersensitivity to coccidioidin early after pulmonary challenge to a level comparable to that of the resistant DBA/2 mouse strain. The two mouse strains showed divergent responses thereafter in that DBA/2 mice maintained their T-cell reactivity, whereas BALB/c mice became anergic (12). The acquired anergy was transferable to syngeneic recipient mice with the soluble fraction obtained from lysed spleen cells (11). In marked contrast to the suppressive effect of spleen cell lysates from infected BALB/c mice, lysates of spleens from infected DBA/2 mice were found to potentiate T-cell responses in syngeneic recipients (8). The cumulative results indicate that these two mouse strains exhibit marked differences in the afferent and efferent arms of the immune response to infection with *C. immitis*. Since cytokines are important for controlling the development and expression of protective cellular immunity, we investigated the in vivo production of gamma interferon (IFN-γ) and interleukin-4 (IL-4) during active coccidioidomycosis. The relevance of these molecules in host defense against *C. immitis* was examined by the complementary approaches of treatment of the mice with recombinant cytokines and in vivo cytokine depletion with specific monoclonal antibodies (MAb).

MATERIALS AND METHODS

Mice. Female BALB/c and DBA/2 mice, 5 to 7 weeks of age, were purchased from Charles River Laboratories (Charleston, S.C.). The mice were maintained for at least 1 week before use. Serologic analyses, performed by Microbiological Associates (Rockville, Md.), showed that the mice were negative for antibodies to common murine pathogens.

Infection. The procedure for infecting mice via a pulmonary route was performed as described previously (11). In brief, arthroconidia were harvested from 6- to 8-week-old mycelial-phase cultures of *C. immitis* Silveira (ATCC 28868), passed over a nylon column to remove hyphal elements, and enumerated by hemacytometer counts. Mice were lightly anesthetized by an intraperitoneal (i.p.) injection of sodium pentobarbital and then infected by intranasal instillation of 20 arthroconidia suspended in 20 μl of pyrogen-free saline. A systemic

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challenge was performed by injecting the mice i.p. with 400 arthroconidia suspended in 0.5 ml of pyrogen-free saline.

Cytokine responses. Cytokine-specific mRNA expression was evaluated by the reverse transcription PCR procedure (24, 27, 29). For these analyses, the lungs were homogenized in 2 ml of a guanidine thiocyanate-phenol buffer (Tri-Reagent; Molecular Research Center, Cincinnati, Ohio). Chloroform (200 μl) was added to 1 ml of lung homogenate, and after vigorously shaking the samples, the RNA was precipitated from the aqueous phase by the addition of isopropanol (500 μl). The precipitate was washed with 75% ethanol–25% diethyl pyrocarbonate-treated water and then air dried. Samples were diluted in diethyl pyrocarbonate-treated water to a concentration of 1 mg/ml, as determined by an optical density at 260 nm, and maintained at −20°C until processed further.

For reverse transcription, the RNA (1 μg) was diluted in 6.5 μl of a reaction mixture containing 2 μl of 5× RT buffer (250 mM Tris-HCl [pH 8.3], 375 mM KCl, 15 mM $MgCl_2$), 100 mM dithiothreitol, 2.0 μl of deoxynucleoside triphosphate (dNTP) mixture, 1 μl (1 μg) of random hexamers, 0.5 μl (20 U) of RNasin (Promega, Madison, Wis.), and 1.0 μl (200 U) of Moloney murine leukemia virus reverse transcriptase (GIBCO, Grand Island, N.Y.). The reaction mixture was incubated at 25°C for 10 min at 42°C for 1 h, and then at 95°C for 10 min to denature the reverse transcriptase.

PCR was performed by adding 2 μl of the cDNA to 44 μl of a PCR mixture containing 5 μl of 10× reaction buffer (100 mM Tris-HCl, pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin, 1 μl (10 mM) of each dNTP, 0.25 μl (1 U) of Amplitaq DNA polymerase (Perkin-Elmer, Norwalk, Conn.), and 34.75 μl of sterile dH_2O. The PCR primer pairs were added in a volume of 4 μl, and the target sequences were amplified in an PTC-100 thermal controller (MJ Research Inc., Watertown, Mass.) programmed for 5 min at 94°C and 5 min at 60°C and then for 25 to 30 cycles of 1.5 min at 72°F, 45 s at 94°F, and 45 s at 60°C, with a final extension of 10 min at 72°C. The PCR products (10 μl) were electrophoresed through a 2.0% agarose gel and visualized by staining with ethidium bromide. Primer sets were synthesized for murine IFN-γ, IL-4, and hypoxanthine phosphoribosyl-transferase, which served as an internal control (27). The primers were designed to span at least one intron and contained the following sequences (5′ to 3′): IFN-γ, sense TACTGCCACGGCACAGTCATTGAA, antisense GCAGCGACTCCTTTTCCGCTTCCT (17); IL-4, sense ACGAGGAT GGATGTGCCAAACGTC, antisense CGAGTAATCCATTTGCATGATGC (28); and hypoxanthine phosphoribosyl-transferase, sense GTAATGATCAGT CAACGGGGGAC, antisense CCAGCAAGCTTGCAACCTTAACCA (27). Controls for the PCR were assayed in parallel with the lung mRNA and included samples of lung RNA that had not been reverse transcribed, to rule out contamination with genomic DNA, and as a positive control, a cDNA provided by the manufacturer.

Cytokine production at the protein level was determined by enzyme-linked immunosorbent assay (ELISA). Mice were sacrificed, and their lungs were removed en bloc and homogenized in a glass tissue grinder (Wheaton 200; Thomas Scientific, Swedesboro, N.J.). The homogenates were centrifuged to remove cellular debris (10,000 × *g* at 4°C for 10 min), filtered through a 0.22-μm-pore-size membrane, and frozen at −70°C until assayed. IFN-γ levels were quantified by an ELISA purchased from Genzyme Corporation (Boston, Mass.); IL-4 was quantified by an ELISA from Endogen, Inc. (Cambridge, Mass.).

Cytokines and anticytokine antibodies. Recombinant murine IFN-γ (rIFN-γ; lot no. M3-RD48, specific activity, 5.2×10^6 U/mg) was a generous gift of Genentech Corporation (South San Francisco, Calif.). Recombinant murine IL-4 (rIL-4; lot no. 2530-029, specific activity, 7.0×10^7 U/mg) was generously provided by Sterling Winthrop (Collegeville, Pa.). The preparations were diluted in Hanks balanced salt solution to a concentration of 10^6 U/ml.

Affinity-purified rat IgG1 MAb to murine IFN-γ (anti-IFN-γ MAb; neutralization activity, 7.2×10^3 U/mg) was purchased from Lee Biomolecular Research (San Diego, Calif.). The MAb was diluted in a buffer composed of 0.1 M NaCl, 10 mM Tris-HCl, and 1 mM Na_2 EDTA (pH 7.2) to contain 5×10^3 neutralizing units per ml. Rat IgG1 MAb to murine IL-4 was prepared from the 11B11 rat hybridoma (30) grown as ascites in pristane-primed BALB/c *nu/nu* mice. The MAb was precipitated with 50% ammonium sulfate, dialyzed against phosphate-buffered saline, and diluted to a concentration of 600 μg of protein/ml of Hanks balanced salt solution. Affinity-purified rat IgG (ICN ImmunoBiologicals, Costa Mesa, Calif.), diluted to 600 μg of protein/ml of Hanks balanced salt solution was used as an irrelevant control.

Assessment of disease severity. Mice were sacrificed at 12 or 13 days after the challenge, and their lungs, livers, and spleens were removed, weighed, and homogenized in 2 ml of sterile saline, as described previously (12). The homogenates were serially diluted and plated on Mycobiotic medium (Difco Laboratories, Detroit, Mich.), and the numbers of CFU were determined after a 3- to 5-day incubation at 30°C.

Statistical analyses. The results were analyzed for statistical significance by the parametric Student's *t* test or nonparametric Mann-Whitney rank-sums test, depending on whether the data fit a normal distribution curve. Probability values of <0.05 are considered significant.

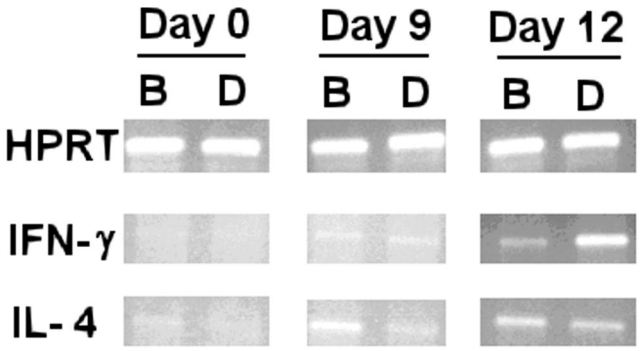

FIG. 1. Expression of mRNA transcripts for hypoxanthine phosphoribosyltransferase (HPRT), IFN-γ, and IL-4 in lung extracts obtained from BALB/c (B) and DBA/2 (D) mice before and at 9 and 12 days after pulmonary challenge. The image was prepared by using Aldus Photostyler for Windows from photographs of gels scanned with a Hewlett-Packard ScanJet IICX.

RESULTS

Cytokine production. Previous data from this laboratory showed that both the resistant DBA/2 and susceptible BALB/c strains of mice develop delayed-type hypersensitivity responses to *C. immitis* antigens by day 9 postinfection (12). Thereafter, between days 12 and 15, BALB/c mice become anergic, whereas DBA/2 mice maintain a strongly positive delayed-type hypersensitivity response. To determine if these differences correlated with differences in cytokine expression by the two mouse strains, RNA was extracted from infected mouse lungs and assayed for the presence of IFN-γ and IL-4 messages. The results, depicted in Fig. 1, showed that IFN-γ mRNA was expressed at comparable levels in lung tissues from both mouse strains at day 9, but by day 12, IFN-γ mRNA expression was increased in the DBA/2 mice relative to that of BALB/c mice. In contrast, the increased expression of IL-4 mRNA was detected in lungs from infected BALB/c mice at day 9, as compared with lungs from infected DBA/2 mice. By day 12, both mouse strains showed increased IL-4 mRNA.

To confirm the results of the PCR amplification and to ensure that mature proteins were produced, we performed experiments to measure IFN-γ and IL-4 protein levels by ELISA at 3-day intervals after the pulmonary challenge. The resistant DBA/2 mice showed increased levels of IFN-γ, when compared with levels produced by infected BALB/c mice, as early as 6 days postinfection ($P < 0.05$) (Fig. 2). The production of this cytokine was not detected in the susceptible mouse strain until day 9 postinfection, and although the levels increased thereafter in both DBA/2 and BALB/c mice, the magnitude of the response was significantly greater in the resistant mouse strain at 12 days ($P < 0.0001$) and 15 days ($P < 0.05$) postinfection.

A reciprocal pattern was observed in the kinetics of the IL-4 production. IL-4 levels were significantly elevated ($P < 0.01$) in lung homogenates from BALB/c mice 9 days after the pulmonary challenge, at which time DBA/2 mice showed only a modestly elevated level of IL-4. Thereafter, on days 12 and 15, both strains showed increased and comparable levels of IL-4 in lung tissues. Cytokine responses were not measured beyond day 15 because of an excessive mortality in the BALB/c mouse group (12).

Treatment of mice with rIFN-γ or anti-IFN-γ. The association between the IFN-γ response and resistance to *C. immitis* prompted studies to determine if rIFN-γ treatment of the mice of the susceptible BALB/c strain might increase their resis-

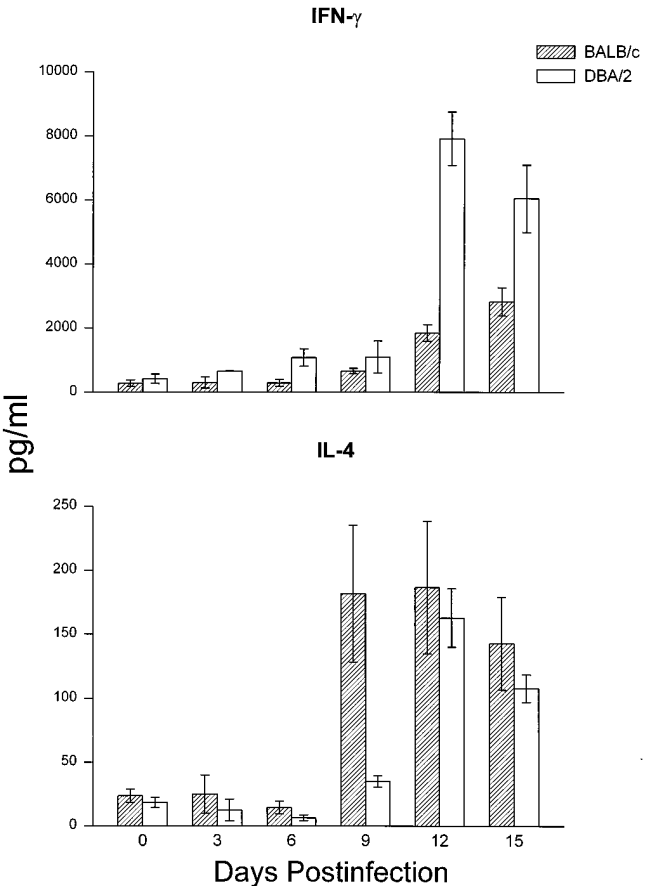

FIG. 2. Levels of IFN-γ and IL-4 in homogenates of lung tissues obtained before and at various times after pulmonary challenge. The bars depict means ± standard errors obtained in groups of seven or more mice.

tance to the disease. For these experiments, the mice were injected i.p. with 10^5 U of rIFN-γ, beginning on the day before infection and then again on the day of pulmonary or i.p. challenge and at daily intervals for 12 days postinfection. The recombinant cytokine did not protect against pulmonary challenge (Fig. 3A) but provided a highly significant level of protection in BALB/c mice against the systemic challenge (Fig. 3B), as measured by a reduction in the number of fungal CFU in their lungs ($P < 0.0001$), livers ($P < 0.0001$), and spleens ($P < 0.003$) at day 13 postinfection when compared with the CFU in sham-treated mice.

A protective role of IFN-γ was confirmed by using a reciprocal approach, wherein we treated the resistant DBA/2 mice with 10^3 neutralizing units of rat anti-mouse IFN-γ MAb. The MAb was administered intravenously beginning on the day before challenge and then repeated on the day of the challenge and at 3-day intervals for 12 days. The results, shown in Fig. 4A, established that the neutralization of endogenous IFN-γ in DBA/2 mice effected a significant decrease in their ability to control the fungus after pulmonary challenge, as evidenced by the increased numbers of fungal CFU in their lungs, livers, and spleens by 13 days postinfection ($P < 0.02$ in each organ). The administration of anti-IFN-γ also potentiated the disease when the mice were challenged via the systemic route ($P < 0.02$ in each organ) (Fig. 4B).

Treatment of mice with rIL-4 or anti-IL-4. The increased production of IL-4 in the susceptible BALB/c mice suggested that this cytokine might be detrimental to host defense against *C. immitis*. To test this, DBA/2 mice were treated with 5×10^4 U of the recombinant IL-4 by daily i.p. injections, beginning on the day before systemic challenge and continuing for 12 days after infection. Treatment with rIL-4 did not reduce the capacity of DBA/2 mice to contain the disease, as evidenced by the comparable numbers of fungal CFU in tissues from rIL-4-treated and nontreated mice (Fig. 5).

By the reciprocal approach, BALB/c mice were treated with a neutralizing anti-IL-4 MAb. The neutralization of endogenous IL-4, via intravenous injections of 300 μg of rat anti-IL-4 MAb on the day before, on the day of, and at 3-day intervals after systemic challenge, significantly protected mice as revealed by a reduction in CFU in the lungs ($P < 0.005$) and livers ($P < 0.02$) at day 13 postinfection as compared with CFU of mice given normal rat IgG (Fig. 6).

DISCUSSION

The results of this investigation establish that differences in the susceptibility and the resistance to *C. immitis* of two inbred mouse strains are expressed by, and possibly regulated through, the preferential production of IFN-γ or IL-4. Analyses of cytokine responses at the molecular and protein levels revealed that mice of the resistant DBA/2 strain mounted a predominant IFN-γ response in their lungs after pulmonary challenge, whereas mice of the susceptible BALB/c strain showed a predominant IL-4 response. The importance of these cytokines as determinants of disease outcome was established by treating the mice with recombinant murine cytokines or neutralizing anticytokine antibodies. Treatment of the susceptible BALB/c mouse strain with rIFN-γ significantly protected

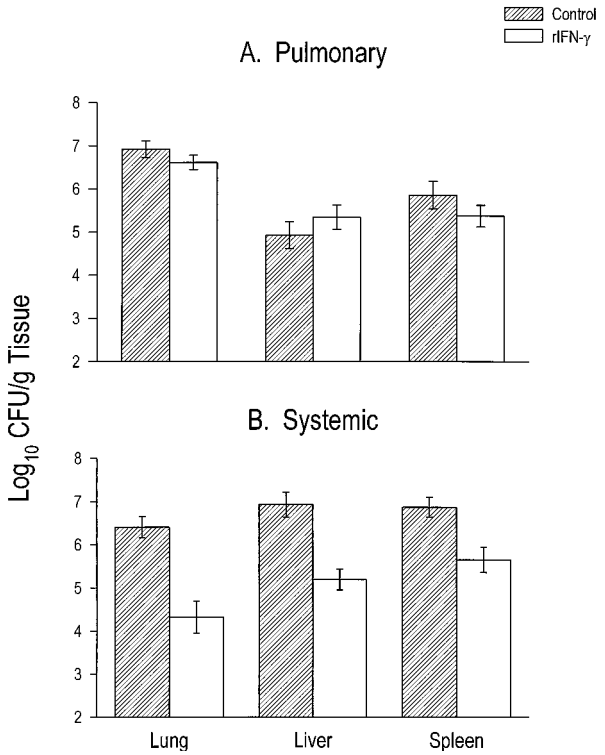

FIG. 3. Therapeutic effects of rIFN-γ treatment of BALB/c mice. The bars depict means ± standard errors of $\log_{10}$ CFU/g of lung, liver, or spleen tissue from groups of 13 mice treated with rIFN-γ or buffer alone and then sacrificed 13 days after intranasal challenge (A) or i.p. challenge (B).

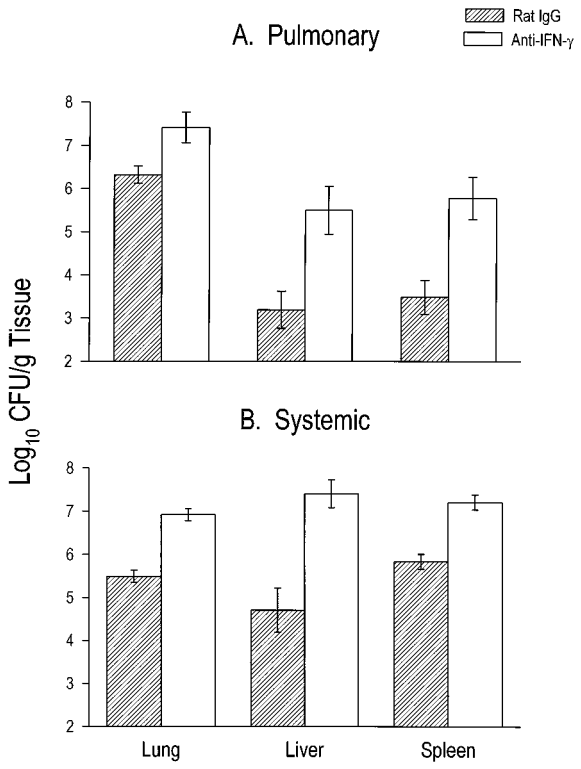

FIG. 4. Adverse effects of neutralizing endogenous IFN-γ in the resistant DBA/2 mouse strain by administration of rat anti-mouse IFN-γ MAb or normal rat IgG. The bars depict means ± standard errors of $\log_{10}$ CFU obtained from groups of 10 mice at 14 days after intranasal challenge (A) or i.p. challenge (B).

animals against systemic challenge, and by the converse approach, treatment of the resistant DBA/2 strains with neutralizing anti-IFN-γ antibodies potentiated the course of the disease. Although the treatment of DBA/2 mice with rIL-4 did not alter the disease, neutralization of endogenous IL-4 in infected BALB/c mice by the administration of a neutralizing

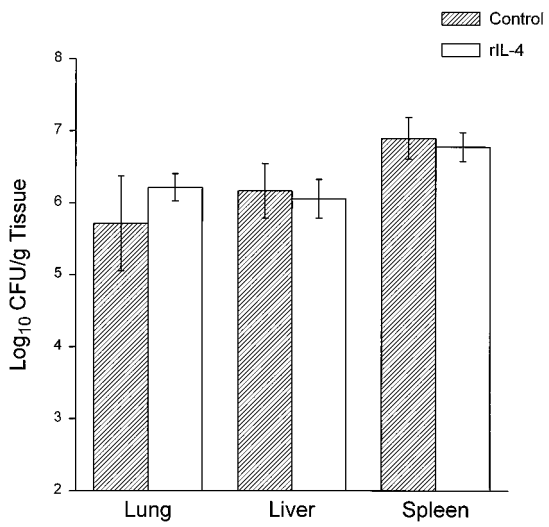

FIG. 5. Lack of an effect of rIL-4 treatment of DBA/2 mice. The bars depict means ± standard errors of $\log_{10}$ CFU obtained from groups of 10 mice given rIL-4 or buffer alone. The mice were sacrificed 13 days after the i.p. challenge.

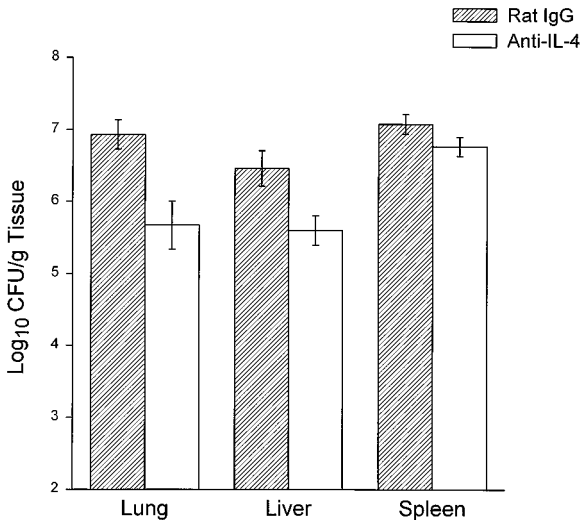

FIG. 6. Enhanced resistance in BALB/c mice treated with anti-mouse IL-4 MAb. The bars depict means ± standard errors of $\log_{10}$ CFU obtained from groups of 10 mice given anti-IL-4 MAb or normal rat IgG. The mice were sacrificed 13 days after the i.p. challenge.

anti-IL-4 antibody led to a significant reduction in the fungal load in their tissues.

The administration of rIFN-γ via the i.p. route protected BALB/c mice against systemic challenge but did not ameliorate the severity of the disease in mice challenged via the pulmonary route. A lack of protection against pulmonary challenge can likely be explained on the basis that systemic administration of IFN-γ does not activate pulmonary macrophages (22). It bears emphasis, however, that depletion of endogenous IFN-γ in mice infected by the pulmonary route reduced their capacity to restrict the disease, a result that validates the use of a systemic challenge for this cytokine. The pulmonary and systemic routes of challenge are also valid for analyses of IL-4. Depletion of endogenous IL-4 by treatment with anti-IL-4 led to a significant reduction in the fungal load in tissues of mice infected by a systemic route (Fig. 6), and in a preliminary experiment, anti-IL-4 treatment of BALB/c mice challenged by the pulmonary route led to a log reduction in the number of CFU in their livers and spleens (data not shown). In addition, we recently demonstrated that both IL-4 and IFN-γ mRNAs are expressed, in a reciprocal manner, in lung extracts from BALB/c mice infected by the systemic route (results not shown) as well as by the pulmonary route (Fig. 1). Future studies should be directed towards evaluating cytokine expression in mice after treatment with neutralizing anti-IFN-γ or anti-IL-4 at various days during the course of disease progression and assessing the effect of cytokine depletion upon survival after the challenge.

Previous investigations have shown that murine $CD4^+$ T cells can be divided into subsets, distinguished by their profile of cytokine production (26, 37, 38). T helper 1 (Th1) lymphocytes produce IFN-γ and IL-2, whereas Th2 cells produced IL-4, IL-5, IL-6, and IL-10. The preferential expression of Th1- or Th2-associated cytokines in vivo has a pronounced effect upon the outcome of disease in murine leishmaniasis (18, 19, 25, 35, 36), listeriosis (21), schistosomiasis (27, 33, 37, 40), trypanosomiasis (20), tuberculosis (31), and candidiasis (34). Protection against these diseases correlates directly with the expression of Th1-associated cytokines, which mediate the induction and expression of CMI responses, whereas suscepti-

bility correlates with the expression of Th2-associated cytokines, which potentiate the production of IgE and IgG1 antibodies while suppressing macrophages and T-cell responses. Our finding that mice of the resistant DBA/2 strain showed an increased production of IFN-γ, whereas the susceptible BALB/c strain mounted an early IL-4 response, would be concordant with the preferential activation of Th1 and Th2 cells in the two strains, respectively. The production of these two cytokines was not, however, mutually exclusive. Rather, both mouse strains produced elevated levels of IFN-γ and IL-4 after 9 days of infection. It bears emphasis, however, that the lack of a dichotomy of cytokine production in the two strains was observed in cells compartmentalized to the site of infection and assayed for cytokine expression in the absence of exogenously added antigen. Whereas the latter may have yielded a clear divergence in the cytokine profile, in situ analyses of cytokine responses generally revealed quantitative rather than qualitative differences in cytokine expression (6, 18, 25, 39). Although IFN-γ production is assigned to Th1 T cells, natural killer cells also produce abundant levels of this cytokine (36). Hence, studies are needed to determine if IFN-γ levels are attributable to Th1 cells, natural killer cells, or both.

Little work has been done to elucidate the pattern of cytokine production in human coccidioidomycosis (1, 2, 14). Ampel et al. (2) showed that peripheral blood mononuclear cells and adherent monocytes from healthy skin-test-positive, but not skin-test-negative, subjects secrete IFN-γ and IL-2 when cultured in the presence of a spherule-phase lysate. Investigations are needed to assess cytokine responses in active coccidioidomycosis, with emphasis on determining if progressive, disseminated coccidioidomycosis might be associated with an increased production of IL-4 and other Th2-associated cytokines which can suppress T-cell function. The latter possibility is supported, albeit indirectly, by reports that patients with severe coccidioidomycosis manifest a hyperproduction of antibodies, including IgE (9, 10).

One mechanism by which IFN-γ might mediate resistance to *C. immitis* is by activating macrophages to inhibit or kill the fungus. Beaman (4) showed that the treatment of murine peritoneal or alveolar macrophages with rIFN-γ potentiated their anti-*Coccidioides* activity. Of particular interest, Beaman (5) also reported that rIFN-γ activated human blood monocytes to kill *C. immitis* arthroconidia and endospores. These results warrant studies to determine if active coccidioidomycosis might alter the capacity of macrophages to respond to activation with rIFN-γ.

The protective effect of rIFN-γ therapy was observed when treatment was initiated before the challenge with *C. immitis*. We have not yet addressed the question of whether therapy will reverse or ameliorate the disease when initiated after a challenge, that is, once the disease is established. This possibility has important implications to immunotherapeutic intervention in human coccidioidomycosis and will be investigated in future studies.

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