

Histoplasma capsulatum Infection in Nude Mice

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Congenitally athymic nude (nu/nu) mice, when injected intraperitoneally with *Histoplasma capsulatum*, developed a rapidly fatal disseminated infection characterized by heavy parasitization of reticuloendothelial tissues. In contrast, their heterozygous (nu/X) littermates, which possessed a functioning thymus, developed only a low-grade infection which was apparently self-limited and rarely fatal. Transplantation of thymic tissue into nu/nu mice diminished greatly the severity of infection and reduced mortality by about 50%. These studies emphasize the importance of cell-mediated immunity in host defense against histoplasmosis and suggest that the nude mouse may be a valuable model for the study of this chronic intracellular infection.

Although there have been a number of studies of histoplasmosis in humans and in experimental animals, the mechanism of host defense against *Histoplasma capsulatum* is not fully understood. Host defense is thought to depend primarily on cell-mediated immunity (CMI). The rationale for this is based largely on the association of histoplasma exposure with both a positive skin test and positive in vitro parameters of CMI (lymphocyte blastogenesis; production of migration inhibition factor (6, 10). These parameters often revert to negative in people with disseminated histoplasmosis (17, 25). Control of histoplasmosis is apparently achieved through T-lymphocyte intervention, which leads to macrophage activation (13-15). However, there are many unanswered questions concerning the character and specificity of cell-mediated immune processes and the role of antibody in host defense in histoplasmosis (8, 10, 17, 19, 25).

The congenitally athymic nude (nu/nu) mouse is known to have deficient CMI and impaired T-lymphocyte-dependent antibody production (22). The present studies were designed to test the suitability of the nude mouse as a model for disseminated histoplasmosis, to compare its susceptibility to that of heterozygous (nu/X) littermates, and to evaluate the effect of thymus transplantation upon the course of infection.

MATERIALS AND METHODS

Mice. Specific-pathogen-free nude mice (nu/nu) on a BALB/c background were obtained from Sprague-Dawley, Inc. (Madison, Wis.) and maintained in the specific-pathogen-free state by using sterilized food, bedding, water, and barrier filter top cages. Breeding

was performed by mating hairless nu/nu males with hairy female heterozygote (nu/X) littermates. Nude animals were checked periodically at autopsy to verify the absence of a developed thymus. Immunodeficiency was further confirmed by the inability of the nu/nu mice to reject homozygous (C1D H-2C) skin grafts and by their inordinate susceptibility to cryptococcosis (J. R. Graybill and D. J. Drutz, Cell. Immunol., in press).

BALB/c mice without the nu gene were obtained from Charles River Laboratories (Wilmington, Mass.), since the original BALB/c strain from which the Sprague-Dawley nude mice were derived is not available in this country (Carl Hansen, personal communication). In the experiments to be described, 5- to 7-week-old animals of both sexes were used.

H. capsulatum. Three strains of *H. capsulatum* were obtained. Strains 1077 and 102 were gifts of Rebecca Cox (San Antonio State Chest Hospital), and strain 877 was a clinical isolate. The identity of the fungi as *H. capsulatum* was confirmed by morphology and growth characteristics of mycelial and yeast-phase organisms.

Strain 1077 was passed by the intraperitoneal route in nu/nu mice with periodic subculture on sheep blood agar. At 37°C it grew as the yeast form. In one experiment, an inoculum which had only partially converted from mycelial to yeast form and which contained both budding yeast cells and hyphae in the process of conversion was used with equivalent results. Subsequently, this strain was maintained in the yeast form. Strains 877 and 102 were grown on brain heart infusion medium (Baltimore Biological Laboratory, Cockeysville, Md.) and maintained in the yeast form by serial passage on brain heart infusion slants at 37°C.

Infective inocula were prepared by diluting yeast or a mixture of hyphal and yeast-form *H. capsulatum* in phosphate-buffered saline and adjusting the number of organisms to 10^5 to 10^6 /ml in a hemocytometer. The viability of yeast-phase organisms was approximately 20%. Infection was produced by intraperitoneal injection of 0.2 ml in each mouse. Animals were followed

until their deaths; animals that survived for 40 to 70 days were sacrificed.

H. capsulatum was verified as the cause of death by removing the spleen and liver (and occasionally other organs) from dead animals and searching for *H. capsulatum* in tissue sections stained with Gomori methenamine silver stain, Giemsa stain, and hematoxylin and eosin stain. Spontaneous death from wasting disease in uninfected nu/nu mice occurred very rarely in our mouse colony. *H. capsulatum* was recovered from cultured liver and spleen in infected animals, but their relatively poor growth prevented quantitative cultures.

Thymic transplantation. Intact thymuses were removed by dissection from 24- to 48-h-old neonatal heterozygous (nu/X) or non-nude gene-containing (i.e., homozygous normal) BALB/c mice and implanted subcutaneously in the flank of nu/nu recipients (nu/thy) 4 weeks before infecting them with *H. capsulatum*. A needle insertion technique was used to minimize trauma (3). Random nu/thy mice were checked at the time of death or sacrifice to verify the presence of transplanted thymic tissue.

Quantitation of *H. capsulatum*. Because of problems encountered in culturing *H. capsulatum* quantitatively from the viscera, a technique was devised whereby silver-stained fungi were counted directly in histological sections of the liver and spleen by light microscopy. To accomplish this, 20 randomly selected high-power fields ($\times 1,000$) 1 mm apart were examined for *H. capsulatum*, using an ocular micrometer. Full-thickness fields were examined in each area, using a grid system to be sure every tissue specimen was thoroughly sampled. In this manner, the mean number of fungi per high-power ocular field was determined for each organ, and the percentage of the 20 fields counted in which organisms were seen was determined. The fields were counted independently in a blinded manner by two observers. Results were nearly identical.

These quantitative studies were carried out in conjunction with experiment 4 (see Table 1), in which 14 nu/nu, 15 nu/X, and 5 nu/thy mice received 3×10^7 *H. capsulatum* 102 intraperitoneally. Five mice from each group were sacrificed on the day that the first nu/nu mouse died spontaneously of *H. capsulatum* infection (day 16). Three nu/X mice and two nu/nu mice were similarly sacrificed at the time the third remaining nu/nu mouse expired at day 40.

Statistics. Statistical analysis was performed by using life table methods for survival; the Wilcoxon rank sum test was used to compare tissue burdens of *H. capsulatum*.

RESULTS

Four experiments were performed in which nu/X and nu/nu mice were inoculated with yeast-phase (three experiments) or mixed yeast/mycelial-phase (one experiment) *H. capsulatum*. In three of these experiments, nu/thy mice were infected at the same time. Table 1 outlines the results of these experiments. In general, nu/nu mice were highly susceptible to lethal infection with either mycelial or yeast-phase *H. capsulatum*, whereas nu/thy mice were less likely to die. In contrast, nu/X mice seemed highly resistant to lethal infection, regardless of the inoculum size or strain of *H. capsulatum* used. By 40 to 70 days after challenge, 35 of 38 nu/nu mice were dead, whereas 39 of 40 nu/X mice were alive and apparently well. Nu/thy mice displayed an intermediate pattern of susceptibility, with 11 of 20 mice surviving to day 50.

Figure 1 depicts graphically the data in experiment 1. Significant differences in survival between nu/nu and nu/X mice were first apparent

TABLE 1. Survival of nu/nu, nu/thy, and nu/X mice after intraperitoneal inoculation of mycelial or yeast-phase *H. capsulatum*

Expt	<i>H. capsulatum</i> strain	Fungal phase	Inoculum size	Mouse	Day of death						
					0-10	11-20	21-30	31-40	41-50	51-60	61-70
1	1077	Yeast/mycelial ^a	1×10^5	nu/nu	0/10 ^b	0/10	0/10	9/10	10/10	— ^c	—
				nu/thy	0/10	0/10	0/10	3/10	5/10	—	—
				nu/X	0/10	0/10	0/10	0/10	0/10	—	—
2	1077	Yeast	1×10^8	nu/nu	0/9	2/9	8/9	9/9	—	—	—
				nu/thy	0/10	3/10	4/10	6/10	6/10	—	—
				nu/X	0/10	0/10	0/10	0/10	0/10	—	—
3	877	Yeast	1×10^5	nu/nu	0/10	0/10	0/10	0/10	0/10	8/10	9/10
				nu/X	0/10	0/10	0/10	0/10	0/10	0/10	0/10
4	102	Yeast	3×10^7	nu/nu	0/14	1/14 ^d	3/9	7/9 ^e	—	—	—
				nu/thy	0/5	0/5 ^d	—	—	—	—	—
				nu/X	0/15	0/15 ^d	1/10	1/10 ^e	—	—	—

^a These data are illustrated in greater detail in Fig. 1.

^b Cumulative deaths to date; ratio expresses proportion of dead animals to total animals inoculated with *H. capsulatum*.

^c Absence of data from a column (—) indicates that experiment had been terminated.

^d Five animals were sacrificed from each group for quantitation of fungi in liver and spleen after the spontaneous death of the first nu/nu mouse on day 16.

^e Two nu/nu and three nu/X mice were sacrificed at the time the seventh nu/nu mouse died on day 40.

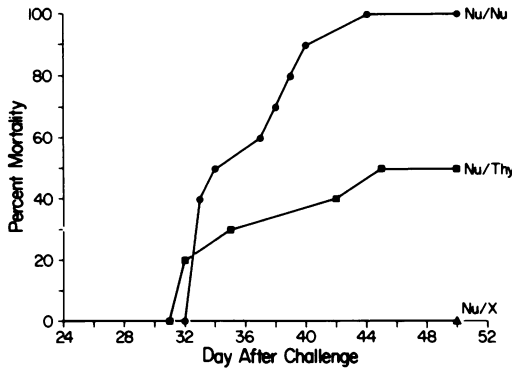


FIG. 1. Cumulative percent mortality of nu/nu, nu/thy, and nu/X mice to 10^5 *H. capsulatum* 1077 injected intraperitoneally.

33 days after challenge ($P < 0.01$). Nu/thy mice were significantly more susceptible to *H. capsulatum* than nu/X mice but significantly less susceptible than nu/nu mice (each $P < 0.01$).

Dead nu/nu animals consistently had disseminated infection involving liver, spleen, heart, lung, brain, and kidney, with histological findings indicating diffuse macrophage parasitization.

Figure 2 compares the results of silver and hematoxylin and eosin stains of the liver from nu/nu and nu/X mice 50 days after infection with strain 1077. The tissue from nu/nu mice showed enormous numbers of organisms with the typical morphology of *H. capsulatum*; there was little inflammatory response. This picture mimics closely that encountered in humans with progressive disseminated histoplasmosis. In rather striking contrast, the tissues of nu/X animals contained very few fungi; the ones pictured in Fig. 2 were found with some difficulty.

Table 2 shows the results of quantitative determinations of *H. capsulatum* in the spleens and livers of nu/nu, nu/X, and nu/thy mice at days 16 and 40 after infection with strain 102 (experiment 4). Fungi were not enumerated in the livers at day 16; here, whole organ homogenates were cultured for *H. capsulatum*. As previously noted, all cultures were positive, but quantitation of fungi was unsatisfactory.

At day 16, the animals were not heavily infected. The mean number of organisms per high-power field is greater in the nu/nu group than in either the nu/thy or the nu/X group, but the difference is not statistically significant ($P < 0.5$). However, if the percentage of the 20 random fields positive for organisms is determined, significantly more "nu/nu fields" had organisms than either of the other groups ($P < 0.05$). The differences at this time period, therefore, al-

though consistent with our mortality data, were not striking.

At day 40, however, the mean number of organisms per high-power field in both spleen and liver was considerably larger in nu/nu than in nu/X mice. Moreover, fungi were seen in the kidney only in the nu/nu mice. The small number of mice surviving to day 40 prevented a meaningful statistical analysis, but the quantitative trends are striking.

DISCUSSION

We have developed a murine model of disseminated *H. capsulatum* infection closely paralleling the progressive form of human infection (25). This infection is often difficult to establish in mice (although there are strain differences in this regard [21]). However, nu/nu mice, which have only a thymic rudiment (11) and impaired T-cell function (2, 20, 22), proved exquisitely susceptible to *H. capsulatum*. That this is a thymus-dependent function is shown by the increased resistance conferred by thymic transplantation—a procedure known to only partially restore T-cell function in nu/nu mice (16).

Studies by others suggest that the critical event terminating *H. capsulatum* replication is macrophage activation (9, 14, 15, 27). Howard found that intact lymphocytes derived from histoplasma-immune mice could activate macrophages from either immune mice or nonimmune mice, enabling them to inhibit intracellular growth of *H. capsulatum* (14, 15). The status of CMI in nu/nu mice is somewhat paradoxical. Specific-pathogen-free nu/nu mice are known to have nonspecifically activated macrophages, making them initially more resistant than nu/X mice to some pathogens such as *Listeria monocytogenes* (7, 12) and *Candida albicans* (5). However, they cannot clear the organisms from their tissues as efficiently as surviving nu/X mice. Accordingly, they develop chronic, persistent infection. On the other hand, nu/nu mice have been shown to be consistently more susceptible than nu/X mice to other intracellular organisms such as mycobacteria (4, 26) and *Toxoplasma gondii* (18), in which CMI is considered of paramount importance in host defense. In these examples, specific CMI may be required for optimal host defense (8). Our results are consistent with the findings in these latter experiments and imply an important role for T-cell/macrophage interaction in successful resistance to *H. capsulatum* challenge.

The role of antibody in the outcome of *H. capsulatum* infection is currently unclear. Precipitating, hemagglutinating, complement-fixing, fluorescent, and latex-agglutinating antibod-

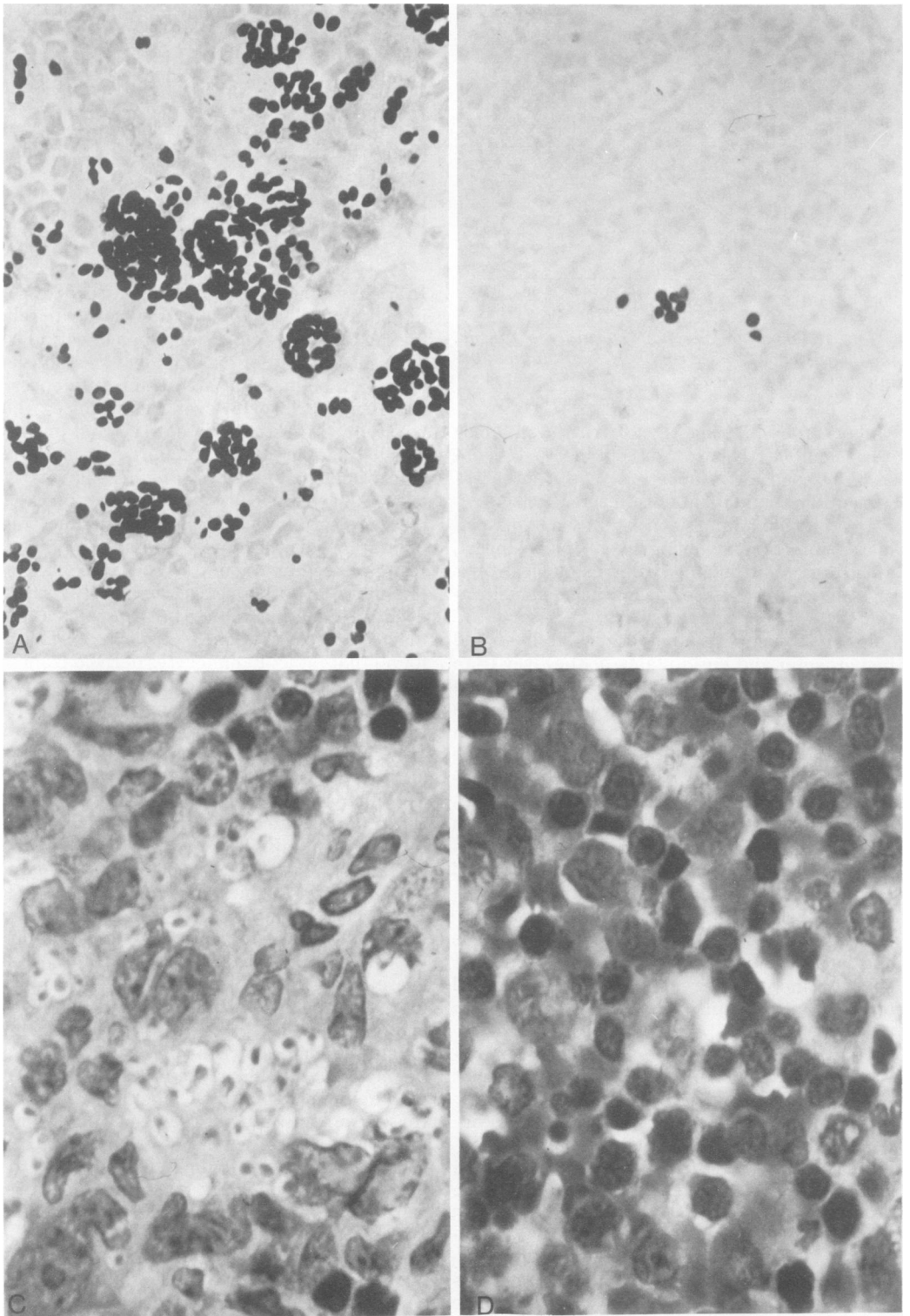


FIG. 2. Results of silver and hematoxylin and eosin stains of spleens from *nu/nu* (A and C) and *nu/X* (B and D) mice 50 days after infection with *H. capsulatum* 1077.

TABLE 2. Mean number of organisms per high-power field in tissue of mice infected with *H. capsulatum* 102 at two time periods^a

Day of infection	Spleen			Liver	
	nu/X	nu/nu	nu/thy	nu/X	nu/nu
16	0.9	2.4	0.9		
	0.6	0.6	0.4		
	0.1	0.4	0.1		
	0	0.2	0		
	0	0	0		
Mean	0.3	0.7	0.3		
40	9.0	116.0		27.0	83.0
	1.7	50.0		0.3	70.0
	1.6	9.0		0.3	14.0
Mean	4.1	58.3		9.2	55.7

^a Twenty random fields were counted in each mouse.

ies are all detectable in histoplasmosis (1, 23). The nu/nu mouse has a known defect in synthesis of T-dependent antibody (2), although other antibodies may be produced normally (28). The possible role of T-dependent antibody against *H. capsulatum* is an issue which will require further study.

In summary, we have developed a model of disseminated *H. capsulatum* infection in the nu/nu mouse, providing further evidence of the importance of T-cell function in resistance to this infection and permitting further studies of the mechanisms of host resistance to *H. capsulatum*.

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