Induction and Expression of Cell-Mediated Immune Responses in Inbred Mice Infected with *Coccidioides immitis*

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Comparisons of the course of coccidioidomycosis in two strains of inbred mice established that BALB/c mice are significantly more susceptible to pulmonary infection with *Coccidioides immitis* than are DBA/2 mice. The susceptibility of BALB/c mice does not reside in their inability to mount a delayed-type hypersensitivity response to *C. immitis* antigen. That is, BALB/c mice manifested footpad hypersensitivity to coccidioidin early during the course of disease, to a level comparable to that of DBA/2 mice. In contrast to the more resistant DBA/2 mouse strain, however, BALB/c mice developed anergy by day 15 postinfection. Suppression of the delayed-type hypersensitivity response was not specific for *C. immitis* antigen, as evidenced by the finding that BALB/c mice immunized with mycobacterial purified protein derivative prior to infection with *C. immitis* were suppressed in their footpad response to mycobacterial antigen at day 15 postinfection. Taken together, these results establish that genetically determined susceptibility to this fungus is associated with an acquired suppression of cell-mediated immune reactivity.

Coccidioidomycosis is a mycotic disease caused by the diphasic fungus *Coccidioides immitis*. Primary infection is acquired via inhalation of mycelial-phase arthroconidia, which convert to endosporulating spherules in host tissue. Manifestations of the disease range from a benign, self-limited pulmonary infection to a severe, progressive, and often fatal mycosis involving pulmonary and extrapulmonary tissues.

Host defense against *C. immitis* is T-cell dependent, transferable by immune T cells, independent of antibody, and correlated with delayed-type hypersensitivity (DTH) to coccidioidal antigens (1–3). Thus, in humans and experimental animals, primary self-limited disease is commonly associated with strong cell-mediated immune responses to *C. immitis*, and conversely, progressive coccidioidomycosis is associated with depressed cell-mediated immunity (CMI) (6–9, 14, 23). The basis for the depressed CMI is not known, nor has it been determined whether depressed CMI is the cause or the effect of the disease.

Genetic factors have been considered to be important determinants in the outcome of primary coccidioidomycosis. This concept is supported by the finding that persons of certain races or ethnic ancestry, in particular Filipinos and Blacks, are predisposed to the development of severe, disseminated disease (11, 13, 21). In recent studies, Kirkland and Fierer (16, 17) documented differences in the susceptibility of inbred mouse strains to *C. immitis*, as evaluated by lethality and numbers of CFU of the fungus in tissues following intraperitoneal infection with arthroconidia. Of the various mouse strains examined, BALB/c mice were the most susceptible, and DBA/2 mice were the most resistant. Both of these mouse strains are of the *H-2*<sup>k</sup> haplotype; hence, susceptibility is not primarily controlled by the *H-2* locus.

The differences in the hereditary patterns of disease severity in coccidioidomycosis, combined with the crucial role of CMI in host defense against *C. immitis*, suggests an immunologic basis for genetically determined susceptibility to this fungus. With this supposition in mind, we evaluated the induction and persistence of DTH responses in BALB/c and DBA/2 mice at various time intervals after pulmonary infection with *C. immitis*. The results establish that both mouse strains mount a DTH response early during the course of disease but manifest divergent reactivity thereafter. Whereas BALB/c mice develop anergy to coccidioidin (CDN), the DTH response persists in the more resistant DBA/2 mouse strain.

**MATERIALS AND METHODS**

**Animals.** Female BALB/c and DBA/2 mice (age, 5 to 6 weeks) were purchased from Jackson Laboratory (Bar Harbor, Maine). The mice were maintained in our laboratory for 1 to 2 weeks before study.

**Antigens.** CDN was prepared as a toluene-induced lysate of mycelia of *C. immitis* Silveira (ATCC 28868), as described by Pappagianis et al. (22). Purified protein derivative (PPD) from *Mycobacterium tuberculosis* was obtained from Connaught Laboratories (Willowdale, Ontario, Canada).

**Infection.** Pulmonary infection with *C. immitis* was induced by intranasal instillation of viable arthroconidia of strain Silveira by previously published methods (18, 19). Briefly, arthroconidia were harvested in physiologic saline from 6- to 8-week-old mycelial-phase cultures grown on 1% glucose–0.5% yeast extract agar. The cell suspension was passed over a sterile cotton column to remove hyphal elements, and the arthroconidia were enumerated by hemacytometer counts. Viability was confirmed by plate cultures on glucose-yeast extract.

Just before infection, mice were lightly anesthetized by an intraperitoneal injection of Nembutol (pentobarbitol sodium) in sterile distilled water (1 mg per mouse). An inoculum of arthroconidia suspended in 20 μl of physiologic saline was dispersed in dropwise increments into the nares. Control animals were treated in an identical manner with sterile saline alone.

**Assessment of disease severity.** Disease severity was assessed by comparing the survival rates and the number of CFU in mice at days 1 through 30 postinfection. Calculations

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of the mean lethal dose of arthroconidia for BALB/c and DBA/2 mice were performed by probit transformation (12). Determinations of CFU were made by homogenizing the lungs, livers, and spleens in bags (Whirlpak; American Scientific Products, Dallas, Tex.) containing sterile saline, as described by Walsh et al. (24). Tenfold dilutions of the suspensions were plated on Mycobiotic medium (Difco Laboratories, Detroit, Mich.), and the plates were examined for mycelial colonies at 3 and 5 days after incubation at 33°C.

Footpad testing. DTH was evaluated by testing mice in the footpad with CDN (100 μg [dry weight]) or PPD (30 μg [dry weight]). Mice were injected in the right or left hind footpads with 50 μl of antigen in phosphate-buffered saline (PBS) or, for a control, PBS alone. Just before and 24 h after injection, footpad thicknesses were measured with a dial caliper (Mitutoyo, Tokyo, Japan). The results were calculated as the difference in footpad thickness of antigen- and PBS-injected pads at 24 h minus the difference in footpad thickness of antigen- and PBS-injected pads before footpad challenge.

Immunization. Mice were immunized with Formalin-killed spherules of C. immitis Silveira grown in modified Converse medium as described by Levine et al. (19). Each mouse was given a total of 2.1 mg (dry weight) of lyophilized spherules in three injections administered three times at weekly intervals. The first injection was given subcutaneously in the nape of the neck; the second and third injections were administered intramuscularly in the right and left hind legs, respectively. Control mice were treated in a similar manner with saline. The mice were challenged with viable arthroconidia via an intranasal route 7 weeks following the last injection.

For immunization with PPD, mice were given a single subcutaneous injection of PPD (500 μg) in complete Freund adjuvant (CFA) or, for a negative control, PBS in incomplete adjuvant 14 days before infection with C. immitis. A positive control group consisted of mice immunized with PPD in CFA and not infected with C. immitis.

Statistical analyses. Differences in survival of mice at days 1 through 30 postinfection were analyzed by the Wilcoxon signed-ranks test (15). Comparisons of CFU and footpad hypersensitivity responses were performed by Student's unpaired t test. Analyses of CFU were performed by using log_{10} transformed data.

RESULTS

Susceptibility of mice to pulmonary infection. The survival rate of BALB/c and DBA/2 mice at days 1 through 30 after intranasal instillation of 10 arthroconidia is depicted in Fig. 1. A 100% mortality was observed in BALB/c mice by day 22 postinfection as compared with a 21% mortality in DBA/2 mice (P < 0.005). The differences in the susceptibilities of the two mouse strains were further documented by comparisons of survival at 30 days following infection with incremental doses of arthroconidia. Less than 5% of BALB/c mice survived infection with 5, 10, or 20 arthroconidia compared with survival in 97, 79, and 50%, respectively, of DBA/2 mice. Analyses of these data by probit transformation established that the mean lethal dose of arthroconidia for DBA/2 mice was 18.6 compared with <2 arthroconidia for BALB/c mice.

The number of CFU of C. immitis in pulmonary and extrapulmonary organs of BALB/c and DBA/2 mice at various times postinfection are shown in Fig. 2. At day 12 postinfection, the two mouse strains showed comparable numbers of CFU in pulmonary and extrapulmonary tissues. Thereafter, BALB/c mice showed a significantly greater increase of CFU in the lungs (P < 0.025), livers (P < 0.0001), and spleens (P < 0.001) when compared with DBA/2 mice.

Correlation of susceptibility with DTH response. To determine if the differences in the susceptibilities of BALB/c and DBA/2 mice were associated with differences in their ability to mount or maintain a cell-mediated immune response to the fungus, the two mouse strains were infected with 10 arthroconidia; and at 3-day intervals postinfection, randomly selected groups of mice were challenged in the footpad with...
CDN. The results are depicted in Fig. 3. Both mouse strains mounted a significant DTH response by day 9 postinfection which increased in magnitude by day 12. The DTH response diverged thereafter, with the development of anergy in the susceptible BALB/c mouse strain and the persistence of DTH in the more resistant DBA/2 mice.

The specificity of the depressed T-lymphocyte reactivity in BALB/c mice was examined by preimmunizing this mouse strain and, for comparison, DBA/2 mice with PPD in CFA 2 weeks before infection with C. immitis and then challenging the mice in the footpad at day 14 postinfection. The data from these experiments are summarized in Table 1. Infection with C. immitis significantly suppressed the cell-mediated immune response to PPD in BALB/c mice, as evidenced by the fact that the mean footpad hypersensitivity of PPD-immunized, C. immitis-infected BALB/c mice was reduced by 57% from that of immunized, noninfected BALB/c mice (groups 3 versus 2, P < 0.001). In contrast, the PPD response in DBA/2 mice was not affected by coccidioidal infection; i.e., PPD-immunized, C. immitis-infected DBA/2 mice manifested a mean DTH response comparable to that of the PPD-immunized, noninfected DBA/2 mice (Table 1).

It is noteworthy that while coccidioidal disease suppressed the footpad hypersensitivity responses of BALB/c mice to PPD, the extent of suppression to PPD was less than that observed with CDN. C. immitis-infected BALB/c mice were nonresponsive to CDN, as judged by the fact that their footpad hypersensitivity response did not differ from those of negative (noninfected) BALB/c controls (3.2 x 10^{-2} ± 0.6 x 10^{-2} and 3.7 x 10^{-2} ± 0.7 x 10^{-2} mm, respectively). In contradistinction, the mean PPD footpad response of PPD-immunized, C. immitis-infected BALB/c mice was significantly greater than that of nonimmunized, C. immitis-infected BALB/c mice (groups 2 versus 1, P < 0.0001). These results establish that infection of BALB/c mice with C. immitis results in a hyporesponsive state to PPD as opposed to anergy to CDN. One finding observed in these experiments was that BALB/c mice exhibited a greater footpad hypersensitivity response to PPD, in the absence of coccidioidal infection, than did DBA/2 mice (22.4 x 10^{-2} ± 2.8 x 10^{-2} and 10.9 x 10^{-2} ± 1.8 x 10^{-2} mm, respectively; P < 0.001). This result may be attributable to differences in the ability of these two strains to mount or maintain cell-mediated immune reactivity to PPD.

**Effect of prior vaccination on DTH and survival.** To determine if vaccination of BALB/c mice with Formalin-killed spherules before infection with C. immitis would alter the development of anergy to CDN, the mice were immunized with killed spherules 7 weeks before intranasal instillation of 10 arthroconidia and then tested with CDN in the footpad at day 14 postinfection. Spherule-vaccinated BALB/c mice maintained footpad hypersensitivity to CDN, to a level comparable to that of nonvaccinated or vaccinated DBA/2 mice (Fig. 4). Vaccinated BALB/c mice were also protected against pulmonary challenge with 100 arthroconidia; i.e., 10 (66%) of 15 spherule-vaccinated mice survived this challenge as compared with only 1 (7%) nonvaccinated BALB/c mouse (P < 0.005; Fig. 5). These results document the ability of BALB/c mice to mount a protective immune response against pulmonary challenge with C. immitis.

**DISCUSSION**

The results of this study establish three new findings in genetically controlled susceptibility to C. immitis. First, BALB/c mice are highly susceptible to a pulmonary route of infection with this fungus, as compared with the susceptibility of DBA/2 mice. Second, the susceptibility of BALB/c mice is not attributed to their inability to mount a DTH response, but is associated with the development of anergy
to the fungus during the course of active disease. Third, the suppressed T-cell response observed in *C. immitis*-infected mice is not totally antigen specific.

The difference in the susceptibility of these inbred mouse strains to infection with arthroconidia administered by the pulmonary route is in accord with previous findings of Kirkland and Fierer (16, 17) that BALB/c mice are significantly more susceptible to an intrapulmonary route of infection with *C. immitis*. In the studies of Kirkland and Fierer (17), resistance was determined to be a dominant phenotype and under the control of a non-major histocompatibility gene given the provisional designation *Cms*. Phenotypic expression of the *Cms* gene does not appear to occur early during the course of disease. This conclusion is based on the fact that neither we nor Kirkland and Fierer (16) observed any difference in the CFU of *C. immitis* in BALB/c and DBA/2 mice within the first 10 to 12 days of infection. Thereafter, fungal growth was unrestricted in the susceptible mouse strain, coincident with the development of anergy. These results, taken together and in the context of the crucial role of CMI in host defense against *C. immitis* (1–3, 7), suggest that the *Cms* gene controls expression (or regulation) of the cell-mediated immune response to this fungal pathogen.

The depressed T-lymphocyte reactivity observed in *C. immitis*-infected BALB/c mice is not totally antigen specific. That is, BALB/c mice immunized with PPD prior to infection with *C. immitis* are suppressed in their response to PPD at day 14 postinfection. There is a distinction, however, in the degree of suppressed T-cell response to *C. immitis* and mycobacterial antigen. Whereas infected BALB/c mice exhibit a T-cell hyporeactivity to PPD, they are nonresponsive to challenge in the footpad with CDN. The differences in the degree of suppression of response to these two antigens may be related to the ability to suppress the DTH response induced by different modes; i.e. the CDN response was induced by active infection, whereas DTH to PPD was induced by administering soluble antigen in CFA. An alternative possibility to account for these results is that suppression to CDN and PPD occurs by different mechanisms; viz., hyporeactivity to PPD may occur by an antigen-nonspecific mechanism(s), whereas anergy to CDN may be attributable to both antigen-specific and nonspecific suppression.

Certain features of the disease in BALB/c and DBA/2 mice bear resemblance to human coccidioidomycosis. Cumulative epidemiologic investigations have established that Filipinos, Blacks, and to a lesser extent, Hispanics are genetically predisposed to developing severe, disseminated coccidiido-

mycosis (11, 13, 21). The susceptibility of these persons is not associated with their inability to mount DTH reactivity to fungus, as evidenced by the acquisition of skin test reactivity and in vitro lymphocyte transformation responses to coccidioidal antigens following vaccination with killed spherules (25). These results are similar to those obtained in the murine model, in that BALB/c mice are able to develop a DTH response to CDN following vaccination with killed spherules and during the early phase of coccidioidal disease. Likewise, the development of anergy, as was observed in BALB/c mice, is a common observation in human coccidioidal disease (6-9, 14, 23). Although the depressed CMI is specific for *C. immitis* antigens in most patients with coccidioidalmycosis (7, 9, 14), a generalized anergy or hyporesponsiveness has been documented, particularly in patients with progressive disseminated disease (6, 23).

The immunologic basis for genetically determined susceptibility to *C. immitis* is not known. A salient finding in the present investigation is that both BALB/c and DBA/2 mice manifest a DTH response to the fungus by day 12 postinfection, at which time the two strains have comparable numbers of CFU in their tissues. Thereafter, BALB/c mice fail to control fungal growth and are nonresponsive by footpad hypersensitivity, whereas DBA/2 mice begin to clear, or at least restrict, fungal growth and exhibit a strong DTH reactivity to CDN. The acquired immunosuppression could be attributable to the activation of an immunoregulatory cell or circuit, as has been documented in other mycoses (4, 5, 10, 20) or could be a sequela of overwhelming disease. In preliminary experiments to explore these possibilities (8), we have demonstrated that intravenous transfer of spleen cell lysates (filtered to remove fungal cells) from infected BALB/c mice suppresses the response of syngeneic recipients to immunization with CDN. No suppression was observed in the transfers of spleen cell lysates from *C. immitis*-infected DBA/2 mice (unpublished data). These results are consistent with suppression by a spleen cell factor or component produced during active disease in the susceptible mouse strain. Further studies of this model should enable the identification and characterization of immunosuppressive mechanisms resulting from, and perhaps contributing to, progressive coccidioidal disease.

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**LITERATURE CITED**


