TISSUE RESPONSES TO THE BLASTOSPORES AND HYPHAE OF CANDIDA ALBICANS IN THE MOUSE

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SUMMARY. Separate groups of mice challenged intravenously with either the blastospore or hyphal forms of the same strain of Candida albicans were examined for comparative mortality rates, organ localisation, and tissue and cellular response to the organisms. Blastospores were more rapidly and consistently fatal to mice than the hyphae. Relatively more hyphal elements than blastospores were initially localised in the lungs but more blastospores than hyphal elements were trapped in the liver. The cells of both forms were more effectively killed in the lungs than in other organs. Blastospores initially found in the kidneys increased rapidly in numbers, but hyphal inocula either grew slowly in the kidneys or were eliminated. After mice were challenged with either hyphae or blastospores the initial inflammatory response in the lungs and liver was predominantly of polymorphonuclear leukocytes, but macrophages were the first inflammatory cells to be seen in kidney sections. Peripheral blood counts showed a leukocytosis in mice of both groups although only blastospores resulted in increased numbers of circulating atypical lymphocytes. The results indicated that lungs may play a more important role than other reticuloendothelial organs in innate resistance to vascular invasion by either of the morphological forms of C. albicans, and macrophages may be crucial to host resistance to renal invasion by this fungus.

INTRODUCTION

The relative pathogenicity of the blastospore and filamentous forms of Candida albicans is not clear because both are commonly found in the infected tissues of humans and experimental animals (Hill and Gebhardt, 1956; Louria, Brayton and Finkel, 1962–63; Winblad, 1975; Parker, McCloskey and Knauer, 1976). Some workers (Young, 1958; Gresham and Whittle, 1961; Louria et al., 1962–63; Saltarelli, Gentile and Mancuso, 1975) have observed that yeast to filamentous transformation precedes the invasive process and postulated that the hyphal form is the more virulent. Other evidence suggests the yeast phase is more virulent (Winsten and Murray, 1956; Simonetti and Strippoli,

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1973; Mardon, Gunn and Robinette, 1975; Evans and Mardon, 1977) but the manner in which blastospores may be more effective in initiating fatal infections has not been explained.

Nonspecific defence mechanisms may be critical in host resistance to systemic candidiasis because neither circulating antibodies nor thymus-dependent cell-mediated immunity has been shown to confer protection (Taschdjian et al., 1969; Rogers, Balish and Manning, 1976). The reticuloendothelial system is an important component of innate resistance to disseminating micro-organisms and a difference in virulence between the two forms of C. albicans may be related, in part, to the role of the reticuloendothelial organs in eliminating the fungus from the infected host. Previously, Evans and Mardon (1977), using two strains of C. albicans, reported that yeast and pseudohyphal cells showed different patterns of localisation in the organs of the mouse. The present studies were undertaken to investigate the comparative roles of reticuloendothelial organs in host resistance to the blastospore and hyphal forms of the same strain of C. albicans and to characterise renal infections and inflammatory responses in mice challenged with the different morphological forms of the fungus.

MATERIALS AND METHODS

MICE. Male AKR/J mice (Jackson Laboratories, Bar Harbor, Maine), 6–8 weeks old at the start of each experiment, were used throughout. They were fed water and standard laboratory pellets (Ralston Purina Co., St Louis, Mo) ad libitum and were allowed to adjust to their new environment for 1 week before the start of an experiment. Room temperature was maintained at 24°C and 12 h of light followed by 12 h of darkness were allowed during each 24-h period.

ORGANISM AND CULTURE CONDITIONS. C. albicans strain ATCC10261 was maintained, with monthly transfers, on Sabouraud's dextrose-agar slants. Blastospore and hyphal suspensions of the organism were prepared by the method of Mardon et al. (1975). Briefly, minimal glucose-salts-biotin (GSB) media supplemented with amino acids were seeded with 22-h-old blastospores and incubated on a gyratory water-bath shaker at 37°C for 6 h. When the cultures were incubated in an oxygen-enriched atmosphere the resulting growth consisted of more than 90% blastospores. When the cultures were incubated in a CO₂-enriched atmosphere more than 95% of the blastospore inoculum germinated and the resulting hyphae grew to a mean length of 33 μm within the 6-h incubation period. The cultures were harvested on membrane filters (0.45 μm porosity, Millipore Corp., Bedford, Mass) and washed with sterile distilled water.

MORTALITY RATES OF MICE. Either blastospores or hyphal cells were suspended in sterile 0·85% nonpyrogenic saline. In one set of experiments blastospore and hyphal suspensions were adjusted to equivalent turbidities resulting in an equivalent cell-mass content of the suspensions (Mardon et al., 1975). In another set of experiments all suspensions were adjusted to contain 3 × 10⁵ colony-forming units (cfu) of either blastospores or hyphal cells in each 0·5 ml as determined by plate counts on Mycosel Agar (BBL, Cockeysville, Md). In these latter experiments the cell-mass content of hyphal suspensions was approximately four times that of blastospore suspensions as determined by dry-weight measurements. Each mouse was inoculated via the lateral tail vein with 0·5 ml of either a blastospore or hyphal suspension. Mice were observed daily and mortalities recorded. Infections were confirmed by recovering C. albicans in cultures of organs excised from dead animals.

ORGAN LOCALISATION. Blastospore or hyphal cultures grown for 6 h were harvested on membrane filters, washed and suspended in sterile distilled water. Eight ml of GSB medium, containing no glucose or amino acids but supplemented with 25 μCi of D-[U-¹⁴C]-glucose (specific activity 284 mCi/mmol; New England Nuclear, Boston, Mass), were seeded with either...
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blastospores or hyphal cells to a turbidity of 50 Klett units in a Klett–Summerson colorimeter (Model 800-3, Klett Mfg Co., New York, NY) equipped with a red filter. This turbidity corresponded to about $9 \times 10^6$ blastospore cfu/ml and $2.3 \times 10^6$ hyphal cfu/ml. The cultures were placed in 25-ml Erlenmeyer flasks and the cells were pulse labelled at $37^\circ C$ for 30 min followed by the addition to each flask of 10 ml of GSB medium containing unlabelled glucose. After incubation for an additional 5 min, the cells were harvested on membrane filters, washed three times with 15-ml volumes of sterile distilled water and suspended in sterile nonpyrogenic saline. Suspensions pulse labelled in this manner showed negligible loss of radioactivity after treatment with cold 5% trichloroacetic acid, indicating that the $^{14}C$ had been incorporated into cellular material. Microscopy showed that there was no alteration in cellular morphology during the labelling procedure and colony counts after incubation of the preparations on Mycosel Agar indicated no measurable loss in viability.

All suspensions were adjusted to a turbidity of 35 Klett units, then diluted 1 in 4 with sterile 0.85% nonpyrogenic saline. Each dose consisted of approximately $7 \times 10^5$ blastospore cfu giving about $2 \times 10^5$ cpm or $2 \times 10^6$ hyphal cfu giving about $2 \times 10^5$ cpm.

Localisation assays were performed as previously described (Evans and Mardon, 1977) at specified intervals from 30 s to 144 h after injection. Replicate blood samples were collected from the retro-orbital plexus, mice were killed by cervical dislocation and liver, lungs, spleen, kidneys, brain, heart, thymus, and lymph nodes (axillary, inguinal, and mesenteric) were excised and weighed. Localisation was determined by the number of cpm recovered from blood or homogenised organs and data were expressed as percentage of the original inoculum present in each organ or ml of blood. Viable cfu were determined by spreading serial dilutions of organ homogenates on Mycosel Agar and counting the colonies that appeared after incubation at $37^\circ C$ for 48 h.

Histopathology. Randomly chosen mice from each group were killed by cervical dislocation at specified intervals from 30 s to 72 h after injection. Liver, lungs, spleen, and kidneys were excised, fixed in buffered 10% (v/v) formalin, and embedded in paraffin. Tissue sections were cut to a thickness of 6–7 μm and stained with haematoxylin and eosin (HE), periodic acid Schiff (PAS), or Gomori’s methenamine silver (GMS).

Peripheral blood leukocytes. Tail blood was taken from mice before injection, 12 h after injection, and daily thereafter until the animal died from infection. With the exception of 12-h samples, all blood was collected between 0900 and 1100 h each day. Blood smears were made and samples were collected in heparinised capillary pipettes (Drummond Scientific Co., Broomall, Pa) from candida-infected mice, and from control mice after intravenous injection of 0.5 ml of nonpyrogenic saline. Samples were diluted, erythrocytes lysed, and total leukocytes counted with an electronic particle counter (Coulter Counter, Model ZBI, Coulter Electronics Inc., Hialeah, Fla). Differential leukocyte counts were performed by classifying 200 white blood cells on each blood smear stained with Wright’s stain.

Statistical methods. The confidence level for all experiments was set at 95%. The Student $t$-test (two-tailed) was used to determine significances of differences between groups.

RESULTS

Mortality rates

When cell suspensions of each form were adjusted to the same turbidity and injected into mice, animals that survived challenge with the hyphal inoculum lived significantly longer than those receiving blastospores (table). Although blastospore and hyphal suspensions of comparable turbidities contained comparable amounts of cellular material in terms of dry weight, they contained different concentrations of colony-forming units, which could account for the different survival rates between the two groups of mice. The survival times of
animals given equivalent doses of blastospores and hyphal elements were therefore compared, and again those challenged with the hyphal preparation survived significantly longer (table).

**Organ localisation**

Less than 10% of either of the radioactive inocula remained in the blood at 30 min after injection and increased levels of radioactivity were not detected in the blood throughout the experimental period. The lungs and liver cleared more organisms from the bloodstream than did other organs (fig. 1). However, as shown in figs. 1A and 1B, significantly greater percentages of the hyphal than the blastospore inoculum initially localised in the lungs ($p < 0.05$ at 0.01 h) while significantly more blastospores were cleared from the blood by the liver ($p < 0.05$ at 0.01 h). Within 30 min after injection the number of blastospores remaining in the lungs was greatly reduced and this reduction was accompanied by a concomitant increase of the number in the liver. Few cells of either morphology localised in the spleen or kidneys (figs. 1C, 1D and 1E). Quantitative estimates of ¹⁴C indicated that only very low percentages of either inoculum were found in brain, heart, thymus or lymph nodes at any time after challenge.

The number of viable units that accumulated per mg of various tissues was determined to ascertain the relative effects of the different tissues on viable organisms. Lung tissue was highly effective in trapping and eliminating viable elements of either morphology and liver tissue was least effective (figs. 2A and 2B); neither lung nor liver tissue supported the growth of *Candida*. Spleen tissue was moderately efficient in killing viable blastospores until 72 h after challenge. Kidneys did not eliminate or limit the growth of blastospores, which began to multiply there between 6 and 12 h after injection (fig. 2A) with both kidneys developing infections of equal severity. However, growth of the hyphal inoculum in kidney tissues was delayed until 12-24 h after challenge and failed to reach the amount of growth found after the blastospore challenge (fig. 2B). Furthermore, after injection of hyphal suspensions, renal infections were asymmetric but without marked predilection for either left or right kidney. When brain, heart, thymus, and lymph nodes were examined,

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Dilution*</th>
<th>Dose (cfu)</th>
<th>Mortality (Dead/total)</th>
<th>Survival time (Mean days ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blastospore</td>
<td>1 in 4</td>
<td>$7 \times 10^5$</td>
<td>21/21</td>
<td>4.9 ± 0.3</td>
</tr>
<tr>
<td>1 in 10</td>
<td>$3 \times 10^5$</td>
<td>16/16</td>
<td>8.0 ± 0.3</td>
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<tr>
<td>Hyphal</td>
<td>1 in 4</td>
<td>$2 \times 10^5$</td>
<td>10/12†</td>
<td>22.5 ± 4.8</td>
</tr>
<tr>
<td>1 in 2:5</td>
<td>$3 \times 10^5$</td>
<td>12/17†</td>
<td>21.6 ± 5.0</td>
<td></td>
</tr>
</tbody>
</table>

* Each suspension was adjusted to a turbidity of 35 Klett units before dilution.
† The hyphal inoculum was not consistently fatal within a 270-day observation period.
increasing numbers of viable units were detected within 48 h after the injection of blastospores but growth of the hyphal inoculum was not detected in these organs or in spleen at any time during the 144-h experimental period.

*Peripheral blood leukocytes.* The mean blood leukocyte count of saline-injected control mice ranged from 6500 ± 200 to 10 300 ± 500/μl throughout a 21-day experimental period. Mice challenged with either blastospores or
FIG. 2.—Colony-forming units per mg of tissue recovered from mice after intravenous injection of (A) blastospores and (B) hyphal suspensions of C. albicans. Means ± SEM from 3 mice. ○—○ = Lung; □—□ = liver; ●—● = spleen; ■—■ = kidney.
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Hyphae developed a leukocytosis although the total leukocyte increase was greater in mice challenged with blastospores. Differential leukocyte counts showed that in both instances the leukocytosis was due primarily to increased numbers of polymorphonuclear (PMN) neutrophils (fig. 3A). Mice challenged with blastospores developed a lymphocytopenia when compared with the saline-treated control group (p < 0.05 at 96 h) but animals that received the hyphal preparations had total lymphocyte counts that were significantly higher (p < 0.01) than those of control mice at 2 and 3 weeks after injection (fig. 3B). Hyphal preparations resulted in a significant monocytosis within 12 h after injection (p < 0.001) but a similar increase in peripheral blood monocytes was delayed until 48 h after blastospores were injected (fig. 3C). The most remarkable observation concerning circulating leukocytes was the highly significant (p < 0.001) increase in numbers of atypical lymphocytes after injection of blastospores but not hyphal suspensions (fig. 3D). Cells classified as atypical lymphocytes were those showing lymphoblast morphology and lymphocytes containing two or more nuclei.

Histopathology after blastospore challenge. Within 30 s after injection, the cells were seen in alveolar capillaries, the portal vein, splenic sinuses, and renal interstitial tissues. After 1 h a few blastospores also appeared in hepatic sinusoids as well as in renal glomeruli and cortical tubules; blastospores were first seen in renal medullary tubules at 2 h after injection. Small foci of inflammation were first detectable at 3–6 h in HE-stained sections and the blastospores in the lungs and liver were accompanied by an inflammatory response of mainly PMN leukocytes. Blastospores were eliminated from the lungs without germinating whereas those that localised in the spleen and liver formed germ tubes within 3 to 6 h but failed to develop into long filaments in these organs during the experimental period (fig. 4).

Kidney sections showed that within 6 h many of the blastospores in the cortical areas had developed germ tubes, and the small inflammatory foci in the renal cortex consisted primarily of macrophages (fig. 5). A progressive renal infection ensued and within 24 h the elongating filaments produced budding cells and extended into the tubular lumen. Micro-abscesses in the kidney developed primarily in the cortex although some lesions were observed in the medulla and niduses of fungal growth were seen at the base of renal pyramids within 48 h. The inflammatory response in the kidney became increasingly intense and at 24 and 48 h was one of mixed mononuclear and PMN phagocytes. Terminal lesions in the kidney consisted of central areas containing microcolonies of fungal growth but few inflammatory cells and surrounded by peripheral areas of mixed infiltrates consisting of macrophages, PMN leukocytes, and lymphocytes.

Histopathology after challenge with hyphal suspensions. There were many hyphal elements in the alveolar capillaries within 30 s of injection (fig. 6), but these appear to have been eliminated without a detectable inflammatory infiltrate, and the fungus could no longer be seen in lung sections by 24 h. Hyphal fragments that localised in liver or spleen were not readily eliminated and after 48 h blastospores budding from hyphal elements were observed in
FIG. 3. Legends at foot of next page.
FIG. 4.—Organisms in liver of mouse 72 h after intravenous injection of *C. albicans* blastospore suspension. Periodic acid-Schiff. × 360.

FIG. 5.—Macrophages in cortex of mouse kidney 6 h after intravenous injection of blastospores of *C. albicans*. Haematoxylin and eosin. × 780.
Fig. 6.—Lung 30 s after intravenous injection of hyphal suspension, showing organism in alveolus. Gomori's methenamine silver. × 360.

Fig. 7.—Kidney 72 h after intravenous injection of hyphal suspension showing large nidus of fungal growth in renal calyx. Gomori's methenamine silver. × 80.
those organs. An inflammatory response was first evident in the liver at 12 h and consisted of predominantly PMN leukocytes.

Hyphal elements that localised in the kidney were seen in the cortical interstitium initially and later in cortical and medullary tubules where they elongated. Blastospores budding from hyphae in the kidneys were first observed at 12 h after injection, and by 48 h yeast cells were seen in lung sections of mice challenged with the hyphal suspension. The inflammatory response to growth of the hyphal inoculum in the renal cortex was not observed until after 12 h and consisted initially of mononuclear phagocytes, but by 72 h many PMN also were present. In contrast to infections initiated by blastospores in which fungal growth occurred primarily in the renal cortex, the hyphal inoculum resulted in a few foci of infection in the cortex and medulla but large bezoars of fungal growth were seen in the renal calyx (fig. 7).

**DISCUSSION**

These studies confirm that blastospores are more virulent for mice than hyphal forms grown from the same strain of *C. albicans* and the greater virulence of blastospores was shown to be independent of cellular mass or viable units. This greater virulence may reside, in part, in less effective host defence mechanisms against the yeast phase of *C. albicans*. Differing cell-wall composition between the two growth forms has been reported (Chattaway, Holmes and Barlow, 1968; Braun and Calderone, 1978; White et al., 1979) and may have a bearing on variations in pathogenicity. Although the structure of the cell wall in *C. albicans* has not yet been fully elucidated, the total thickness of germ-tube walls has been found to be approximately one-half that of the walls of the blastospore (Cassone et al., 1973). In addition, differences in protein and polysaccharide content between the two forms together with differences in digestion by β-glucanase indicate that considerable differences are likely to be present in the tertiary structure of the cells walls (Chattaway et al., 1968). Differences in structural integrity could have an influence on whether host defence mechanisms are more effective against the hyphal than the blastospore phase. To elucidate further the differential virulence of the two forms of *C. albicans*, the in-vivo mechanisms by which the host deals with the dissemination of this opportunistic pathogen have been examined.

The most critical organ in vascular clearance should be the first encountered by the fungi after their entry into the bloodstream. Baine, Koenig and Goodman (1974) reported evidence to this effect and showed that *C. albicans* yeast cells injected into a peripheral vein were cleared from the blood predominantly by the lungs but those injected into a mesenteric vein were cleared largely by the liver. In the present investigations both morphological forms showed substantial trapping by lung tissue immediately after injection.

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**FIG. 3.**—Leukocytes in peripheral blood of mice after intravenous injection of nonpyrogenic saline or suspensions of *C. albicans*. (A) Neutrophils; (B) total lymphocytes; (C) monocytes; (D) atypical lymphocytes. Means ± SEM from five mice. ○—○ = Blastospore inoculum; ●—● = hyphal inoculum; □—□ = saline.
into a peripheral vein, but a greater percentage of hyphal elements localised initially in the lungs whereas blastospores localised initially in the liver. 

Particle size as well as route of injection has a bearing on initial organ localisation. Munson, Regelson and Woolles (1970) showed that after intravenous injection into mice, pulmonary uptake of sheep erythrocytes, which have a mean particle diameter of 5.5 µm, was much greater than that of a lipid emulsion which had a mean particle diameter of less than 1 µm. In the present studies, the filamentous forms, because of their larger size, may have been more readily trapped in the lung, the first organ encountered after intravenous injection. More blastospores appear to have passed through the alveolar capillaries to be trapped in other organs. The data (fig. 1) suggest this sequence of events. Furthermore, radioactive and viable-unit data considered together indicate that after the initial movement of candida cells from lungs to other organs, there was little relocation of the original inoculum. Trapping of most candida cells within various organs appeared to be essentially complete and irreversible within 1 h after injection. This finding is contrary to the suggestion of Stanley and Hurley (1969) that reticuloendothelial cells may potentiate candida infections by acting as vectors in its dissemination. If reticuloendothelial cells do sequester candida cells and transport them to other organs, this cannot be in appreciable numbers.

Lung tissue was more effective than other tissues in destroying both morphological forms of the fungus, and the less virulent hyphal form localised mainly in the lungs. Lung tissues also appeared to filter out organisms that reached the bloodstream after the challenge dose had multiplied in other organs because yeast cells were seen in lung sections of mice at 48 h after injection of hyphal suspensions. The effective candidacidal activity of lung tissue apparently is not restricted to the mouse because Damodaran and Chakravarty (1973) reported that lungs of rabbits given intratracheal injection of the yeast cells of C. albicans are resistant to infection. If the effectiveness of lung tissue in killing C. albicans is a general phenomenon, this might partially explain observations that the organism rarely produces primary pulmonary infections in the human host (Rippon, 1974).

Liver tissue was least effective of the reticuloendothelial organs in killing organisms of either morphology but, due to the greater mass of the liver in comparison with other parenchymal organs, it accounted for substantial bloodstream clearance. Hurtrel, Lagrange and Michel (1980a) also found that the number of C. albicans reaching the liver of mice remained fairly stable for 3 days after injection of yeast cells.

Injection of blastospores into mice elicited a prompt inflammatory response of PMN leukocytes in liver and lung tissues. By contrast, injection of hyphal cells resulted in a minimal infiltrate of PMN cells in the lung and liver which was not detectable until 12–24 h. These results are consistent with the in-vitro studies of others (Denning and Davies, 1973; Weeks et al., 1976) in which yeast cells were more highly chemotactic for PMN neutrophils than filamentous preparations.

The kidney has been identified as the organ most severely affected during
human candidiasis (Louria, Stiff and Bennett, 1962; Lehner, 1964; Parker et al., 1976) as well as in experimental infections (Louria et al., 1962–63; Winblad, 1975; Rogers and Balish, 1976; Hurtrel et al., 1980a) and the findings in the present investigations confirm this view although growth of Candida was more extensive in mice challenged with blastospores. The initial inflammatory response in kidneys was qualitatively different from that seen in other tissues. Although some PMN leukocytes were present, the earliest cellular response in the kidneys to challenge with either morphological phase consisted primarily of macrophages. Other investigators (Louria et al., 1962–63; Winblad, 1975; Rogers and Balish, 1977) have reported renal infiltrates of predominantly PMN leukocytes in experimental animals but these observations were made 24 h or more after challenge with C. albicans blastospores.

Analysis of peripheral blood leukocytes after blastospore challenge showed that the greatest increase in neutrophils occurred at the time of micro-abscess formation in the kidneys and thus may reflect the quantitative recruitment of these cells in response to progressive infections. A sharp increase in peripheral-blood monocytes was observed soon after injection of hyphal suspensions. Such a response was also seen within 48–72 h after injection of blastospores when these organisms had germinated and produced filaments in the kidneys. It is tempting to speculate that this monocytosis could reflect a general mobilisation of mononuclear phagocytes by hyphae because many macrophages were seen in kidneys, the organs showing the most extensive growth of C. albicans. A profound increase in atypical lymphocytes was observed in the blood within 12 h after mice were challenged with blastospores but not after hyphal challenge. Further experiments are planned to determine the origin and significance of these cells.

Polymorphonuclear leukocytes and macrophages are known to participate in host resistance to systemic infection with Candida but the relative importance of these phagocytic cells is not clear. Evidence is available that PMN neutrophils kill yeast cells (Lehrer and Cline, 1969) and damage pseudohyphae (Diamond, Krzesicki and Wellington, 1978) and PMN leukocytes are often considered to be of primary importance in resistance to candidiasis (Rogers and Balish, 1977; Hurtrel et al., 1980b). However, macrophages have been shown to inhibit growth of Candida (Peterson and Calderone, 1977) although some engulfed yeast cells germinate and grow out of macrophages (Ozato and Uesaka, 1974; Arai, Mikami and Yokoyama, 1977). Activated macrophages have been shown to kill a large percentage of ingested candida yeast cells (Maiti, Kumar and Mohapatra, 1980) and Evron (1980) has suggested that macrophages play a protective role in systemic candidiasis. The results reported here indicate that macrophages may be important to host resistance during early stages of renal invasion because macrophages were the first inflammatory cells found in the kidneys after mice were challenged systemically with either morphological form of C. albicans. If macrophages can inhibit initial growth of organisms that reach the kidneys, Candida could subsequently be eliminated from the host by additional resistance mechanisms such as PMN activity and pulmonary destruction of
organisms that enter the bloodstream from infected areas. Such a sequence of events would explain the survival of some animals that were challenged with the hyphal form of *C. albicans*.

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