Inefficiency of In Vivo Candidacidal Mechanisms in Experimental Subcutaneous Infections with *Candida albicans* in Mice

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A murine model of subcutaneous *Candida albicans* infections was used to evaluate host defenses against inocula of from 10^1 to 10^8 yeast cells. In these experiments, small inocula did not produce abscesses that drained to the skin surface, whereas larger ones did. Also, small numbers of organisms often remained at the infected sites for up to 21 days after inoculation with either small or large numbers of organisms. The data from these studies suggest that the in vivo candidacidal mechanisms in these infections are relatively inefficient and that they therefore may require some additional mechanism to control proliferation of the remaining organisms.

Microbicidal activity is generally considered the mechanism by which neutrophils directly participate in the in vivo resolution of infections (1, 7, 13). However, neutrophils have recently been found to have significant in vitro growth-inhibitory capacity against a variety of microorganisms as well (4, 5, 9). This microbistatic activity appears to be distinct from that of macrophages (2) and from the usual microbicidal mechanisms of neutrophils in that it resides in the cytoplasm of these cells (4, 5) and is present in active form in abscess fluid supernatants (10). Since inflammatory cells may sometimes not be able to kill all invading pathogens promptly, we have previously postulated that some form of microbial growth control, such as this microbistatic protein, may be necessary to prevent the remaining organisms from proliferating and overwhelming the host (8). On the other hand, most infections begin with relatively small inocula, so it is possible that the microbicidal activities of infiltrating leukocytes may be able to eliminate the organisms rapidly enough to prevent their proliferation. In the present study we have evaluated in vivo candidacidal activity in experimental subcutaneous *Candida albicans* infections in mice in order to determine for different numbers of infecting organisms whether growth control of unkillable microorganisms might be needed for the defense against tissue infections with relatively small numbers of this organism.

The strain of *C. albicans* used in these studies was originally obtained as a clinical isolate at the Milwaukee VA Medical Center, with the organisms being maintained by repeated subculture on Mycosel agar slants (BBL Microbiology Systems, Cockeysville, Md.). Before use, *C. albicans* yeast cells were removed from slants inoculated 4 to 6 days earlier, washed three times, and then suspended in normal saline at a range of concentrations of 10^1 to 10^8 yeast cells per 0.5 ml (the volume to be injected). The animals used in these studies were C57BL/6 mice obtained from Sasco King Animal Labs (Omaha, Nebr.) and housed in the Milwaukee VA Medical Center Veterinary Medical Unit; this unit is fully approved by the American Association for Accreditation of Laboratory Animal Care. The animals were females and were used at 8 to 13 weeks of age.

Experimental infections were produced as previously de-

scribed (11) by the subcutaneous injection on each side of the upper back of various numbers of *C. albicans* yeast cells suspended in 0.5 ml of saline. Control animals received only saline. The subcutaneous site of material delivered by this technique was confirmed in preliminary experiments by injection of 0.5 ml of a methylene blue solution. The gross appearance of abscesses resulting from subcutaneous inoculation of *C. albicans* yeast cells was examined daily for animals injected with either a small (10^3) or a large (10^8) inoculum. Abscesses were considered intact if they did not show breaks in the skin or signs of leakage of the abscess contents.

Groups of inoculated mice were killed 3, 10, and 21 days after inoculation, at which time the entire abscess, with surrounding skin and underlying muscle, was excised, fixed in 10% buffered formalin, and embedded in paraffin for sectioning. The resulting sections were stained with periodic acid-Schiff stain and examined microscopically to evaluate the morphology of the abscesses and their contents. For each of the inocula studied in this manner, slides from four to six subcutaneous infections were examined for each of the three time points. The numbers of live organisms present in the abscesses were estimated by removing all of the involved subcutaneous tissue under the injection site, grinding it with saline in a mortar with a pestle, and plating appropriate dilutions of the resulting suspensions on neopeptone-glucose agar. The plates were incubated at 37°C, and the resulting colonies were counted 24 h later. The abscess fluids were dispersed in 1 ml of normal saline, and the numbers of leukocytes in the resultant suspensions were determined by counting in a hemacytometer. These specimens did not undergo grinding in a mortar with a pestle as did those used for determination of CFU.

By using the technique described above, injection of *C. albicans* yeast cells produced two separate subcutaneous infections, one over each side of the upper back. Inoculation with at least 10^6 yeast cells per site was required to produce palpable nodules at the site of injection. By microscopic observation, these nodules represented well-circumscribed but irregularly shaped and nonencapsulated abscesses containing both yeast and filamentous cells, as well as a dense infiltrate consisting primarily of neutrophils. In agreement with the gross observations, it was found that inoculum sizes of more than 10^6 yeast cells elicited increased numbers of

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TABLE 1. Numbers of leukocytes present in abscesses

Inoculum size ^a	No. of leukocytes/abscess (10 ⁶) present on ^b :	
	Day 1	Day 4
None (control)	3.0 ± 0.7	3.1 ± 0.7
1	2.5 ± 0.9	2.3 ± 0.5
2	1.5 ± 0.3	3.7 ± 1.0
3	2.8 ± 0.5	3.1 ± 0.7
4	2.5 ± 0.6	6.0 ± 3.3
5	4.2 ± 1.0	7.7 ± 2.4
6	15.1 ± 3.4	49.4 ± 14.8
7	12.0 ± 2.9	44.9 ± 8.2
8	28.7 ± 8.7	94.1 ± 12.0

^a Log₁₀ number of *C. albicans* yeast cells injected.^b Data are means ± standard errors from 8 to 10 abscesses per point, obtained from four to five animals that were each studied on different days.

leukocytes in the abscess fluids, whereas smaller inocula did not (Table 1). Cultures of homogenized tissues from the infected sites generally revealed growth of *C. albicans*, even with the smaller inocula. The numbers of colonies obtained on culture at 1 and 4 days after inoculation generally represented a percentage of the original number of organisms injected, except that proportionately larger numbers of colonies were obtained for the two smaller inocula (Table 2). Colony counts exceeding the number of organisms injected were obtained at day 4 for 5 of the 8 infected sites where 10¹ organisms were injected and for 6 of the 10 sites where 10² organisms were injected.

The subcutaneous infections produced with the larger number of organisms (10⁸) began to drain to the skin surface as early as 5 or 6 days after inoculation; however, those produced with smaller numbers did not show evidence of drainage (Table 3). Drainage of the abscesses continued for some time afterwards, with resolution of the infections apparently occurring by about 14 days after inoculation, as judged by visual inspection. However, when the sites were cultured at 21 days after inoculation, many were still found to contain small numbers of viable organisms (for the inoculum of 10³ organisms, six of eight sites were positive, with a mean ± standard error of 896 ± 762 CFU, compared with the inoculum of 10⁸ organisms, for which four of eight sites were positive, with 732 ± 529 CFU; data were from two sites each in four animals for each dose, with the animals injected on different days).

TABLE 2. Numbers of *C. albicans* CFU present in abscesses

Inoculum size ^a	Log ₁₀ CFU/abscess on ^b :	
	Day 1	Day 4
None (control)	0.0 ± 0.0	0.0 ± 0.0
1	1.2 ± 0.1	2.0 ± 0.3
2	1.8 ± 0.2	2.1 ± 0.3
3	2.2 ± 0.2	2.1 ± 0.1
4	2.6 ± 0.2	2.5 ± 0.2
5	3.9 ± 0.2	3.3 ± 0.2
6	5.3 ± 0.2	4.6 ± 0.2
7	6.1 ± 0.2	5.8 ± 0.2
8	0/24	20/24
8	7.7 ± 0.2	7.8 ± 0.1

^a Log₁₀ number of *C. albicans* yeast cells injected subcutaneously.^b Data are means ± standard errors from 8 to 10 abscesses per point, obtained from four to five animals that were each studied on different days.

TABLE 3. Drainage of abscesses

Day after inoculation	No. of abscesses that drained/no. observed with inoculum of ^a :	
	10 ³ organisms	10 ⁸ organisms
3	0/24	0/24
4	0/24	6/24
5	0/24	12/24
6	0/24	17/24
7	0/24	20/24
8	0/24	20/24
9	0/24	23/24
10	0/24	23/24
13	NE	24/24

^a Data are from two experiments of six animals each with two abscesses being monitored for each animal (total of 24 for each inoculum size). Inoculum sizes are numbers of *C. albicans* yeast cells injected. NE, not examined.

In these studies, an experimental murine model of subcutaneous *C. albicans* infections was used to evaluate the efficiency of in vivo candidacidal mechanisms in the normal host, particularly for situations in which only small numbers of viable organisms are introduced into the tissues. It was found that inocula of 10⁶ organisms or more elicited significant leukocyte infiltration, development of palpable abscesses, and spontaneous drainage of inflammatory exudates containing organisms from the infected tissues, whereas smaller inocula did not. It appears that the chemotactic stimuli in the latter infections were insufficient for abscess formation. Viable organisms were generally found at the infected sites even with small inocula and for up to 21 days after inoculation, indicating that the in vivo candidacidal mechanisms are clearly not capable of rapidly killing the organisms in these infections.

Previous work with this model system has demonstrated significant proliferation of the organisms in both subcutaneous tissues and kidneys in leukopenic animals (11). In addition, *C. albicans* has previously been shown to be capable of significant proliferation in homogenized dermis from normal animals (3), as well as in the intact dermis of leukopenic ones (3, 4). Therefore, with the persistence of viable organisms observed in the present study, one might expect very large numbers of progeny to be produced in the absence of some means to control their growth. Although it is possible that the leukocyte microbicidal processes managed to kill all of these progeny, it would seem more practical for the host to have some way of preventing their development. Indeed, previous studies with this system indicate that proliferation of the injected yeast cells into multicelled filaments does begin in normal animals but then progresses much more slowly than in leukopenic ones (11).

The mechanism by which neutrophils prevent proliferation of microbial cells appears to be entirely separate from the processes used by these cells to kill pathogens. The microbistatic system requires disruption of the cells to release calprotectin, the apparently responsible protein, from their cytoplasm (4, 5, 9, 10, 12). Calprotectin probably functions by depriving microorganisms of the zinc they need for enzymes involved in cell division, although it is also possible that this protein works through a different mechanism and is merely inactivated by zinc (6, 10, 12). In any event, it appears likely that sites of acute inflammation may contain high concentrations of neutrophil cytoplasm and the calprotectin it contains, because of the high numbers of these cells present in such locations and the relatively short

life span of neutrophils in tissues. However, with smaller infections, the number of neutrophils recruited may not be adequate to either kill the infecting organisms or prevent their proliferation, as is suggested from the data concerning small-inoculum infections discussed above. Because of the apparent inefficiency of the *in vivo* candidacidal processes in this system, some mechanism may be needed by normal animals to control the growth of organisms not killed by the microbicidal activities of their inflammatory cells.

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REFERENCES

1. Gallin, J. I. 1988. The neutrophil, p. 737-788. *In* M. Sampter (ed.), Immunological diseases. Little, Brown and Co., Boston.
2. Granger, D. L., J. R. Perfect, and D. I. Durack. 1986. Macrophage-mediated fungistasis *in vitro*: requirements for intracellular and extracellular cytotoxicity. *J. Immunol.* **136**:672-679.
3. Hahn, B. L., and P. G. Sohnle. 1988. Characteristics of dermal invasion in experimental cutaneous candidiasis of leucopenic mice. *J. Invest. Dermatol.* **91**:233-237.
4. McNamara, M. P., J. H. Wiessner, C. Collins-Lech, B. L. Hahn, and P. G. Sohnle. 1988. Neutrophil death as a defence mechanism against *Candida albicans* infections. *Lancet* **ii**:1163-1165.
5. Murthy, A. R., S. S. L. Harwig, K. T. Miyasaki, and R. I. Lehrer. 1990. Fungistatic components of human neutrophils, abstr. 940. Program Abstr. 30th Intersci. Conf. Antimicrob. Agents Chemother. American Society for Microbiology, Washington, D.C.
6. Murthy, A. R., K. T. Miyasaki, and R. I. Lehrer. 1991. Human neutrophil cytosolic factors inhibit *C. albicans* growth. *Clin. Res.* **39**:176A.
7. Root, R. K., and M. S. Cohen. 1981. The microbicidal mechanisms of human neutrophils and eosinophils. *Rev. Infect. Dis.* **3**:565-598.
8. Sohnle, P. G., and C. Collins-Lech. 1990. Comparison of the candidacidal and candidastatic activities of human neutrophils. *Infect. Immun.* **58**:2696-2698.
9. Sohnle, P. G., C. Collins-Lech, and J. H. Wiessner. 1991. Antimicrobial activity of an abundant calcium-binding protein in the cytoplasm of human neutrophils. *J. Infect. Dis.* **163**:187-192.
10. Sohnle, P. G., C. Collins-Lech, and J. H. Wiessner. 1991. The zinc-reversible antimicrobial activity of neutrophil lysates and abscess fluid supernatants. *J. Infect. Dis.* **164**:137-142.
11. Sohnle, P. G., and B. L. Hahn. Inhibition of pseudohyphal growth as a neutrophil-mediated host defense mechanism against experimental deep *Candida albicans* infections in mice. Submitted for publication.
12. Steinbakk, M., C. F. Naess-Andresen, E. Lingaas, I. Dale, P. Brandtzaeg, and M. K. Fagerhol. 1990. Antimicrobial actions of calcium-binding leucocyte L1 protein, calprotectin. *Lancet* **336**:763-765.
13. Thomas, E. L., R. I. Lehrer, and R. F. Rest. 1988. Human neutrophil antimicrobial activity. *Rev. Infect. Dis.* **10**(Suppl. 2):S450-S456.