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Genetic Control of Susceptibility to *Cryptococcus neoformans* in Mice

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Inbred mice injected intravenously with 5×10^6 cells of *Cryptococcus neoformans* showed two patterns of survival: sensitive (A/WySn, A.BY, A/J, DBA/2J, NZB/BlNJ, and SWR/J) and resistant [C57BL/10Sn, B10.A, B10.A (2R), B10.S (7R),C57BR/cdJ, C58/J, C3H/HeJ, BALB/c, DBA/1J, and SJL/J]. Relative susceptibility based on survival time was shown to correspond to differences obtained for 50% lethal dose values. Either decreasing the dose of organisms or changing to the intraperitoneal route of inoculation resulted in prolonged survival times, but neither change affected the observed patterns of survival. F1 hybrids between different sensitive strains were also sensitive, whereas F1 hybrids between sensitive and resistant strains were resistant, indicating a dominant mode of inheritance. Sensitivity and resistance were shown to be under single gene control by segregation analysis in F2 progeny produced by inbreeding (B10.A × A/WySn)F1 hybrids and in (F1 × A/WySn) backcross progeny. Blood obtained from parental strains, F1, F2, and backcross hybrids was tested for the presence or absence of hemolytic complement. Mice lacking hemolytic complement activity in their sera are homozygous for the Hc^0 allele at the *Hc* locus on chromosome 2 and are deficient in the complement component C5. A 1:1 correspondence was found between C5 deficiency and sensitivity to *C. neoformans*. Resistance was shown to cosegregate with the presence of hemolytic complement in the F2 and the backcross progenies.

Cryptococcus neoformans is an encapsulated yeast of worldwide distribution. The most common clinical manifestation of cryptococcosis is meningitis, usually with dissemination to extraneural sites. Epidemiological evidence indicates that the number of people infected with *C. neoformans* may be far greater than the number who subsequently develop meningitis (16, 34). Therefore, host defense systems, such as the complement (C′) cascade and phagocytosis, as well as antigen-directed humoral and cellular mechanisms, must be relatively efficient in conferring resistance to severe cryptococcosis. In this study, we have examined differences in susceptibility mediated by the C′ system.

There have been numerous investigations of natural differences in the susceptibility of inbred strains of mice to infectious agents, including *C. neoformans* (1-4, 11, 12, 14, 19-23, 25, 29, 33; P. A. Morozumi and D. A. Stevens, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, F63, p. 373). Genetic control of natural resistance to certain microbes has been reported (1, 2, 11, 12, 21-23). In three cases (3, 14, 29), the relative susceptibility of the mice studied correlated with their hemolytic C′ phenotype. Hemolytic C′ activity in inbred strains of mice is determined by the presence or absence of the C′ component C5 in the sera (17). Strains of mice carrying the Hc^1 allele at the hemolytic C′ locus (*Hc*) on chromosome 2 have C5 in their sera, while strains homozygous for the alternate allele, Hc^0, have C5-deficient sera (10, 30). The presence of serum C5 affords prolonged survival to mice infected with pneumococcus (29), *Corynebacterium kutscheri* (3), and *Candida albicans* (14) when compared with C5-deficient mice.

In this study, we report that inbred strains of mice can be divided into two groups based on survival time: (i) those which are sensitive to and (ii) those which are resistant to *C. neoformans*. This relative difference in susceptibility correlates with C′ phenotype of the animals and is a stable, inheritable trait which appears to be under the control of a single gene. Segregation analysis of F2 and backcross (BX) progenies shows that sensitivity and resistance cosegregate with the absence and presence of hemolytic C′, respectively. Because C′ and antibody alone cannot kill yeast cells of *C. neoformans* (8), opsonization is discussed as the basis for the differences in susceptibility seen in this study.

MATERIALS AND METHODS

Organism. The strain of *C. neoformans* (NIH-B3502) used in this study originated from a single

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basidiospore isolate of mating type **a** of *Filobasidiella neoformans*. This culture was generously provided by K. J. Kwon-Chung, Laboratory of Clinical Investigations, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md. The culture was maintained with bimonthly transfer on glucose-peptone agar slants containing 1% glucose, 2% proteose peptone no. 3 (Difco Laboratories, Detroit, Mich.), and 2% agar (Difco). Yeast cells for animal inoculation were grown for 48 h at room temperature on a rotary shaker (New Brunswick Scientific Co., New Brunswick, N.J.) in 1% glucose–2% yeast extract (Difco) broth. The cells were harvested by centrifugation at 2,000 rpm for 5 min (International centrifuge model BE-50, International Equipment Co., Boston, Mass.), washed once, and suspended in saline (0.85% NaCl). The washed cells were counted in a hemacytometer and adjusted to give the desired concentration of cells in a 0.25-ml volume. Viability of the organism was determined by triplicate plate counts on glucose-peptone agar.

Animals. The following strains of inbred mice were obtained from the Jackson Laboratory, Bar Harbor, Maine: A.BY, A/J, A/WySn, C57BL/10Sn (B10), B10.A, B10.A (2R), C57BR/cdJ, C58/J, C3H/HeJ, DBA/1J, DBA/2J, NZB/BlNJ, SJL/J, and SWR/J. The F1 hybrids between C57BL/6J (B6) and A/J were also obtained from the Jackson Laboratory. The BALB/c mice (Simonsen Laboratory, Gilroy, Calif.) were the gift of B. Bonavida of this department. The original B10.S (7R) breeding stock was provided by J. Stimpfling. The F1, F2, and BX hybrids described in the text were bred in this laboratory. All animals were housed in the Microbiology and Immunology departmental facilities and were given lab chow (Ralston Purina Co., St. Louis, Mo.) and tap water ad libitum. Groups of four to eight animals, selected for each experiment, were matched for age as closely as possible.

Mice were injected intravenously in a lateral tail vein or intraperitoneally with the appropriate dose of *C. neoformans* contained in 0.25 ml. Brain tissue was examined directly for encapsulated yeast cells and cultured on glucose-peptone agar to confirm that deaths of experimental animals were due to *C. neoformans*. Isolates were confirmed as *C. neoformans* by their ability to produce a brown pigment when grown on *Guizotia* seed agar (28).

The 50% lethal dose. The 50% lethal dose was estimated by the method of Reed and Muench (24).

Statistical analysis. The results of the experiments measuring survival time were expressed as the mean survival time in days ± the standard error of the mean. Closeness of fit in the segregation analyses was tested using the χ^2 test (9).

Hemolytic C′ assay. The procedure of Rosenberg and Tachibana (26) was used to assay hemolytic C′. Briefly, the test is as follows. A 2.5% suspension of sheep erythrocytes (Mission Laboratory Supply, Inc., Rosemead, Calif.), labeled with radioactive chromium (New England Nuclear Corp., Boston, Mass.), was sensitized with a 1:50 dilution of rabbit anti-sheep hemolysin (State of California Department of Health, Berkeley, Calif.) at ice-bath temperature. To the sensitized cells were added 3 to 4 drops of fresh blood directly from the tail artery of the mouse. The test mixture was incubated at 37°C for 60 min, and then the reaction was stopped by adding 2 ml of cold Veronal-buffered saline. The tubes were centrifuged, and 1 ml of supernatant was removed. The radioactivity in the supernatant and cells was counted (Searle model 1185 Automatic Gamma System, Searle Analytic Inc., Des Plaines, Ill.), and these counts were used to determine the percent lysis from the formula: $[(2S - ns)/(C + S - ns)] \times 100$, where S = counts in supernatant, C = counts in the cells, and ns = counts attributable to nonspecific leakage of chromium. All mice were tested in duplicate, and appropriate controls were included in each series of tests. Less than 3% lysis was considered negative.

RESULTS

Survey of strains of inbred mice. Mean survival time was calculated for 16 strains of inbred mice after intravenous injection with 5×10^6 cells of *C. neoformans* (Table 1). Two patterns of survival were observed. The sensitive strains (A background, DBA/2J, NZB/BlNJ, and SWR/J) all had mean survival times of 4

TABLE 1. *Relative susceptibility of inbred mouse strains to C. neoformans*[a]

Strain	No. of mice	Mean survival (days) ± SEM[b]	*H-2*[c]	C5[d]
A/WySn	25	3.9 ± 0.5	*kkdd*	–[e]
A.BY	11	3.8 ± 0.8	*bbbb*	–[f]
A/J	9	2.3 ± 0.2	*kkdd*	–
DBA/2J	14	2.2 ± 0.1	*dddd*	–
NZB/BlNJ	14	2.4 ± 0.4	*dddd*	–
SWR/J	7	2.1 ± 0.2	*qqqq*	–
(A/WySn × A.BY)F1	11	4.0 ± 1.0	*k/b k/b d/b d/b*	–[f]
(DBA/2J × A/WySn)F1	3	3.0 ± 0.0	*d/k d/k d/d d/d*	–[f]
B10.A	23	20.4 ± 1.4	*kkdd*	+[e]
B10	20	25.8 ± 4.4	*bbbb*	+
B10.A (2R)	6	30.0 ± 7.1	*kkdb*	+[f]
B10.S (7R)	5	20.4 ± 2.5	*sssd*	+[f]
C58/J	6	21.3 ± 2.1	*kkkk*	+
C57BR/cdJ	8	25.2 ± 1.6	*kkkk*	+
C3H/HeJ	7	20.6 ± 0.8	*kkkk*	+
BALB/c	21	13.5 ± 0.8	*dddd*	+
DBA/1J	7	15.4 ± 1.0	*qqqq*	+
SJL/J	11	31.3 ± 2.0	*ssss*	+
(B10.A × A/WySn)F1	25	22.2 ± 4.9	*kkdd*	+[e]
(B10 × A.BY)F1	6	21.3 ± 1.2	*bbbb*	+[f]
(B10 × A/WySn)F1	6	20.5 ± 1.7	*b/k b/k b/d b/d*	+[f]
(B6 × A/J)F1	6	14.3 ± 0.5	*b/k b/k b/d b/d*	+[f]

[a] Mice received 5×10^6 cells intravenously.
[b] SEM, Standard error of the mean.
[c] *H-2* haplotype is given for *K*, *I*, *S*, and *D* regions, respectively.
[d] C5 designation is based on MuB1 determination by Cinader et al. (5), except where indicated.
[e] C5 based on typing done in this study.
[f] C5 designation is presumed from other determinations on strain backgrounds and from mode of inheritance (5).

days or less. The resistant strains (B10 background, C58/J, C57BR/cdJ, C3H/HeJ, BALB/c, DBA/1J, and SJL/J) had mean survival times of more than 13 days, with most mice surviving 21 days.

The effect of changing the route of inoculation was examined in a small group of mice which were injected intraperitoneally and observed for 42 days. In the sensitive group, 8 of 9 mice died in this period, whereas only 2 of 11 of the resistant mice died. Those mice which survived for 42 days were then sacrificed; cultures of brain tissue were positive in all but one mouse, a member of the resistant group.

The histocompatibility (*H-2*) haplotype of the surveyed strains showed no correlation with relative sensitivity or resistance. However, the presence and absence of C5, a trait controlled by alternate alleles at the *Hc* locus, was in complete agreement with assignment of *C. neoformans*-resistant and -sensitive phenotypes, respectively (Table 1).

Dose response. Survival times for both sensitive and resistant strains increased when mice were inoculated with decreasing doses of *C. neoformans* (Table 2). Mean survival time after inoculation with standard test dose was shown to correspond to a 2 $\log_{10}$ difference in 50% lethal dose values between the sensitive A/WySn and the resistant B10.A mice. In addition, the values obtained for the F1 hybrid were equivalent to those shown for the resistant parent.

F1 analysis. Inheritance of patterns of susceptibility to *C. neoformans* was examined in selected F1 hybrids (Table 1). The progenies of matings between two sensitive strains, (A/WySn × A.BY)F1 and (DBA/2J × A/WySn)F1, were also sensitive. Mice produced by mating sensitive and resistance strains, (B10.A × A/WySn)F1, (B10 × A.BY)F1, (B10 × A/WySn)F1, and (B6 × A/J)F1, were all resistant, indicating a dominant mode of inheritance of resistance to *C. neoformans*.

Segregation analysis in F2 and BX generations. The segregation of sensitive and resistant phenotypes was examined in F2 and BX generations. The F2 hybrids were produced by inbreeding (B10.A × A/WySn)F1 mice. The BX hybrids were produced by backcrossing the F1 to the sensitive A/WySn strain. In Fig. 1, the accumulated mortality of parent inbred strains and of the F1, F2, and BX hybrids is shown. The numbers of sensitive and resistant phenotypes seen in the F2 and BX generations were compared with the values which would be expected if the trait were under control of a single gene by the χ^2 test (Table 3). The values of χ^2 obtained were consistent with the hypothesis of single gene control. There was no indication of linkage of relative sensitivity to *C. neoformans* with either sex or coat color.

TABLE 2. *Dose response of A/WySn, B10.A, and (B10.A × A/WySn)F1 mice to intravenous inoculation of C. neoformans*[a]

Strain	Dose	% Survival	Mean survival[b]	Log_{10} LD_{50}[c]
A/WySn	5×10^6	0 (0/6)[d]	2.8 ± 0.3	4.00
	1×10^6	0 (0/6)	16.3 ± 0.7	
	1×10^5	0 (0/6)	27.0 ± 2.5	
	1×10^4	50 (3/6)	28.0 ± 4.4	
	1×10^3	100 (6/6)		
B10.A	5×10^6	0 (0/7)	18.0 ± 1.1	6.03
	1×10^6	86 (6/7)	23	
	1×10^5	86 (6/7)	35	
	1×10^4	100 (7/7)		
F1	5×10^6	0 (0/6)	20.5 ± 0.9	6.00
	1×10^6	67 (4/6)	22.5 ± 0.7	
	1×10^5	67 (4/6)	31.0 ± 5.7	
	1×10^4	100 (6/6)		

[a] The mice were observed for 35 days post-inoculation.
[b] Given in days ± standard error of the mean.
[c] LD_{50}, Fifty percent lethal dose.
[d] (No. survivors/total).

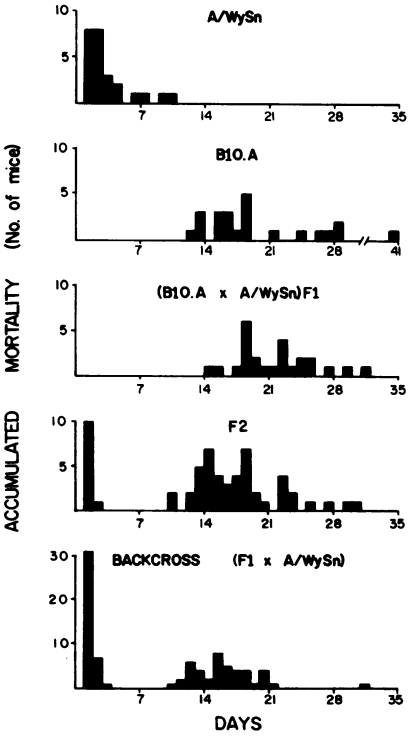

FIG. 1. *Accumulated mortality of parental strains, A/WySn and B10.A, and F1, F2, and BX hybrids after intravenous inoculation with 5×10^6 cells of C. neoformans.*

Assay for hemolytic C′. Because of the correlation of C′ phenotype and the relative sensitivity to *C. neoformans* observed in the survey of inbred mice, testing for hemolytic C′ was performed on parental strains and F1, F2, and BX hybrids. The mice were then injected with *C. neoformans* and survival times were recorded. These results (Table 4) showed that survival time corresponded to the C′ type in all of the mice tested. The resistant phenotype cosegregated with the presence of hemolytic C′, which is coded by the Hc^1 allele (10).

DISCUSSION

Differences in the natural resistance of mice to microorganisms has been studied in bacterial (3, 21-23, 25, 29), parasitic (1, 2, 20), and fungal (4, 14, 19; P. A. Morozumi and D. A. Stevens, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, F63, p. 373) systems. Examination of the inbred strains of mice used in these studies indicates that relative susceptibility to different microbes does not often run parallel. Although inheritance of resistance to *Salmonella typhimurium* is complex (33), early net growth rate in vivo is under the control of a single autosomal gene or gene cluster (11, 12). This locus, which appears identical to the *ity* locus, (23), maps to chromosome 1 and is linked to the locus for leaden coat color (2, 23). The *lsh* locus, which controls liver parasite burden in the early phase of *Leishmania* infection (1), also maps to chromosome 1 and is linked to the locus for leaden coat color (1). In this report, we have used survival time as the basis for delineating sensitive and resistant strains of mice. In Table 2, the prolonged survival seen in resistant animals corresponds to a 50% lethal dose value that is 2 $\log_{10}$ greater than that obtained for sensitive mice. Finally, we have shown that relative susceptibility to *C. neoformans* in inbred mice is an inherited trait controlled by alternate alleles of a single gene. Sensitivity and resistance cosegregate in the F2 and BX progenies with the absence and presence, respectively, of hemolytic C′.

TABLE 3. *Distribution of sensitive and resistant phenotypes in F2 and BX progenies*[a]

Progeny	Expected[b]/ total	Observed/ total	χ^2
F2			1.126[c]
Sensitive	14.5/58	11/58	
Resistant	43.5/58	47/58	
Backcross			0.195[d]
Sensitive	41/82	39/82	
Resistant	41/82	43/82	

[a] Mice received 5×10^6 *C. neoformans* intravenously.
[b] Expected if resistance is controlled by a dominant allele at a single locus.
[c] $0.3 > P > 0.2$.
[d] $0.7 > P > 0.5$.

TABLE 4. *Mean survival time of parental strains and hybrid progeny of different C′ phenotypes*[a]

Strain	No. of mice	C′[b]	Mean survival time (days) ± s.e.m.[c]
A/WySn	8	−	2.5 ± 0.2
B10.A	9	+	20.4 ± 1.2
(B10.A × A/WySn)F1	11	+	23.2 ± 1.2
Backcross	39	−	2.4 ± 0.1
	43	+	17.1 ± 0.7
F2	11	−	2.5 ± 0.2
	47	+	18.4 ± 1.1

[a] Mice received 5×10^6 *C. neoformans* intravenously.
[b] C′ phenotypes were determined by the hemolytic C′ assay described in the text and are given as (+) hemolytic C′ detected, or as (−) hemolytic C′ not detected.
[c] s.e.m., Standard error of the mean.

Hemolytic C′ activity in mice has been shown by Herzenberg et al. (10) to be dependent on a locus (*Hc*) on chromosome 2 (30) at which there have been two alleles described, Hc^0 (hemolytic C′ absent) and Hc^1 (hemolytic C′ present). Subsequent work has identified C5 as the protein missing from the sera of mice homozygous for Hc^0 and has shown that this C′ component is identical to the mouse serum antigen MuB1 (5, 17). Recently, the absence of C5 in the sera of C5-deficient mice has been shown to result from the failure of macrophages to secrete the protein, not from a failure to synthesize its precursor, pro C5 (18). The presence in sera of C5 is inherited in both inbred and outbred mice as a simple mendelian dominant trait (5, 10, 32). In a report of human C5 deficiency, the study of a large kindred indicated that there was codominant inheritance of the trait (31), but the results were equally compatible with an explanation of gene dosage of a simple dominant character. This mode of inheritance correlates well with that obtained for sensitivity and resistance to *C. neoformans* in this study (Table 3). The expected versus the obtained values of sensitive offspring in the F2 and BX mice (14.5 versus 11 and 41 versus 39, respectively) were in very good agreement for the sample size. In addition, there was a 1:1 correspondence between the presence of hemolytically active C′ and resistance to *C. neoformans* in both the progenitor strains and their hybrid progenies (Table 4).

During activation of the C′ cascade by either the classical or alternate pathways, C5 is split into two fragments, C5b which remains complexed with other complement components and C5a which is released from the complex (27).

The C5a molecule is active as an anaphylatoxin and as a powerful chemotactic factor for polymorphonuclear leukocytes. Lack of C5 would block complement activation by either pathway, thereby decreasing the opsonization and recruitment of phagocytic cells that the intact system affords. This is reflected in reports of human deficiencies in C5 in which the patients had recurrent bacterial infections and their sera showed impaired opsonic activity (13, 31).

The role of C5 in host defense previously has been studied in mice infected with pneumococcus (29), *Corynebacterium kutscheri* (3), and *Candida albicans* (14, 15). Although the presence of hemolytic complement conferred increased survival time in all three models, the most striking effects, similar to those shown here with *C. neoformans*, were seen with another encapsulated organism, the pneumococcus. This may be related to the antiphagocytic properties common to the capsules of these microbes, properties which would mandate an efficient opsonization system. In addition, repeated episodes of disseminated infection with the gonococcus, another encapsulated organism, have been reported in humans with C5 deficiency (31).

Opsonization of *C. neoformans* yeast cells has been shown to be primarily a function of the alternate C′ pathway (7). Although the classical pathway is necessary for optimal kinetics of opsonization, the main role of the classical C′ cascade in this system seems to be activation of the alternate scheme. Decreased survival time was seen in guinea pigs depleted of late C′ components (C3 through 9) before infection with *C. neoformans*. Those animals deficient in C4, however, had survival times comparable to normal animals (6). These results would support the importance of the alternate C′ pathway in in vivo host defense against cryptococcosis, as well as its role in those in vitro phenomena measured in opsonization-phagocytosis assays. In the C5-deficient strains of mice, alternate C′ pathway activation would be blocked after generation of C3b (27). The resultant decrease in opsonization could impair phagocytosis, an effect shown with C5-deficient sera in the pneumococcus (29) and *Candida* (15) systems, and subsequent clearance of yeast cells from the blood. In the guinea pig model of cryptococcosis, it has been suggested that the number of organisms reaching the brain early in the course of infection is a critical factor in survival (6). If so, then the rapid deaths seen in the C5-deficient strains of mice infected with *C. neoformans* may represent the relative inability of these mice to sequester yeast cells from the central nervous system.

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