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Experimental Hematogenous Candidiasis Caused by *Candida krusei* and *Candida albicans*: Species Differences in Pathogenicity

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Hematogenous infections caused by *Candida krusei* have been noted with increasing frequency, particularly in cancer patients receiving prophylaxis with antifungal triazoles. Progress in understanding the pathogenesis of this emerging infection has been limited by the lack of an animal model. We developed a CF_1 mouse intravenous inoculation model of candidiasis to evaluate the pathogenicity of *C. krusei* in normal and immunosuppressed mice and to compare it with that of *Candida albicans*. Several inocula (10^6 to 10^8 CFU per animal) of two clinical strains of *C. krusei* and three American Type Culture Collection strains of *C. albicans* were tested. Groups of 20 mice each were injected with a single intravenous dose of one inoculum. Animals randomized to receive *C. krusei* were immunosuppressed by intraperitoneal injection of cyclophosphamide or the combination of cyclophosphamide plus cortisone acetate or they did not receive immunosuppressive agents (normal mice). One hundred percent mortality was observed in normal mice injected with 10^6 CFU of *C. albicans* per mouse compared with no mortality in normal mice that received 10^8 CFU of *C. krusei* per mouse ($P < 0.01$). Resistance to *C. krusei* infection was markedly lowered by immunosuppression, particularly by the combination of cyclophosphamide plus cortisone acetate, with a significantly shorter survival and a higher organ fungal burden in immunosuppressed than in normal animals ($P < 0.01$). Tissue infection was documented by culture and histopathologic findings in all examined organs.

There has been a significant increase in life-threatening opportunistic mycoses in immunocompromised patients (1). As a result, there is growing interest in developing new antifungal agents, particularly those of the triazole family. Several agents such as fluconazole, itraconazole, and saperconazole are currently being evaluated (1, 7, 9, 11, 21). These agents have significantly broadened the antifungal spectrum of the imidazoles but have limited or no activity against *Candida krusei* (16). *C. krusei* has been associated with significant morbidity and mortality in immunocompromised patients who have a variable response to amphotericin B (12, 14, 23, 26). Since the newer triazoles are currently used for prophylaxis in cancer patients (9), it is possible that *C. krusei* superinfections may emerge. Little is known about the optimal management of infection with *C. krusei*. We therefore developed a mouse model of disseminated *C. krusei* infection in which the establishment of organ infection and mortality required immunosuppressive therapy and a very large infecting inoculum. This contrasted with the higher and more rapid rate of mortality observed when much smaller inocula of *Candida albicans* were used to infect nonimmunosuppressed mice.

MATERIALS AND METHODS

Organisms. Two randomly selected human isolates of *C. krusei* (2506 and 4935) recovered from the blood of cancer patients cared for at The University of Texas M. D. Anderson Cancer Center were used as infecting inocula for the *C. krusei* model. These isolates were identified with the Vitek System 1860 (Vitek Systems, Inc., Hazelwood, Mo.). In addition, three randomly selected isolates of *C. albicans* (ATCC 64544, 64546, and 64549) were used as inocula for the *C. albicans* control model. All isolates were maintained in water stocks and subcultured onto Sabouraud dextrose agar plates. Before inoculation, cells were incubated overnight at 35°C. Eighteen hours after incubation, suspensions were made with 0.9% sterile saline and adjusted spectrophotometrically to 5% transmittance (Spectronic 20; Bausch & Lomb, Inc., Rochester, N.Y.). Suspensions were inspected microscopically and manually counted undiluted in a hemacytometer. Counts were verified by serial dilution on Sabouraud dextrose agar plates, which were incubated at 35°C for 24 h.

Animals. CF_1 male mice (CF_1-Harland Breeding Laboratories, Indianapolis, Ind.) weighing 25 g were used for all experiments. Normal and immunosuppressed mice were housed in standard cages with corncob bedding and given food and water ad libitum. Five mice were housed per cage.

Infection model. Twenty-four hours before fungal inoculation, one group of mice received one dose of 125 mg of cyclophosphamide per kg (body weight) (Bristol Laboratories, Syracuse, N.Y.) injected intraperitoneally. Another group of mice received subcutaneous injections of cortisone acetate (Merck Sharp & Dohme, Rahway, N.J.) at a dose of 125 mg/kg for 3 consecutive days and cyclophosphamide at the same dosage schedule as indicated above. Blood was collected from the orbital venous plexus.

To verify the induction of neutropenia, leukocytes and differential counts were monitored 48 h after the injection, when the total peripheral leukocyte count fell to approximately 500 cells per ml (from a baseline of around 8,000 cells per ml), with a polymorphonuclear content of less than 10% of the total leukocyte count. Peripheral leukocyte count was monitored daily thereafter until neutrophil recovery (>500

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polymorphonuclear leukocytes per ml). Mice were neutropenic for 6 days. Animals were not given antibacterial antibiotics because of the short duration of neutropenia, which is unlikely to be associated with bacterial superinfection in this mouse strain. One dose of 10^8 CFU of either *C. krusei* 2506 or 4935 per mouse was inoculated into the lateral tail veins of groups of 20 normal (nonimmunosuppressed) mice or 20 mice immunosuppressed with either cyclophosphamide or a combination of cyclophosphamide plus cortisone acetate. Similar groups of mice were injected with the same dose of immunosuppressive agents but did not receive an infectious inoculum and thus served as controls. One dose of 10^6 CFU of *C. albicans* (either ATCC 64544, 64546, or 64549) per mouse was inoculated into the lateral tail veins of groups of 20 normal mice. All animals were monitored for 30 days, and mortality was recorded. An autopsy was performed on approximately 20% of randomly selected animals that died, and cultures of various organs (liver, spleen, kidneys, lungs, brain, heart) were obtained on blood agar and Sabouraud dextrose agar plates and incubated at 35°C. No attempt was made to quantitate fungi in these organs.

Organ clearance. Two groups of 20 mice each were studied, namely, a normal group and a group immunosuppressed by both cyclophosphamide and cortisone acetate as previously described. Mice were inoculated intravenously with 10^8 spores of *C. krusei* 2506. On days 2, 6, and 8, five mice per experimental group were selected at random and killed by cervical dislocation. The kidneys, liver, and lungs of each of these animals were removed aseptically, transferred into sterile polyethylene bags, and homogenized in 5 ml of sterile saline. Samples were removed from each homogenate, serially diluted in 0.9% NaCl, plated onto Sabouraud dextrose agar plates, and incubated at 35°C for 48 h. The colonies were then enumerated, and CFUs were calculated per gram of each organ. All experiments were performed in duplicate.

Histopathology. Representative formalin-fixed portions of kidneys, liver, spleen, lungs, heart, and brain obtained at autopsy were stained with hematoxylin and eosin to detect areas of inflammation and with Gomori methenamine silver stain to search for fungi in all organs of neutropenic mice and in organs with abscesses shown by hematoxylin and eosin stain. Only animals injected with *C. krusei* were examined histopathologically.

Statistical analysis. All differences in survival depending on concentration of inoculum administered and the presence or absence of immunosuppression were analyzed by the Mann-Whitney U test (13). Statistical significance was defined as a P of <0.05.

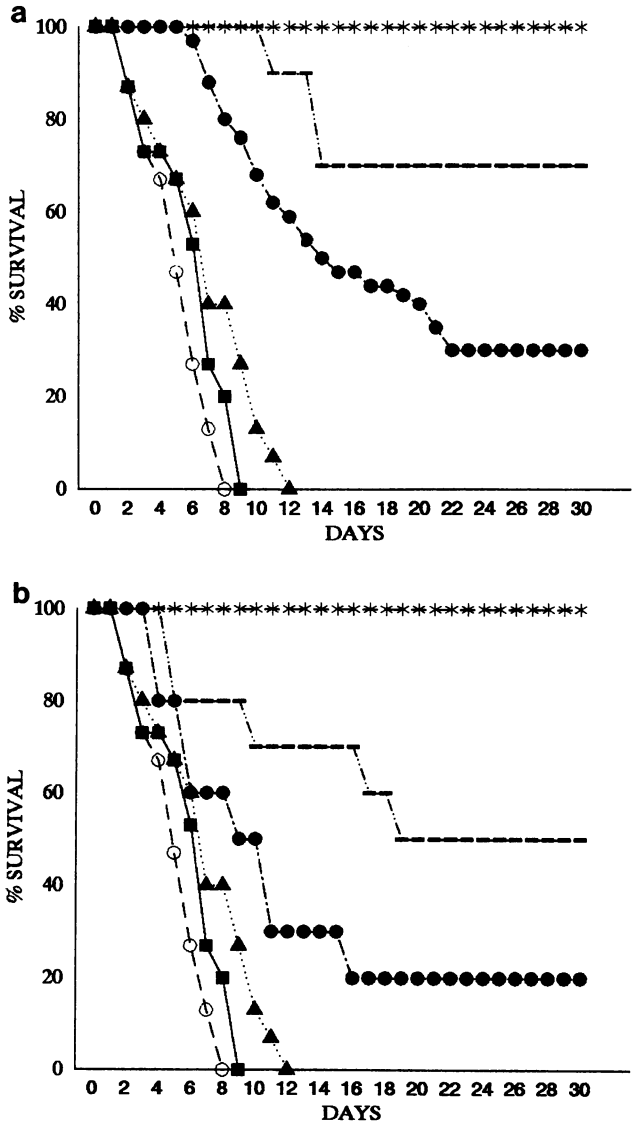

FIG. 1. Mortality studies. Cumulative mortality of lethally infected mice by day after inoculation for three strains of *C. albicans* (ATCC 64544 [○], 64546 [■], and 64549 [▲] [10^6 CFU per mouse]) and two strains of *C. krusei* (*, — ·· —, ●) (strains 2506 [a] and 4935 [b] [10^8 CFU per mouse]). Animals inoculated with *C. krusei* were either normal (*) or were immunosuppressed with cyclophosphamide alone (125 mg/kg once intraperitoneally) (— ·· —) or with a combination of cyclophosphamide at the same dose and cortisone acetate given subcutaneously at a dose of 125 mg/kg for 3 consecutive days (●).

RESULTS

Experimental infection. Survival was significantly shorter for nonimmunosuppressed mice infected with *C. albicans* (100% mortality by day 8 to 12 for all three strains) than for normal mice infected with *C. krusei* (no mortality) ($P < 0.05$) or immunosuppressed mice infected with *C. krusei* (30 to 80% mortality by day 30) ($P < 0.05$) (Fig. 1). A 100% survival rate was noted for control animals that received immunosuppressive agents but no fungal inoculation. Animals infected with *C. krusei* and immunosuppressed with cyclophosphamide alone had a 30 to 50% mortality by day 30, which was lower than the 70 to 80% mortality of animals receiving combination immunosuppressive therapy ($P < 0.05$) but higher than that of normal mice inoculated with *C. krusei* ($P < 0.05$) (Fig. 1). Cultures from various organs yielded *C. krusei* as the only microbial pathogen.

Organ clearance. Quantitative cultures taken from organs of normal CF_1 mice infected with *C. krusei* 2506 showed that during the first 6 days of infection, the organisms had disseminated to the various organs that were examined. Subsequently, the fungus gradually cleared from the liver and lungs of normal mice but persisted in their kidneys (Fig. 2). In all three organs, a higher fungal burden was observed in immunosuppressed than in normal animals ($P < 0.01$).

Histopathology. There were numerous collections of fungal organisms in all organs of immunosuppressed mice

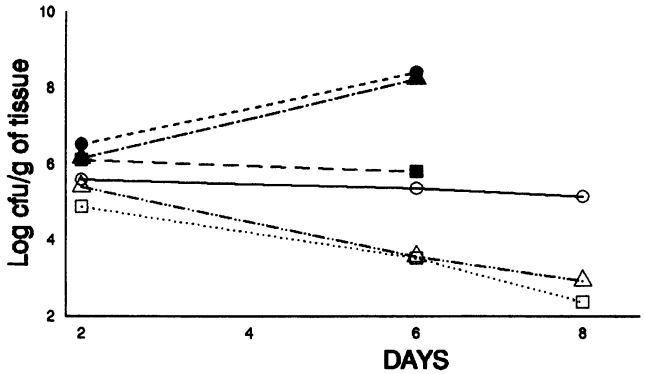

FIG. 2. Effect of immunosuppression on organ clearance of mice infected with *C. krusei* 2506. Mice were inoculated with 10^8 CFU per animal. Twenty-four hours before fungal inoculation, one group of mice was immunosuppressed with an intraperitoneal injection of 125 mg of cyclosphamide per kg and a subcutaneous injection of cortisone acetate at 125 mg/kg (which was continued for 2 additional days). Statistically significant differences were seen between normal and immunosuppressed animals ($P < 0.01$). Symbols: ○, kidney (normal); ●, kidney (immunosuppressed); □, lung (normal); ■, lung (immunosuppressed); △, liver (normal); ▲, liver (immunosuppressed). Results shown represent data accumulated from two experiments with five mice each.

infected with *C. krusei*. However, no inflammatory lesions could be seen (Fig. 3). Except in the kidneys, no fungal lesions could be found in the organs of normal nonimmunosuppressed animals.

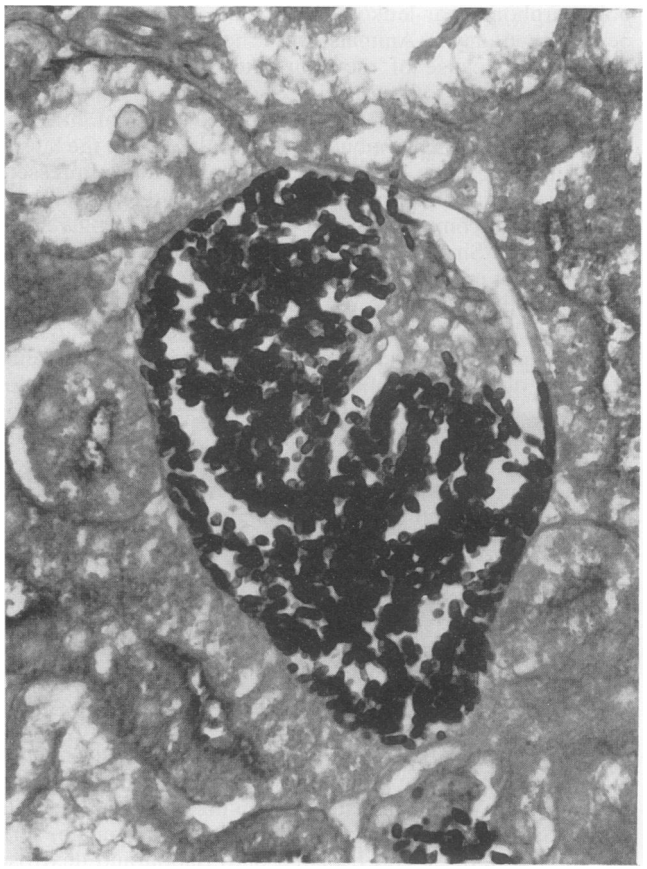

FIG. 3. Response of an immunosuppressed CF_1 mouse 4 days postinoculation with *C. krusei* 2506. Collections of fungal organisms can be seen within a glomerulus. No inflammatory cells were seen. Gomori-methenamine-silver stain; magnification, ×250.

DISCUSSION

We established a simple and reproducible murine model of disseminated *C. krusei* infection and demonstrated the importance of immunosuppression in the pathogenesis of this infection. A combination of a large inoculum and immunosuppression was needed to establish severe infection. The immunosuppressive therapy used in this model could lead to various defects in the murine immune system. Cyclophosphamide causes transient neutropenia and monocytopenia (2), marked decreases in spleen weight and cellularity, and depletion of B lymphocytes (24) and helper and suppressor T lymphocytes (15, 20). The administration of cortisone acetate to CF_1 mice has been associated with splenic, lymphatic, and thymic atrophy (8, 22); leukopenia, monocytopenia, and lymphopenia (2, 6); and macrophage dysfunction leading to a decrease in phagocytic activity (10). The need for combination immunosuppressive therapy to achieve greater than 50% mortality underlines the low pathogenicity of *C. krusei*. In contrast, a much smaller inoculum of *C. albicans* was sufficient by itself and without immunosuppression to induce 100% mortality and a much shorter survival time. The multiorgan involvement by *C. krusei* documented by both culture and histopathology reflects the hematogenous dissemination frequently observed in humans (14). The risk factors for hematogenous *C. krusei* infection include the use of broad-spectrum antibacterial agents and severe immunosuppression (14). It is also possible that antifungal prophylaxis with fluconazole is a risk factor for infection (26). However, other factors such as nosocomial transmission of *C. krusei* may have accounted for the apparent increase in superinfections with this yeast in patients receiving prophylaxis with triazoles (25a). The shorter survival and higher fungal burden noted in immunosuppressed animals suggest a potential role for neutrophils and macrophages as defense mechanisms in disseminated *C. krusei* infection. The role of murine phagocytic cells in defending against *C. krusei* has been studied in vitro (25). The most effective candidacidal phagocytes (i.e., polymorphonuclear and bone marrow cells) were able to kill *C. krusei* more promptly and at a significantly lower effector-to-target ratio than they could *C. albicans* or *Candida tropicalis*. In addition, peritoneal resident macrophages and spleen cells showed significant activity against *C. krusei* while they were practically ineffective against *C. albicans* and *C. tropicalis*. On the other hand, bronchoalveolar lavage fluid was not as effective against *C. krusei* as it was against *C. tropicalis* and *Torulopsis glabrata* (17).

The low pathogenicity of *C. krusei* has been previously demonstrated in normal and cyclophosphamide-treated mice (3) and in normal rabbits (5). In these studies, mice were resistant to challenge with *C. krusei* despite receiving increasing doses of cyclophosphamide. Those findings are somewhat different from ours. However, we used different strains of mice, different *C. krusei* isolates, a larger infecting inoculum, and combination immunosuppressive therapy with both cortisone acetate and cyclophosphamide. These differences in experimental design may explain the differences in results obtained by other investigators. A relative resistance of rabbit tissues to disseminated infection with *C. krusei* was also demonstrated in other studies (5). However,

no immunosuppression was used in the latter model. However, *C. krusei* was found to cause kidney and liver infection when injected intravenously into outbred male Sprague-Dawley rats (7).

In conclusion, a mouse model of disseminated *C. krusei* infection with an immunosuppression-dependent survival was established with two clinical isolates. The low pathogenicity of *C. krusei* contrasted with the high pathogenicity of *C. albicans*. Potential applications of this animal model include testing of novel antifungal agents and studying the mechanisms of disease production and the role of the immune system in protecting against this infection.

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