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Genetic Resistance to Murine Cryptococcosis: Increased Susceptibility in the CBA/N XID Mutant Strain of Mice

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In a survey of 301 normocomplementemic inbred mice (belonging to nine different strains: BALB/cN *nu/nu* and *nu/+*, CBA/N, C57BL/KsJ, C57BR/cdJ, CBA/CaJ, BRVR, DW/+, and C57BL/6J) for natural resistance to *Cryptococcus neoformans*, cumulative survival values were found to range from 12 to 22 days. When the average organ weights of infected animals were compared with reference values obtained in uninfected mice of the same age and genetic lineage, the following changes were documented. In the CBA/N strain, the mean spleen and brain weights increased 313 and 13.5%, respectively, whereas the mean liver weight remained unchanged. In the CBA/Ca strain, cerebral cryptococcosis was the dominant clinical feature, and a 54% increase in mean brain weight was recorded at the time of death. The averaged liver weight was drastically lower, whereas spleen weight values evinced a biphasic pattern of transient splenomegaly followed by involution. At the median time of death, CBA/N mice had significantly more cryptococci in the liver and spleen than corresponding CBA/Ca mice. In the (CBA/N × CBA/Ca)F_1 mice, susceptibility to *C. neoformans* segregated according to the sex-linked inheritance of the X-linked immunodeficiency (*xid*) gene. It is concluded that (i) susceptibility to cryptococcosis is under multigenic control, (ii) the *xid* locus on the X chromosome influences susceptibility to cryptococcosis, and (iii) *xid* mice behave differently than CBA/Ca mice in their organ response during the course of the infection.

Cryptococcosis is an acute, subacute, or chronic pulmonary, systemic, or meningeal fungal disease. Disseminated infection is seen in compromised hosts but also in apparently normal individuals with subtle defects in cell-mediated immunity (9). Studies on the differential susceptibility of inbred mice to *Cryptococcus neoformans* have so far led to the identification of two loci which control host resistance, the *Hc* locus on chromosome 2, which determines the presence or absence of complement component C5 in the serum (7), and the *nu* locus on chromosome 11, which, in the homozygous animal, determines a hairless athymic state with absence of mature T lymphocytes and cell-mediated immunity (2, 3). In a systematic attempt to provide a better understanding of the role of genetic factors in experimental murine cryptococcosis, we investigated the survival, organ weight profile, tissue colony counts, and selected histopathological features in strains of inbred mice. We established that host resistance is randomly distributed according to coat color haplotypes and that the *xid* locus on the X chromosome has a major influence on susceptibility to cryptococcosis. The *xid* locus (X-linked immunodeficiency) determines in the homozygous CBA/N mouse the absence of a late-maturing B-cell subpopulation, serologically characterized as *Lyb* 5^+, and a marked defect in antibody-forming ability (8).

MATERIALS AND METHODS

Animals. Inbred mice of the following strains were obtained from the Jackson Laboratory, Bar Harbor, Maine: CBA/CaJ, C57BL/6J, C57BL/KsJ, C57BR/cdJ, and DW/+. They were maintained by brother-sister mating. BRVR mice were bred from a parental stock obtained from C. S. David, Mayo Clinic and Foundation, Rochester, Minn. CBA/N and BALB/cN *nu/nu* and *nu/+* mice were obtained from breeding colonies maintained by Micheline Pelletier, Department of Pathology, Université de Montreál. The animals were housed and handled in accordance with National Institutes of Health guidelines.

Organism. *C. neoformans* ATCC 13690 was obtained from the American Type Culture Collection and was maintained by passage in BALB/c nude mice. Quantitative assessment of growth was performed by the standard plate count technique on serial dilutions of the brain homogenates. Plates showing the highest log titer of growth were used for seeding on brain-liver-heart agar semisolid medium (Difco Laboratories, Detroit, Mich.). The culture was harvested after incubation at 37°C for 48 h, dispensed in 1.0-ml aliquots into screw-capped vials, and stored at −85°C until needed. This frozen stock was periodically renewed. India ink preparations of the isolate revealed a rather thin capsule.

Preparation of inocula. Brain-liver-heart agar medium (50 ml) was inoculated with the thawed contents of a culture vial. The culture was harvested after incubation at 37°C for 72 h. The organism was pelleted by centrifugation at 500 × *g* and repeatedly washed with sterile 0.9% saline until residual agar from the medium had been flushed away. A sample containing the resuspended organism was diluted 100-fold with 0.9% saline. This preparation was counted in a hemacytometer and further diluted to obtain a final concentration of 10^6 yeast cells per ml. The viability of blastospores was greater than 99% as established by a dye-exclusion method (1).

Animal inoculation. Mice were infected at 40 to 44 days of age by injecting 0.1 ml of the standardized yeast cell suspension into the right or left lateral tail vein. Experimental groups routinely comprised 25 to 45 animals of various genetic subsets. Pairs of animals of related genetic lineage

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were infected simultaneously. A population for which survival data were already available was always included with any new population to be tested. The high susceptibility of the CBA/N strain was recognized early in the study, and this strain was used in each subsequent experiment to monitor the virulence of the organism. Infected animals were housed in a physically separate facility. Mortalities were recorded at least on a daily basis.

Monitoring the infection. Upon death, animals were autopsied and their brain, liver, and spleen were separately dispensed into sterile, tared, 30-ml, capped polycarbonate tubes, each tube containing 10 stainless steel ball bearings (1/4 in. [ca. 0.64 cm]). Net organ weights were determined with an analytical balance. A volume of saline equal to 10 times the recorded organ weight was then dispensed into each tube. Grinding was achieved by vigorous mechanical shaking (S8220 mixer; American Scientific Products, McGaw Park, Ill.). Serial 10-fold dilutions of homogenates were prepared. After mixing, 10-μl samples were removed from selected dilutions and uniformly spread on brain heart infusion agar in 90-mm petri dishes. Bacterial contamination was prevented by the addition of penicillin (20 U/ml) and gentamicin (10 μg/ml) to the culture plates. Counts were recorded after 72 h of incubation at 37°C. When animals could not be processed at once, they were immediately frozen in sealed packages at −20°C. Colony counts obtained from these frozen cadavers were similar to those obtained from animals which were processed immediately.

Histopathological studies. Infected animals were sacrificed at various times, and specimens were fixed in 10% buffered formaldehyde solution. Tissues were embedded in paraffin, sectioned (5 μm thickness), and stained with hematoxylin and eosin (H&E) or periodic acid-Schiff.

Statistical analysis. The data were collected over a 2-year period. They were meticulously checked for internal consistency and fed to a computer database. Uninfected animals were sacrificed at the median survival time of corresponding experimental subgroups to obtain reference values for organ weights. Infected CBA/Ca mice were sacrificed at the median survival time of CBA/N mice to compare tissue colony counts in respective strains. Whenever appropriate, the variations in organ weights were compared after adjustment for multiple comparison of all pairs of means including experimental and control populations (Bonferroni test). The Kaplan-Meier product-limit estimate was used to analyze survival data and plot the survival function. The Breslow (generalized Wilcoxon) and Mantel-Cox (generalized Savage) statistics were applied to compare the survival functions of genetically distinct subgroups; there were no censored observations. The Student *t* test procedure for unpaired samples was used to compare mean weights, mean tissue counts, and mean survival between subgroups, the standard error of the mean being presented with these values in the text, tables, and figures.

RESULTS

Susceptibility in relation to other known haplotypes. In Table 1, mouse strains are ranked by mean survival, and their haplotypes for the *Hc*, *H-2*, and coat color loci are presented. Among these *Hc*-positive strains, survival ranged from 12 to 22 days. BALB/c nude and CBA/N mice were highly susceptible; C57BL/Ks mice were intermediate in susceptibility; and C57BR/cd, BALB/c *nu*/+, CBA/Ca, BRVR, and DW/+ mice were least susceptible to the infection. There was no significant difference in mean survival time (MST) between BALB/c *nu*/*nu* and CBA/N mice.

TABLE 1. Differential susceptibility of inbred mouse strains to *C. neoformans*

Mouse strain	No. of mice	MST (days)	SEM	Haplotypes		
				Hc	*H-2*	Coat color
BALB/c *nu*/*nu*	24	12.7	0.557	1	d	AbcD
CBA/N	93	13.2	0.195	1	k	ABCD
C57BL/Ks	23	17.0	0.516	1	d	aBCD
C57BR/cd	27	19.9	0.471	1	k	abCD
BALB/c *nu*+	31	21.0	0.528	1	d	AbcD
CBA/Ca	56	21.1	0.313	1	k	ABCD
BRVR	18	21.3	0.713	1	k	c
DW/+	13	21.9	0.775	1	b	In
C57BL/6	16	22.3	1.174	1	b	aBCD

Between 19 and 22 days, differences in mean survival time were not statistically significant except when comparing C57BR/cd mice with either CBA/Ca or DW/+ mice ($P = 0.03$). All other comparisons had P values of less than 0.001. Survival was not clearly related to *H-2* or coat color haplotypes. Animals which were homozygous at the *nu* or *xid* locus had a much shorter lifespan.

Survival analysis. Plots of cumulative mortalities (Fig. 1) show nonoverlapping distributions among strains and discriminate the C57BL/Ks strain as a truly intermediate population (C57BL/Ks versus CBA/N, $P < 0.0001$; C57BL/Ks versus C57BR/cd, $P = 0.0002$). When comparing BALB/c *nu*/*nu* mice with *nu*/+ mice, CBA/N mice with CBA/Ca mice, or C57BL/Ks mice with C57BL/6 mice, P values were found to be less than 0.0001.

Organ weight profile. CBA/N and CBA/Ca uninfected mice were sacrificed at the median survival time of each strain. The average normal spleen weight was greater in the CBA/Ca than in the CBA/N strain (53.8 ± 3 mg versus 37.1 ± 3 mg; $P = 0.01$), but mean normal weights for brain and liver were found to be in close agreement (403.1 ± 2 mg versus 410.7 ± 8 mg and 1,239.2 ± 41 mg versus 1,222.9 ± 45 mg, respectively). When experimental groups were compared with their respective control population, i.e., age-matched uninfected animals, the following weight profiles emerged: infected CBA/N mice showed a 13.5% increase in brain weight ($P < 0.001$), a 5% decrease in liver weight ($P = 0.27$), and a 213% increase in spleen weight ($P < 0.001$); infected CBA/Ca mice had a 54% increase in brain weight ($P < 0.001$), a 63% decrease in liver weight ($P < 0.001$), and a 44% decrease in spleen weight ($P < 0.001$). At the median survival time of CBA/N mice, the following profile was found for the CBA/Ca strain compared with values obtained in age-matched uninfected animals: a 29% increase in brain weight (520.9 ± 6 mg versus 466.1 ± 7 mg for CBA/N; $P < 0.001$), a 4.7% increase in liver weight (1,297.3 ± 50 mg versus 1,162.9 ± 29 mg for CBA/N; $P = 0.03$), and a 38% increase in spleen weight (74.1 ± 4 mg versus 116.1 ± 4 mg for CBA/N; $P = 0.001$).

For infected black mice (C57BL/Ks and C57BL/6), there was, at the time of death, a 26 to 39% increase in brain weight (545.6 ± 17 mg versus 575.2 ± 19 mg; $P = 0.25$), a 65 to 67% decrease in liver weight (480 ± 23 mg versus 435.2 ± 13 mg; $P = 0.10$), and a 59 to 61% decrease in spleen weight (36.9 ± 4 mg versus 35.2 ± 2 mg; $P = 0.70$).

Tissue counts. Figure 2 presents the cumulative results of quantitation of cryptococci in tissues of CBA/Ca and CBA/N mice on days 13 and 14 of the infection. CBA/Ca mice had lower counts per organ for both the liver (mean, 1.05 log units; $P < 0.0001$) and the spleen (mean, 0.91 log units; $P <$

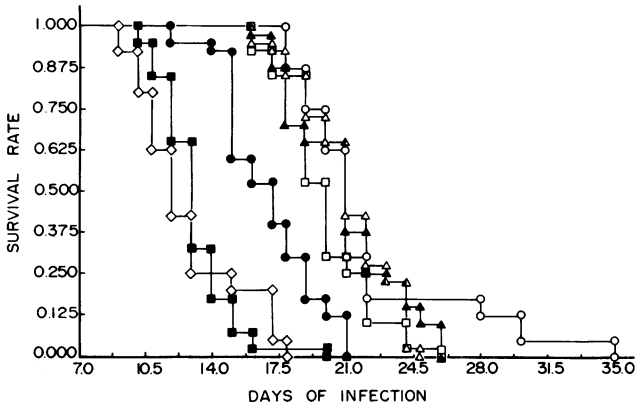

FIG. 1. Percent mortalities in inbred strains of mice infected at 6 weeks of age with 10^5 *C. neoformans* ATCC 13690 cells. Symbols: ◇, BALB/cN *nu/nu*; ■, CBA/N; ●, C57BL/KsJ; □, C57BR/cdJ; △, CBA/CaJ; ▲, BALB/cN *nu*/+; ○, C57BL/6J.

0.0001) as compared with CBA/N mice; CFU in the brain were not statistically different. When tissue colony counts on days 13 and 14 were expressed on a per gram basis, CBA/Ca mice were found to have significantly lower values for the brain ($P < 0.0001$), liver ($P < 0.004$), and spleen ($P < 0.008$). Quantitation of cryptococci in tissues was also performed at the time of death in the CBA/Ca strain. When mean tissue counts were compared with mean tissue counts obtained upon death in the CBA/N strain, these mice were found to harbor a higher burden of organisms in the brain (7.957 ± 0.06 versus 7.506 ± 0.05; $P < 0.001$) and spleen (7.375 ± 0.25 versus 6.464 ± 0.07; $P = 0.002$) but not in the liver (6.637 ± 0.18 versus 6.469 ± 0.06; $P = 0.38$).

For infected black mice (C57BL/Ks and C57BL/6), tissue counts at the time of death ranged from 7.678 ± 0.09 to 7.737 ± 0.12 ($P = 0.69$), from 6.206 ± 0.25 to 6.672 ± 0.09 ($P = 0.08$), and from 6.283 ± 0.18 to 6.842 ± 0.19 ($P = 0.05$) for brain, liver, and spleen, respectively.

Histopathology. Well-defined, cystic collections of fungi were scattered in the cerebrum and cerebellum of CBA/N mice. No inflammatory cells were demonstrated in or around the cystic cavities except for some necrotic macrophages in the center of the cysts. In the leptomeninges, there were isolated or focally clustered cryptococci with few polymorphonuclear leukocytes, lymphocytes, and macrophages. In the CBA/Ca mice, lesions were more severe. Brain parenchyma was diffusely replaced by multiple, confluent, cryptococcal cysts. However, no inflammatory reaction was seen.

Histological study of the liver revealed that both CBA/N and CBA/Ca mice developed a granulomatous hepatitis, although a larger number of granulomas were found in CBA/N mice. Two types of lesions were observed. Clusters of macrophages containing engulfed blastospores were seen in distended sinusoids (Fig. 3b). Other lesions were well-defined granulomas: accumulations of macrophages containing only few cryptococci surrounded by lymphocytes and polymorphonuclear leukocytes (Fig. 3a). In the spleen, lymphoid follicles were well-preserved in the CBA/Ca mice but were depleted and ill-defined in the CBA/N animals. A small number of granulomatous formations containing capsulated yeasts were noted.

The lesions demonstrated upon histological examination of the brain, liver, and spleen of BALB/c *nu*/+ mice were quite similar to those previously observed in CBA/Ca mice (Fig. 4a and c). In BALB/c *nu/nu* mice, there was limited

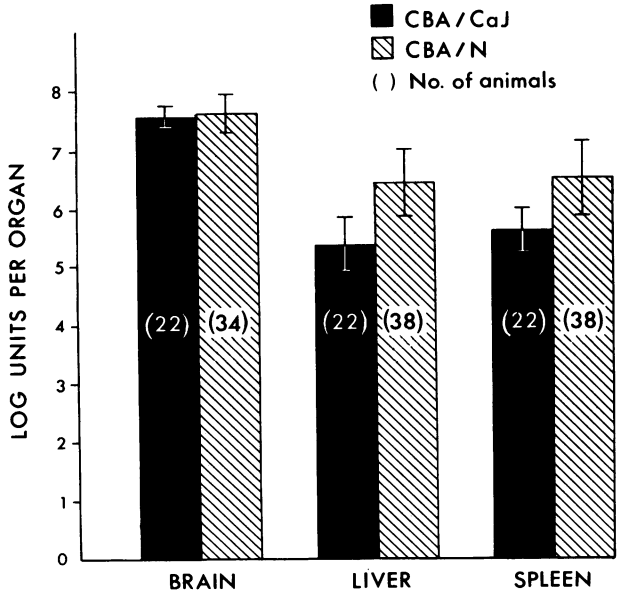

FIG. 2. Mean tissue colony counts of cryptococci on days 13 and 14 of the infection for CBA/N and CBA/CaJ mice. Standard deviations are illustrated by bars.

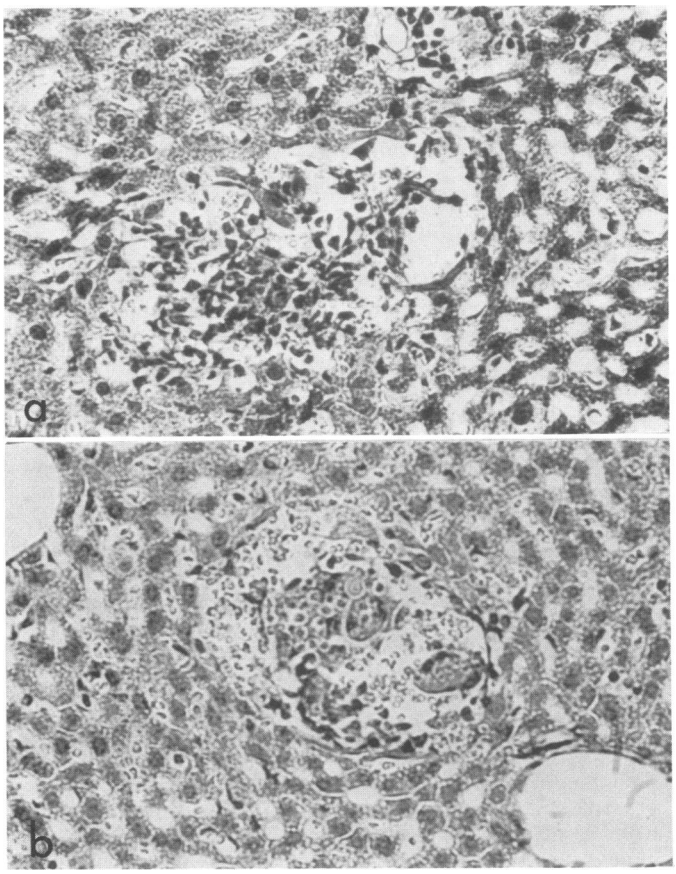

FIG. 3. (a) Well-defined granuloma. Macrophages containing a few yeast cells are surrounded by lymphocytes and polymorphonuclear leukocytes (×1,200; H&E). (b) Clusters of macrophages containing numerous engulfed blastospores (×1,200; H&E).

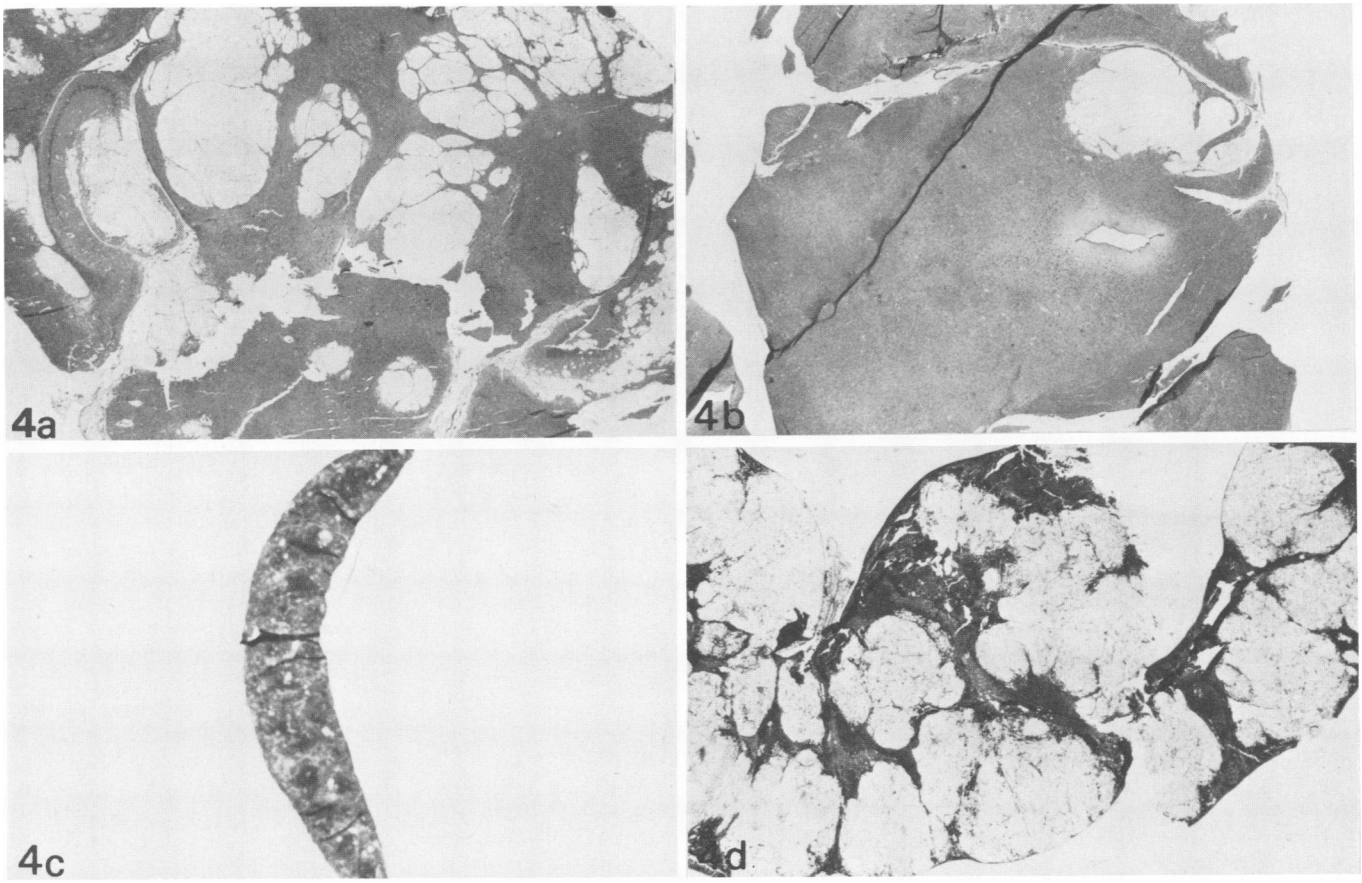

FIG. 4. (a) Photomicrograph of the brain of a BALB/cN *nu*/+ mouse showing multiple cryptococcal cysts without inflammatory reaction (×48; H&E). (b) Photomicrograph of the brain of a BALB/cN *nu*/*nu* mouse showing a small cystic collection of fungi (×48; H&E). (c) Spleen from a BALB/cN *nu*/+ mouse. Lymphoid follicles are well preserved (×48; H&E). (d) Spleen from a BALB/cN *nu*/*nu* mouse. Numerous cystic collections of fungi are replacing the parenchyma (×48; H&E).

involvement of the cerebrum with severe lesions of the spleen and liver. Only a few cystic collections of fungi were seen in the brain, and these cysts were usually smaller than in thymic mice (Fig. 4b). However, the normal splenic architecture was almost completely obliterated by large cryptococcal cysts (Fig. 4d). The hepatic parenchyma was studded with large, cystlike lesions containing numerous yeast cells. Granulomas were not observed.

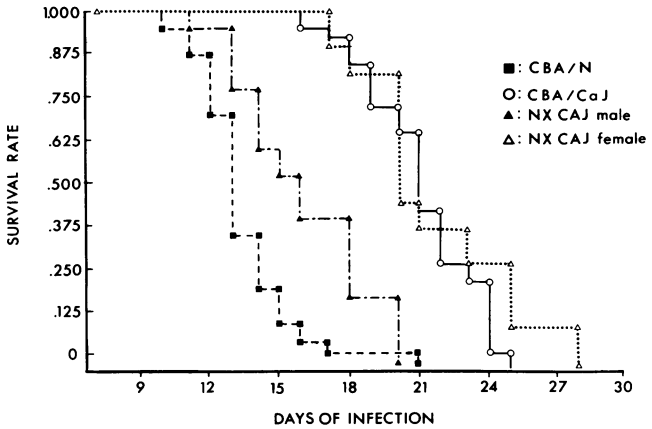

FIG. 5. Plots of survival for the CBA/N, CBA/CaJ, and (CBA/N × CBA/CaJ)F_1 hybrids, male and female. MSTs (in days) ± the standard error of the mean were as follows: 13.2 ± 0.195 (CBA/N); 15.9 ± 0.689 (F_1 male); 21.1 ± 0.313 (CBA/CaJ); 21.5 ± 1.00 (F_1 female).

Influence of the *xid* mutation on susceptibility to cryptococcosis. Six-week-old male and female F_1 offspring from CBA/N × CBA/Ca crosses were infected with cryptococci, and their survival functions were compared with those of each of the parent strains (Fig. 5). Male F_1 mice derived from CBA/N females are known to express the *xid* mutation, whereas their female littermates are phenotypically normal. Female F_1 mice had a MST of 21.5 days versus 15.9 days for males, a 5.6-day difference ($P < 0.001$). Under our defined conditions, we found that, for the CBA/N strain, sexual differences in susceptibility could only account for a modest 1.5-day difference in MST (male, 12.9 days; female, 14.4 days; $P = 0.007$). Therefore, the recorded 5.6-day difference in MST in the F_1 mice is in accordance with a major influence of the *xid* gene on host resistance to cryptococcosis.

DISCUSSION

In our study of the differential susceptibility of inbred mouse strains to *C. neoformans*, we identified a third genetic locus which controls host resistance. The *Cryptococcus* model is therefore analogous to the *Salmonella typhimurium* model of genetically determined resistance since, in both models, three loci were found to influence susceptibility: the *Hc*, *nu*, and *xid* loci on the one hand, and the *Ity*, *lps*, and *xid*

loci on the other (6, 7). Our data and those of Rhodes et al. (7) show that resistance to *C. neoformans* is not likely to involve the *Bcg-Ity-Lsh* gene complex on chromosome 1 because the strain distribution of the Ity^r and Ity^s alleles is, overall, almost opposite the observed murine resistance to cryptococcosis (10). Natural resistance to *C. neoformans* was not linked to coat-color haplotypes, and although mice with the $H\text{-}2^b$ allele had the longest survival time in this study, available data from a previous investigation (7) failed to indicate any survival advantage for this haplotype when C5 deficiency was present. It should also be noted that our data for the nude mouse model fully corroborate results published by Graybill et al. (3) and Cauley et al. (2) despite variations in experimental design and the use of different strains of infecting organism.

Brain weight values of infected animals at the time of death consistently exceeded the values observed in age-matched uninfected animals by more than 25% in all inbred mice tested, except for BALB/c *nu/nu* and CBA/N mice (3.4 and 13.5%, respectively). These two strains were equally at variance as they had massive splenomegaly at the time of death (261 and 313% of control values, respectively). This biological behavior is in striking contrast to the organ weight profiles of CBA/Ca or C57BL mice at the time of death. Moreover, a biphasic evolution of spleen weight was noted for the CBA/Ca strain: from a 38% increase on day 13 to a 44% decrease upon death. Overall, these findings indicate that inbred animals display considerable heterogeneity in their biological response to infection, even under the most carefully standardized conditions. Futhermore, the degree of genetic relatedness had no clear counterpart in term of variations in organ weights, i.e., the changes observed were either alike (C57BL, CBA/N and BALB/c *nu/nu* mice) or unlike (CBA/N and CBA/Ca mice).

Quantitation of cryptococci in tissues failed to uncover major strain differences in relation to actual burden of organism in the target organs at the time of death; overall standard deviations of growth in log units at the time of death for all inbred mice were 0.499, 0.716, and 1.07 for brain, liver, and spleen, respectively. As there was a continuum in survival time over a 10-day period, these results indicate that the in vivo growth rate of *C. neoformans* varies from strain to strain. Even in the least susceptible animals, a steady increase in cryptococcal counts was demonstrated. This point is exemplified by our results for the CBA/Ca strain: at 13 and 14 days, colony counts on the liver and spleen were a log unit lower than those of CBA/N mice, whereas at the time of death, equal or higher counts were achieved. To account for these findings, we suggest that the effectiveness of host defense mechanisms determines the time lapse required to reach a given level of pullulation, which is seemingly the same for all the strains.

The small variation in brain weight for the two susceptible strains (BALB/c nude and CBA/N) can be explained by the low number of cryptococcal cysts found upon histological examination (Fig. 4b). In addition, the splenomegaly observed in nude mice may be ascribed to severe infiltration of the spleen by large cystic collections of fungi (Fig. 4d). However, there was no evidence of overwhelming cryptococcal involvement in the spleens of CBA/N mice; granulomatous lesions could not be distinguished from those of CBA/Ca mice and fungal cystic lesions were consistently absent. These results suggest that a difference in the cellular proliferative response of the host is responsible for the occurrence of massive splenomegaly in CBA/N mice.

One striking observation was the relatively low degree of infection in the central nervous system of susceptible mice, whereas there was more prominent involvement of the liver and spleen. In contrast, large multiple collections of fungi were present in the brains of mice with the resistant phenotype, approaching the "soap-bubble" appearance sometimes observed in human cases (Fig. 4a). These results suggest that the meningoencephalic barrier significantly delays the involvement of the central nervous system, i.e., that the course of the infection in susceptible animals was so rapid that not enough time was allowed for the development of cryptococcal cysts in the brain. In a recent study (4), systemic spread to the cerebrum after experimental cutaneous inoculation in mice was found to occur much less frequently than dissemination to other organs, in accordance with our hypothesis. However, investigation of the experimental pathogenicity and tissue localization of a large number of *C. neoformans* isolates in mice has revealed a rather heterogeneous behavior (11). In a previous study on cryptococcosis in nude mice (5), severe lesions of the brain were documented in BALB/c *nu/nu* animals after infection with a strain of lower virulence than ATCC 13690, based on average period of survival. Nevertheless, there is a solid agreement with the histopathological findings, i.e., the paucity of the inflammatory reaction in the central nervous system, the absence of cellular host response in athymic mice, and the formation of granulomas in the liver and spleen of thymus-bearing littermates.

Comparison of the survival functions of male and female (CBA/N × CBA/Ca)F1 mice by a nonparametric procedure gave a P value of 0.0002 (28 observations, Mantel-Cox). Female F_1 mice had a slightly longer MST than CBA/Ca mice (21.55 versus 21.05 days; $P = 0.55$), whereas male F_1 mice fared significantly better than the overall CBA/N population (15.9 versus 13.2 days; $P = 0.001$). This last observation is consistent with a model in which other autosomal genes present in the CBA/Ca background also contribute to prolonged survival and heightened resistance. The 5.3-day difference in MST for the two strains of black mice in this study (C57BL/Ks and C57BL/6) is another piece of evidence which points to a possible role of other autosomal genes in controlling natural resistance to *C. neoformans*. Indeed, our data indicate that, despite the similitude of organ weight profiles and tissue colony counts at death, C57BL/Ks and C57BL/6 mice were much further apart in survival functions and MST than could have been expected given their relatedness in genetic makeup.

In conclusion, host resistance to *C. neoformans* is decisively influenced by mutations which are located on different murine chromosomes and which, as a rule, are known to affect the immune system. However, a meaningful understanding of the genetic factors which control susceptibility to cryptococcosis can only be achieved by careful investigation of the role of other normal or mutant genes, of which only a very small number are known to affect the immune system. The participation of other autosomal genes in determining the expression of host resistance is strongly favored and should be further explored.

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