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Inbred Mouse Strains Differ in Resistance to Lethal *Coccidioides immitis* Infection

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Inbred strains of mice were infected intraperitoneally with *Coccidioides immitis*, and the mean lethal dose was determined after 28 days. DBA/2N mice had a mean lethal dose of $>10^5$ arthroconidia, whereas BALB/cAnN, C57BL/6N, and C57L/J mice had a mean lethal dose of $\leq 10^3$. Since both BALB/c and DBA/2 mice are the *H-2*^{d} haplotype, resistance is not primarily determined by the major histocompatibility locus. Resistance was the dominant phenotype. The pattern of *C. immitis*-resistant strains does not correspond to the strain distribution of the *lsh* gene or to the pattern of resistance to *Blastomyces dermatitidis* or *Cryptococcus neoformans*. Both resistant and susceptible mice, however, could be successfully immunized with a killed spherule vaccine, and susceptible BALB/cAnN mice were protected from an otherwise lethal infection by prior immunization with an attenuated mutant of *C. immitis*. Despite the evidence that BALB/cAnN mice could respond to immunization, nonimmune mice did not control the later phase of intraabdominal infection as well as DBA/2N mice. Dissemination of *C. immitis* to the lung occurred frequently in BALB/cAnN but not in DBA/2N mice. This suggests that BALB/cAnN mice cannot mount an effective immune response to *C. immitis* during the course of infection.

Coccidioidomycosis, one of the major systemic mycoses in the Western hemisphere, is endemic in the southwestern United States from Texas to California. Inhalation of airborne arthroconidia (arthrospores) results in a primary respiratory infection. Almost 100,000 people are infected this way each year (16). Although less than half of these infections produce symptoms, those that do account for much morbidity and mortality (7). Mortality is essentially limited to those unfortunate people who suffer extrapulmonary spread of infection. Although there is no evidence that the incidence of infection, or even the incidence of symptomatic infection, is genetically influenced, there is very good epidemiological evidence that the risk of dissemination after infection is, in large part, genetically controlled (16). Dissemination occurs in approximately 1% of white men who are newly infected. However, the rate of dissemination in black men and women is 10 to 20 times higher (11). Data from the military, collected under conditions in which the degree of exposure was equivalent, indicate that the rate of dissemination in blacks is 10 times that in whites (20). Filipinos (and probably other Asians) seem to be even more susceptible. Thus, although coccidioidomycosis resembles the other systemic mycoses in affecting previously healthy people, it is unique in that the risk of serious infection is strongly influenced by the racial (genetic) background of the host.

Since mice are susceptible to infection with *Coccidioides immitis* (2) and a great deal is known about the mouse genome, we chose to use a murine model of coccidioidomycosis to try to elucidate the genetics of resistance. We reasoned that by studying experimental coccidioidomycosis in different strains of inbred mice, we might detect genetically determined differences in resistance that could provide insights into the mechanisms of resistance to *C. immitis*. A similar approach has been used to study genetic resistance to facultative and obligate intracellular pathogens (3, 5).

In this paper, we report that inbred mouse strains vary as much as 1,000-fold in their resistance to *C. immitis*.

MATERIALS AND METHODS

Mice. Female BALB/cAnN, DBA/2N, C57BL/6N, C57BL/10N, (BALB/cAnN × DBA/2)F1, (C57BL/6 × DBA/2)F1, and (BALB/cAnN × C57BL/6)F1 mice were obtained from Simonsen Laboratories, Gilroy, Calif. Female C57L/J mice were obtained from Jackson Laboratories, Bar Harbor, Maine.

Mycology. *C. immitis* R.S. was initially isolated 8 years ago from a patient with coccidioidomycosis. It

has been maintained since then by H. Walch (San Diego State University, San Diego, Calif.) who kindly gave us the R.S. strain and a mutant of this strain, denoted 95-271. This mutant is auxotrophic for para-aminobenzoic acid and riboflavin. It is also temperature sensitive, being unable to grow only at temperatures less than 37°C, and nonpathogenic for mice (21). We maintained both strains in their mycelial form at 25°C on Mycophil agar (BBL Microbiology Systems, Cockeysville, Md.). The spherule form of *C. immitis* 95-271 was grown at 31°C on Brosbe's modification of Converse medium (4) in an atmosphere of 5% O_2–10% CO_2–85% N_2. At each passage, endospores were enriched by the method of Levine et al. (13), and, after five passages, all growth was in the spherule form.

Mycelia were harvested by flooding the surface of the agar with distilled water and gently scraping the surface. Arthroconidia were liberated from mycelia by vortexing the suspension with 5-mm glass beads and washing three times by centrifugation at 500 × *g* at 4°C. Formalin-fixed spherules and arthroconidia were prepared by treatment with 0.5% Formalin (Mallincrodt Inc., St. Louis, Mo.) in phosphate-buffered saline at 4°C for 3 days, followed by extensive washing.

Infection model. Age-matched female mice, 2 to 5 months of age, were inoculated intraperitoneally with a suspension of arthroconidia in 0.145 M saline. The number of organisms was estimated by counting in a hemacytometer and confirmed by colony counts. The mice were housed in a Germfree isolator (Germfree Laboratories, Inc., Miami, Fla.) for the duration of the experiment. The mean lethal dose (LD_{50}) values were calculated by probit transformation (8), using mortality rates obtained with at least four different doses of arthroconidia and groups of 5 to 10 mice per dose. To estimate the number of viable *C. immitis* in the organs, groups of mice were infected intraperitoneally with 5 × 10^2 R.S. arthroconidia. On the day of sacrifice, organs were removed, homogenized in 1% Triton X-100 in water, diluted in water, and plated on Mycosel agar containing 50 μg of gentamicin per ml. The number of colonies was counted after 3 days of incubation at 25°C. We did not do colony counts on the organs of mice that died before the scheduled day of sacrifice. In some cases, tissues for biopsy were taken from intraabdominal organs and lungs at the time of sacrifice for histological examination.

In vitro proliferation assay. A modification of the method developed by Corridin et al. was used for the in vitro proliferation assay (6). Mice were immunized in the hind footpads and base of the tail with 1.5 × 10^6 Formalin-treated 95-271 spherules in complete Freund adjuvant (H37Ra) (Difco Laboratories, Detroit, Mich.). After 10 to 17 days, the inguinal and paraaortic lymph nodes were removed and teased into single cell suspensions. Enrichment for T lymphocytes was accomplished by nylon wool passage (12). Nylon wool-purified T cells were plated at 1.25 × 10^6 lymphocytes per ml in RPMI media (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum (MA Bioproducts, Walkersville, Md.) and 5 × 10^{-5} M 2-mercaptoethanol with an equal number of syngeneic, irradiated, nonimmune spleen cells. The cells were plated in a total volume of 200 μl in 96-well plastic tissue culture trays (Costar Data Packaging, Cambridge, Mass.). The purity of the nylon wool-enriched T lymphocytes was assessed by the proliferative response at 3 days to 5 μg of concanavalin A (Difco Laboratories) per ml and 10 μg of endotoxin from *Escherichia coli* 0111:B4 (lipopolysaccharide) per ml. Nylon wool-purified cells consistently had a proliferative response to lipopolysaccharide which was less than 20% of the response of unfractionated cells, without significant loss of concanavalin A reactivity. This indicates that there was a very small number of B cells in the nylon wool-purified cell preparation. Preliminary studies demonstrated that the peak proliferative response to Formalin-killed anthroconidia and spherules in BALB/cAnN and DBA/2N mice occurred on day 5 of culture. Therefore, triplicate cultures were incubated with or without antigen for 5 days, pulsed for 18 h with 1 μCi of [^{3}H]thymidine, and harvested with an automatic harvester. Results are expressed as the stimulation index, which equals mean counts per minute with antigen divided by mean counts per minute without antigen.

RESULTS

Susceptibility of inbred mouse strains to intraperitoneal *C. immitis* infection. When mice were inoculated intraperitoneally with a suspension of arthroconidia, deaths began to occur 10 days after infection, and survival time was inversely related to the infecting dose. Over the range of 10^2 to 10^6 organisms per infecting dose, most deaths occurred within 28 days after infection. Therefore, this time period was chosen to determine cumulative mortality.

Initially, we compared the LD_{50} in BALB/C and DBA/2N mice, two inbred strains that have in common the *H-2*^{d} haplotype. We discovered that the LD_{50} for DBA/2N mice was 1,000 times higher than the LD_{50} for BALB/cAnN mice (Fig. 1). These observations strongly suggested that there was a genetic basis for resistance to *C. immitis* in mice, but that resistance was not

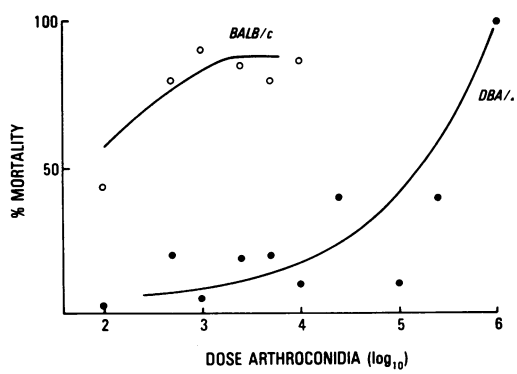

FIG. 1. Relationship of the infecting dose to mortality for both BALB/cAnN and DBA/2N mice. These curves are derived from the results of seven separate experiments. Each point represents a minimum of 10 mice.

determined by genes in the *H-2* (major histocompatability) locus. We then determined the LD_{50} for C57BL/10N and C57BL/6N mice. These two closely related strains were quite susceptible to lethal infection (Table 1), as were C57L/J mice. The progeny of the cross between two susceptible strains (BALB/cAnN × C57BL/6) were susceptible, indicating that BALB/cAnN and C57BL/6 mice did not have complementing genes capable of determining resistance. We also infected (BALB/cAnN × DBA/2N)F1 and (C57BL/6 × DBA/2N)F1 mice to determine which phenotype was dominant; in both instances, resistance was the dominant phenotype (Table 1).

Dissemination of *C. immitis* in resistant and susceptible mice. We infected BALB/cAnN and DBA/2N mice with 500 arthroconidia to learn whether the natural resistance of DBA/2N mice to *C. immitis* correlated with their ability to inhibit multiplication of the fungus and at what point in the course of infection this would become manifest. Groups of at least five mice were killed at intervals after infection; at each point, we determined the number of viable fungi in the peritoneal cavity, the spleen, and the lung. It was difficult to enumerate the number of organisms in the peritoneum because the peritonitis that resulted from intraperitoneal injections was usually focal. However, since the lesser omentum was always infected, we removed and quantitatively cultured this structure as an indicator of the number of organisms in the peritoneum. The numbers of organisms in the omentum 4 days after infection were below the limits of detection, and there was no detectable infection of spleens or lungs at that time (Fig. 2). By day 7, *C. immitis* was recovered from the omental tissue of both strains of mice, and neither the percentage of infected mice nor the mean number of CFU was significantly different in the two strains. However, by day 10 there were more fungi in the omenta and spleens of BALB/cAnN mice than in those of DBA/2N mice. That difference was even more apparent on days 14 and 19, even though the number of CFU in BALB/cAnN mice on day 19 was a minimal estimate, because many BALB/cAnN mice had died and were not scored.

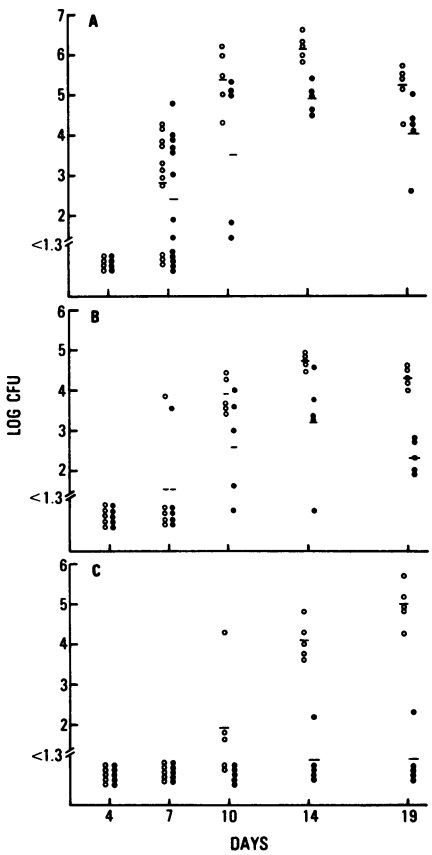

FIG. 2. Numbers of viable *C. immitis* in the omentum (A), spleen (B), and lung (C) after intraperitoneal infection with 5×10^2 arthroconidia. Each point represents a single animal, and the lines represent means. ○, BALB/cAnN mice; ●, DBA/2N mice.

TABLE 1. Susceptibility of inbred mice to intraperitoneal *C. immitis* infection

Mouse strain	LD_{50} (log_{10}) ± SE
BALB/cAnN	1.67 (0.60)
C57BL/10N	2.77 (0.33)
C57BL/6N	2.83 (0.23)
C57L/J	1.65 (0.56)
(BALB/cAnN × C57BL/6)F1	1.82 (0.45)
(C57BL/6 × DBA/2N)F1	4.20 (0.19)[a]
(BALB/cAnN × DBA/2N)F1	4.95 (0.18)[a]
DBA/2NX1	5.25 (0.36)[a]

[a] Significantly different from all other strains ($P < 0.01$) but not significantly different from each other.

The most striking difference between the two strains was that BALB/cAnN mice developed pneumonia and DBA/2N mice did not. In fact, we recovered *C. immitis* from the lungs of only one of five mice on days 14 and 19, whereas on those days all the BALB/cAnN mice had pneumonia which was extensive enough to explain their high mortality.

Histological studies were done on the organs of both mouse strains as well. There were no qualitative differences in the pathology of BALB/cAnN and DBA/2N mice. Mice of both strains formed granuloma in response to the infection; the number of granuloma correlated with the number of CFU.

TABLE 2. Protection of BALB/cAnN mice from lethal *C. immitis* infection by preimmunization with arthroconidia from *C. immitis* 95-271

Response	Mortality (no. of deaths/total no.)	
	Expt 1	Expt 2
Nonimmune	10/10	10/10
Immune[a]	0/10	2/10

[a] BALB/cAnN mice were immunized subcutaneously with 10^5 95-271 arthroconidia in 0.2 ml of saline 30 days before challenge. The mice were challenged intraperitoneally with 5×10^4 (experiment 1) or 5×10^3 (experiment 2) R.S. arthroconidia and observed for 28 days.

Response of BALB/c mice to immunization. Since it appeared that infected BALB/cAnN mice did not mount an effective immune response during infection, we examined their ability to respond to immunization with *C. immitis*. Mice were immunized subcutaneously with 10^5 viable arthroconidia of the attenuated mutant, strain 95-271. Vaccination with this mutant strain has been reported to protect outbred mice from an otherwise lethal inoculum of *C. immitis* (21). BALB/cAnN mice were also protected by vaccination (Table 2). Since T lymphocytes confer resistance to *C. immitis* (1), the ability to immunize BALB/cAnN mice suggests that their T lymphocytes respond to *C. immitis* antigens and that the effector arm of their immune response is intact.

Response of lymphocytes from BALB/cAnN and DBA/2N mice to coccidioidal antigens in vitro. To compare the in vitro immune responses of susceptible and resistant mouse strains to *C. immitis*, we immunized both strains with Formalin-killed spherules of *C. immitis* 95-271 emulsified in complete Freund adjuvant. After 10 to 17 days, draining lymph nodes were removed and teased into a single cell suspension. We then tested the ability of the lymphocytes to proliferate in response to Formalin-fixed arthroconidia and spherules. The results of two representative experiments are shown in Table 3. T lymphocyte-enriched lymph node cells from immunized BALB/cAnN and DBA/2N mice made equivalent proliferative responses to both forms of the fungus.

DISCUSSION

The principal finding of this study was that, despite having the same *H-2* haplotype, DBA/2N mice were 1,000 times more resistant to intraperitoneal coccidioidomycosis than were BALB/cAnN mice. Resistance was shown to be the dominate phenotype. We also found that, despite their marked susceptibility to fatal infections, BALB/cAnN mice could be successfully immunized with *C. immitis*; after immunization, their T cells responded to coccidiodal antigen in vitro, and the mice were completely protected from an otherwise lethal infection. These observations suggested that the increased susceptibility of BALB/cAnN mice was not due to an absolute inability of their T cells to respond to *C. immitis* nor to an absolute defect in effector cell (macrophage) function. However, the fact that *C. immitis* produces a progressive local infection that disseminates and kills BALB/cAnN mice in less than 4 weeks suggests that these mice do not mount an effective immune response during infection, even though they can respond to spherule antigens when presented either in the form of a killed vaccine or as spherules produced in vivo after immunization with a live vaccine. Since the severity of the initial stage of infection (through day 10) was nearly identical in DBA/2N and BALB/cAnN mice, the resistance of DBA/2N mice was not due to their unique ability to prevent germination of the arthroconidia to spherules or to prevent multiplication of spherules during the time before specific immunity had developed.

Although we tested only five mouse strains, we know that the distribution of susceptible and resistant strains does not correspond to the strain distribution resistance to *Leishmania tropica* (10), *Listeria monocytogenes* (5), *Blastomyces dermatitidis* (15), or *Cryptococcus neo-*

TABLE 3. In vitro proliferative response of nylon wool-enriched T lymphocytes from mice immunized with Formalin-killed spherules of *C. immitis* 95-271 in complete Fruend adjuvant

Antigen[a]	Stimulation index[b]			
	Expt 1		Expt 2	
	BALB/cAnN	DBA/2N	BALB/cAnN	DBA/2N
Arthroconidia				
10^6	7.84	3.93	7.22	8.22
10^5	7.83	3.60	5.84	8.31
10^4	ND[c]	ND	2.37	4.21
Spherules				
10^6	3.23	5.70	6.96	8.46
10^5	7.09	5.58	7.04	11.72
10^4	ND	ND	2.49	3.23
Purified protein derivative[d]	25.1	22.15	38.29	62.09

[a] Organisms per ml.

[b] Background values: experiment 1, BALB/cAnN = 1,515 cpm and DBA/2N = 6,336 cpm; experiment 2, BALB/cAnN = 718 cpm and DBA/2N = 1,354 cpm.

[c] ND, Not done.

[d] Purified protein derivative, 25 μg/ml.

formans (18). Because C57L/J mice are susceptible, the distribution of susceptible and resistant strains does not correspond to the strain distribution of the *lsh* gene, which appears to determine resistance to *Leishmania donavoni, Salmonella typhimurium* (17), and *Mycobacterium bovis* (19). Therefore, resistance to *C. immitis* inoculated intraperitoneally is due either to a previously undescribed gene or to a combination of resistance genes which have not been recognized in other experimental systems.

It is important to note that what we have learned about primary *C. immitis* peritonitis may not apply to pneumonia in the murine model, let alone to human infection. It has been reported that mouse strains which are resistant to peritonitis caused by *B. dermatitidis* are susceptible to *B. dermatitidis* respiratory infection (14). Similarly, some mouse strains that are resistant to subcutaneous infection with *S. typhimurium* are killed by intravenous challenge (9). Other investigators have found that respiratory infections with *C. immitis* can be established in DBA/2N mice with relatively few arthroconidia (1). It remains to be determined whether this means that DBA/2N mice are not more resistant to respiratory infection than are BALB/cAnN mice, or that other strains of *C. immitis* are more virulent than the R.S. strain.

Even though the mice were not infected by the respiratory route, our model of coccidioidomycosis has several parallels with the human disease. In both cases, resistance is related to the ability of the host to confine the infection to the portal of entry, and lethal infections are the result of progressive dissemination. Both susceptible and resistant mice responded to the fungus with granuloma, indistinguishable from the human response. Considering these parallels, we are hopeful that what we learn about natural resistance to *C. immitis* peritonitis in the mouse may be applicable to human coccidioidomycosis.

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