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Cutaneous Cryptococcosis in Athymic and Beige-Athymic Mice

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The dermotropism of *Cryptococcus neoformans* SLHA in congenitally athymic (*nu/nu*) and doubly immunodeficient beige-athymic (*bg/bg-nu/nu*) mice is described. Both *bg/bg-nu/nu* and *nu/nu* mice developed cutaneous cryptococcosis within 7 to 12 days following intravenous challenge with 10^4 encapsulated yeast cells. Macroscopically, cutaneous lesions appeared as small subcutaneous nodules without ulceration. Cryptococcal skin lesions were observed primarily on the flank of *nu/nu* mice, whereas skin lesions in *bg/bg-nu/nu* mice were distributed over the trunk, abdomen, and face. While *bg/bg-nu/nu* mice had four times as many macroscopic skin lesions as *nu/nu* mice on day 14 after intravenous challenge, the skin lesions in *nu/nu* mice were larger. Histopathology revealed large foci of encapsulated yeasts extending from the basement membrane of the epidermis through the dermis to the underlying musculature. Yeasts in these lesions evoked a minimal inflammatory response that consisted primarily of macrophages. Interestingly, yeast cells appeared to be degrading collagen bundles located in the dermis. The dermotropic strain used in this study produced gelatinase and other proteases in vitro. These results indicate that *C. neoformans* can be dermotropic in a T-cell-deficient host and that proteases may be a virulence factor(s).

Cryptococcus neoformans is an encapsulated yeast of increasing clinical importance as a human pathogen, particularly in immunocompromised individuals. Human cryptococcal infections range from mild pulmonary disease to severe disseminated cryptococcosis. While *C. neoformans* has a predilection for the central nervous system, systemic spread to any organ system including the kidneys, liver, spleen, bone, and skin is not uncommon.

Cutaneous cryptococcosis occurs in 10 to 15% of patients with disseminated cryptococcosis (8) and has been a generally unappreciated feature of this fungal disease (26). Most cases of cutaneous cryptococcosis occur in patients with underlying defects in cell-mediated immunity, and skin manifestations have been primarily associated with long-term immunosuppressive therapy (particularly prednisone) following renal transplantation or for the treatment of cancer, systemic lupus erythematosus, and AIDS (2, 4, 8, 22, 25, 26). The increasing incidence of patients with cutaneous cryptococcosis is likely due to wider use of immunosuppressive drugs and an increasing number of patients with AIDS (2, 4, 22).

While studying the susceptibility of various congenitally immunodeficient mouse strains to systemic cryptococcosis, we observed that mice with congenital defects in T-cell-mediated immunity developed cutaneous cryptococcosis (23, 24). In this report, we describe the course of cutaneous cryptococcosis in congenitally immunodeficient mice and detail the histopathology and *C. neoformans* characteristics which may be involved in the development of cryptococcal skin lesions.

MATERIALS AND METHODS

Mice. Germfree beige-athymic (*bg/bg-nu/nu*) and beige-euthymic (*bg/bg-nu/+*) N:NIH(S) III mice and athymic (*nu/nu*) and euthymic (*nu/+*) BALB/c mice between 8 and 10 weeks of age were used in this study. Animals were obtained from the University of Wisconsin Gnotobiotic Research Laboratory (Madison) and were maintained in accordance with National Institutes of Health guidelines. On the day each experiment was started, mice were removed from the germfree isolator and maintained in sterile cages with filter bonnets in a laminar flow hood.

Verification of immunodeficiencies. To confirm T-cell defects in athymic mice, we assayed spleen cells from immunocompetent and immunodeficient mice in vitro for their ability to respond to the T- and B-cell mitogens concanavalin A and lipopolysaccharide, respectively, as previously described (1). In contrast to spleen cells from mice with T-cell function (8,000 to 14,000 cpm/10^5 spleen cells), spleen cells from *nu/nu* BALB/c and *bg/bg-nu/nu* N:NIH(S) III mice responded poorly to concanavalin A (400 to 1,000 cpm/10^5 spleen cells). All genotypes had strong responses to lipopolysaccharide (12,000 to 17,000 cpm/10^5 spleen cells). To confirm natural killer (NK) cell defects in *bg/bg-nu/nu* and *bg/bg-nu/+* mice, we compared splenic NK cell activities in a standard 4-h ^{51}Cr release assay as previously described (3). All mice homozygous for the beige gene (*bg/bg-nu/nu* and *bg/bg-nu/+*) had very low levels of splenic NK cell activity (<3% at an effector:target cell ratio of 100:1), whereas NK cell-competent *nu/nu* and *nu/+* mice had elevated levels of splenic NK cell activity (>11% at an effector:target cell ratio of 100:1).

Yeast cultures and animal inoculations. Encapsulated *C. neoformans* SLHA (serotype A) was maintained on Sabouraud dextrose agar. The encapsulated strain was a human clinical isolate obtained from the State Laboratory of Hygiene at the University of Wisconsin. Before inoculation into mice, yeast cells were transferred to Sabouraud dextrose broth and incubated at 37°C for 48 h. Cryptococci were harvested, washed three times by centrifugation (1,000 × *g*, 15 min), and resuspended in injectable saline. Yeast cells were counted on a hemacytometer and adjusted to 10^5 cells per ml. Mice were infected by injecting 0.1 ml of yeast cell suspension into the tail vein. To verify the number of viable cells, the inoculum was serially diluted in phosphate-buffered saline, plated on Sabouraud dextrose agar, and incu-

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bated at 37°C for 48 h and CFU were determined. Ten *nu/nu*, 8 *nu/+*, 13 *bg/bg-nu/nu*, and 13 *bg/bg-nu/+* mice were used in mortality studies for strain SLHA, and deaths were recorded on a daily basis. We have previously described the course of systemic cryptococcosis in the internal organs of both BALB/c and N:NIH(S) III mice (23, 24).

Histopathology. Skin biopsy specimens were collected from three mice per group 14 days after intravenous (i.v.) challenge with 10^4 *C. neoformans* SLHA. After fixation for 48 h in Hollande-Bouin's fixative, specimens were dehydrated through increasing concentrations of ethanol (50, 70, 80, 95%) and embedded in glycol methylacrylate (Bio-Rad Laboratories, Richmond, Calif.). Sections (2 to 2.5 μm) were cut on a JB-4 microtome (Ivan Sorvall, Inc., Norwalk, Conn.) and stained with periodic acid-Schiff followed by azure A-eosin B, and at least three sections per sample were examined by light microscopy.

Proteolytic activity. To assay for proteolytic activity, we used nutrient gelatin stabs to assess the capacity of *C. neoformans* to liquify gelatin, a denatured form of collagen. After inoculation, tubes were incubated at room temperature and monitored daily. To assess whether glucose or pH altered expression of gelatinase, in some experiments we supplemented nutrient gelatin with 2% glucose at either pH 7.0 or pH 4.5. Inoculated tubes were compared with uninoculated controls. Media were prepared as described by Ray and Payne (20). An agar base containing 2% glucose, 0.1% KH_2PO_4, 0.05% $MgSO_4$, and 2% agar was prepared, sterilized, and tempered to 50°C. Agar base was supplemented with either filter-sterilized bovine serum albumin or casein and minimal essential medium vitamins to a final concentration of 1% or 1×, respectively. Plates were inoculated with *C. neoformans* prepared as described above and incubated at 37°C. Plates were examined for clear zones around *C. neoformans* colonies 2 to 3 weeks after inoculation.

RESULTS

Mortality. The differential susceptibility of T-cell-competent and T-cell-deficient mice was assessed by mortality following i.v. challenge with 10^4 *C. neoformans* SLHA (23, 24). The mean survival time of *bg/bg-nu/nu* and *nu/nu* mice was 14.8 ± 0.3 and 22.1 ± 0.9 days, respectively. *nu/nu* mice survived significantly longer ($P < 0.01$) than *bg/bg-nu/nu* mice. The mean survival time of T-cell-competent *bg/bg-nu/+* and *nu/+* mice was 31.4 ± 3.3 and 33.5 ± 4.4 days, respectively. Both *bg/bg-nu/+* and *nu/+* mice survived significantly longer ($P < 0.05$) than their athymic counterparts.

Skin lesions. Following i.v. challenge with *C. neoformans* SLHA, *bg/bg-nu/nu* mice developed visible skin nodules 7 to 10 days postinfection, while *nu/nu* mice developed visible lesions 10 to 12 days postinfection (Table 1). Macroscopically, lesions were similar in both *bg/bg-nu/nu* and *nu/nu* mice and appeared as small subcutaneous nodules (Fig. 1). No ulceration of skin nodules was observed in either *bg/bg-nu/nu* or *nu/nu* mice over the 14-day study. Skin lesions in *nu/nu* mice appeared primarily on the flank (Fig. 1A) or the top of the head (between the ears), and no skin lesions were observed on the abdomen. Skin nodules in *bg/bg-nu/nu* mice were distributed over the trunk of the mouse (Fig. 1B), including the abdomen of two of five mice. In addition to skin lesions on the trunk, *bg/bg-nu/nu* mice also developed facial nodules (Fig. 1C). Beige-athymic (*bg/bg-nu/nu*) mice had significantly more ($P < 0.01$) skin lesions than *nu/nu* mice (21.8 versus 5.2 skin lesions per mouse 14 days postinfection; Table 1). Despite an increased number of skin lesions in *bg/bg-nu/nu* mice, the average size of skin lesions was significantly larger ($P < 0.05$) in *nu/nu* mice (Table 1). India ink preparations from biopsied skin lesions showed numerous encapsulated budding yeasts, and a pure culture of *C. neoformans* grew from lesion aspirates. Interestingly, T-cell-competent *nu/+* and *bg/bg-nu/+* littermates did not develop skin lesions.

TABLE 1. Cutaneous cryptococcosis in beige-athymic (*bg/bg-nu/nu*) and athymic (*nu/nu*) mice

Genotype	Time to onset (days)	Skin lesions	
		Avg no. of lesions/mouse (range)[a]	Avg size (mm) (range)[a]
nu/nu	10–12	5.2 ± 1.4 (2–10)	3.5 ± 0.3 (1–5)
bg/bg-nu/nu	7–10	21.8 ± 4.4 (10–33)[b]	2.3 ± 0.1 (1–5)[c]

[a] Skin lesion number and size were assessed 14 days after i.v. challenge with 10^4 viable *C. neoformans*, and data are expressed as the mean ± standard error of the mean from five mice.

[b] *bg/bg-nu/nu* mice had significantly more ($P < 0.01$) skin lesions than *nu/nu* mice.

[c] *bg/bg-nu/nu* mice had significantly smaller ($P < 0.05$) lesions than *nu/nu* mice.

Histopathology. Microscopically, skin lesions in *bg/bg-nu/nu* and *nu/nu* mice appeared similar. Large numbers of encapsulated yeasts were distributed throughout the dermis and extended from the papillary dermis to the underlying musculature (Fig. 2A and B). In large lesions, focal areas of muscle necrosis were evident. While yeast cells occasionally extended to the basal level of the epidermis, no yeast cells were observed either in the epidermis or disrupting the epidermal basement membrane (Fig. 2C). Yeast cells could also be observed in the stratum corneum (Fig. 2C). Collagen bundles at the edge of yeast foci were disrupted. Yeast cells appeared to be degrading the collagen since there was a loss of stainable collagen around yeast foci and pitting of collagen bundles adjacent to invading yeasts (Fig. 2D). Yeast cells almost completely replaced the connective tissue in the center of these dermal lesions, and some yeasts were observed adhering to the remaining strands of collagen (Fig. 2E). While little to no inflammatory response was evoked at the periphery of dermal lesions, some macrophages could be observed in the center of lesions (Fig. 2E).

Proteinase activity. To assess whether *C. neoformans* was capable of degrading gelatin, a denatured form of collagen, we tested dermotropic strain SLHA for its capacity to liquify gelatin, a test indicative of collagenase activity. Strain SLHA produced visible gelatin liquification on nutrient gelatin 2 to 3 weeks after inoculation after incubation at 21°C (Fig. 3B). The capacity of strain SLHA to liquify gelatin was not glucose or pH dependent; however, greater gelatin liquification was observed in the presence of glucose at pH 7.0. Strain SLHA produced clear zones around colonies that were grown on casein but not albumin agar plates. Proteolytic activity for casein was not pH dependent and was observed at both pH 4.5 and 7.0.

DISCUSSION

In the present study, the dermotropic nature of *C. neoformans* SLHA was expressed only in congenitally T-cell-deficient mice. In contrast, their isogenic T-cell-competent *bg/bg-nu/+* and *nu/+* counterparts did not develop cutaneous cryptococcosis. Both athymic and beige-athymic mice

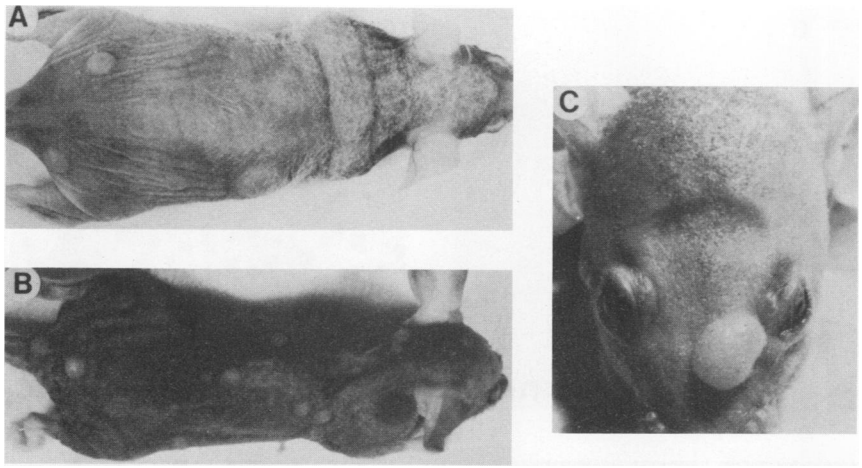

FIG. 1. Cutaneous cryptococcosis in *bg/bg-nu/nu* (A and C) and *nu/nu* (B) mice. Skin nodules 14 days after i.v. challenge with 10^4 *C. neoformans* SLHA.

developed cutaneous manifestations of cryptococcosis between 7 and 12 days after i.v. challenge. Skin lesions appeared as subcutaneous nodules that enlarged with time. The cutaneous involvement in both athymic and beige-athymic mice was observed with progressive disseminated infection in these mice (23, 24). These results suggest that in mice, as has been observed in humans (2, 4, 22, 25, 26), cutaneous manifestations of disseminated cryptococcosis are associated with defects in cell-mediated immunity.

From these studies, it is evident that the level of immune competence is a key component in the expression (or suppression) of *C. neoformans* dermotropism. T-cell function, and presumably T-cell lymphokine production, is apparently required to control cutaneous cryptococcosis since athymic (*nu/nu*, *bg/bg-nu/nu*) and not euthymic (*nu/+*, *bg/bg-nu/+*) mice developed the cutaneous manifestations. In addition, the enhanced susceptibility of *bg/bg-nu/nu* mice to cutaneous cryptococcosis implies that competent phagocytic cells are important in preventing the dermotropic manifestations of cryptococcosis. Previous studies in both *nu/nu* (23) and *bg/bg-nu/nu* (24) mice have demonstrated that T cell-phagocytic cell interactions are important for the production and progression of in situ inflammatory responses and resistance to *C. neoformans*. The enhanced susceptibility of *bg/bg-nu/nu* mice to cutaneous cryptococcosis may also be related to inherent differences in the relative susceptibility of BALB/c and N:NIH(S) mice to cryptococcal infection.

A review of the literature on experimental cutaneous cryptococcosis suggests, however, that skin manifestations are not always linked to suppressed host immunity. Development of cutaneous lesions coincident with systemic cryptococcosis has not been reported by other investigators using athymic mice (7, 13, 15, 19). Conversely, investigators have established cutaneous cryptococcal lesions in immunocompetent mice following i.v. challenge with some human clinical isolates. Dixon and Polak (9) and Fromtling et al. (11) described isolates of *C. neoformans* that were rhinotropic in immunocompetent mice. These isolates also produced cutaneous lesions on the ears, feet, and tail (9, 11). Using a guinea pig model, van Cutsem et al. (30) were able to establish granulomatous skin ulcerations following i.v. challenge with 10^5 cryptococci. Interestingly, the same cryptococcal isolates that produced skin lesions in the guinea pig failed to produce cutaneous lesions in mice despite a 15-fold increase in the inoculum (30). Attempts to establish primary cutaneous cryptococcal lesions following subcutaneous inoculation or scarification, on the other hand, have not been as successful. In the latter studies (10, 17), cutaneous lesions usually regressed despite limited growth at the site of inoculation. Song (27) reported that cortisone pretreatment was necessary to establish persistent primary cutaneous lesions in mice. These studies suggest that some strains of *C. neoformans* possess a characteristic (2) which is required for expression of the dermotropic phenotype.

Several histopathology observations led us to suspect that *C. neoformans* produces proteases capable of degrading the extracellular matrix and/or collagen in the skin. First, skin nodules were caused by large foci of yeasts that replaced collagen bundles in the dermis rather than displacing the overlying tissue. Second, at the edge of lesions, collagen strands appeared to be disorganized and degenerated. Finally, yeast cells were observed adhering to collagen bundles. The capacity of *C. neoformans* SLHA to liquify gelatin indicates that this strain may produce proteases capable of degrading collagen and/or other components of the extracellular matrix. Eukaryotic proteases capable of degrading extracellular matrix proteins that have been identified to date have been classified into three groups on the basis of their substrate specificity. These include (i) collagenase, which degrades interstitial collagens (types I to III); (ii) type IV collagenase-gelatinase, which degrades collagen types IV, V, and VII, gelatin, and fibronectin; and (iii) stromelysins, which degrade collagen types III, IV, and V, gelatin, fibronectin, laminin, and proteoglycans (reviewed in reference 16). Future work will be directed at further characterizing the substrate specificity of this newly described cryptococcal gelatinase-collagenase.

Numerous studies have indicated that the capsule is a major virulence factor (6, 14). Other putative virulence factors described for *C. neoformans* include growth at 37°C and the ability to produce phenoloxidase (14, 21). Also, in vitro studies have shown that some strains of *C. neoformans* have proteolytic activity for casein and fibrinogen (5, 18). Our studies suggest that gelatinase-collagenase production by *C. neoformans* is also a virulence factor. A unique feature of *C. neoformans* is its capacity to cross the blood-brain

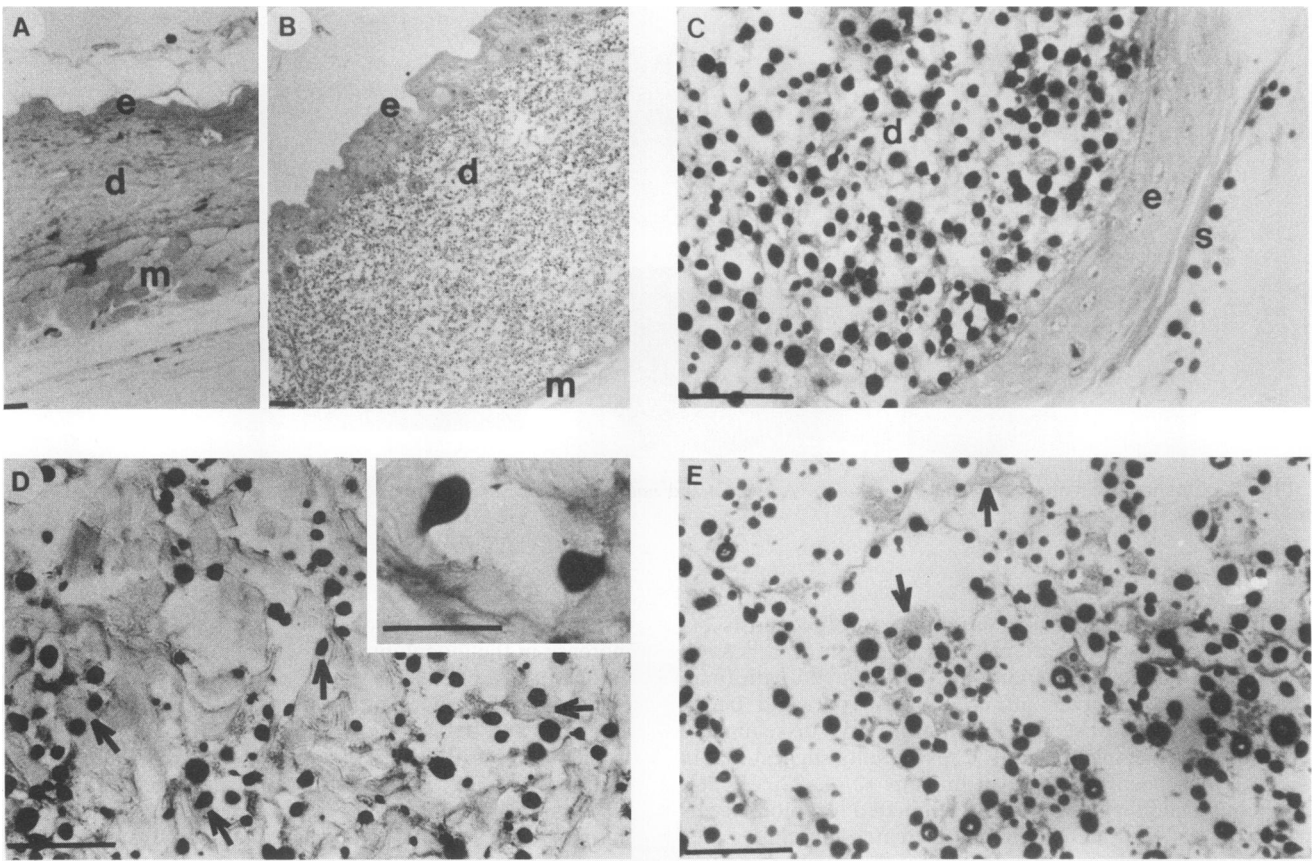

FIG. 2. Histopathology of *nu/nu* and *bg/bg-nu/nu* skin nodules from *C. neoformans*-infected mice. Unless noted, bar = 50 μm. (A) Normal skin, *nu/nu* mouse. (B) Skin nodule, *nu/nu* mouse. Numerous encapsulated yeasts are distributed throughout the dermis. (C) Skin nodule, *nu/nu* mouse. Numerous encapsulated yeasts extend from the basement membrane of the epidermis through the dermis to the underlying musculature. Note yeasts adhering to stratum corneum. (D) Skin nodule, *bg/bg-nu/nu* mouse. Yeasts are in close association with collagen fibers (arrow). Inset: Close-up of yeasts adhering to collagen fibers. Bar = 25 μm. (E) Skin nodule, *bg/bg-nu/nu* mouse. Encapsulated yeasts and some macrophages (arrow) are seen in the center of dermal lesions. Yeasts adhere to remaining strands of collagen. e, Epidermis; d, dermis; m, muscle layer; and s, stratum corneum.

barrier and establish foci in the meninges and brain parenchyma. To date, the mechanism by which *C. neoformans* traverses the endothelium of blood vessels is not understood. Numerous investigators have correlated the expression of collagenase and protease activity with tumor invasion and metastasis (12, 28, 29). Type IV collagenase-gelatinase is frequently elevated in human and mouse tumors, and the levels of enzyme production correlated with the metastatic potential of a series of mouse melanoma cells (28). It is possible that in vivo production of gelatinase-collagenase by *C. neoformans* will explain not only its capacity to multiply in the skin and bone but also its ability to traverse the blood-brain barrier. The development of isogeneic strains of *C. neoformans* with single and/or combined mutations in these putative virulence factors is necessary to sort out the role they play in the virulence of *C. neoformans*.

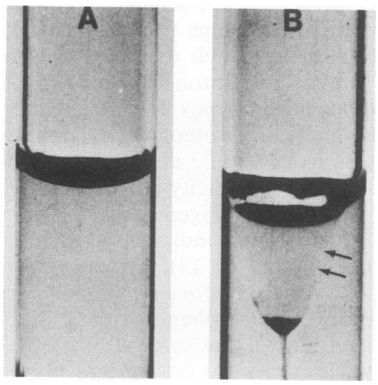

FIG. 3. Gelatin liquification assay for collagenase production. (A) Uninoculated nutrient gelatin. (B) Inoculated with *C. neoformans* SLHA. Arrows denote area of liquified gelatin.

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