Acta Derm Venereol (Stockh) 1992; 72: 241–244

The Fate of Individual Organisms During Clearance of Experimental Cutaneous *Candida albicans* Infections in Mice

PETER G. SOHNLE and BETH L. HAHN

Section of Infectious Diseases, Department of Medicine, Medical College of Wisconsin, Milwaukee, and Medical and Research Services, VA Medical Center, Milwaukee, Wisconsin, USA

A mouse model of acute cutaneous *Candida albicans* infections was used to study the manner in which these infections are cleared. Results of histological examination were correlated with determinations of the viability by acridine orange staining of superficial *C. albicans* pseudohyphae retrieved from the surface of the infected skin. The number of organisms retrieved from the skin surface was highest on the third and fourth day after inoculation, a finding which appeared to relate to a loss of Candida foci observed histologically to occur after the second day. Viability was high (approximately 80%) for at least 1–2 days after the organsims were seen histologically to have become associated with neutrophils and extruded from the stratum Malpighi into the stratum corneum; however, at later time points (fourth and fifth day after inoculation), the viability of the retrieved organisms did decline. Pseudohyphae germinated in vitro and applied to the skin of mice were found to be non-viable when retrieved 24 h later. These data suggest that the microbicidal processes of neutrophils may not be required for resolution of these infections. They are most consistent with clearance through an epidermal proliferative response which relocates the infecting organisms to a very superficial site, from which they can be either lost in a viable state, or subjected to killing by other factors at the skin surface. ***Key words: Neutrophils; Epidermal proliferation; Stratum corneum; Viability of pseudohyphae.***

(Accepted December 2, 1991.)

Acta Derm Venereol (Stockh) 1992; 72: 241–244.

P. G. Sohnle, Research Service/151, VA Medical Center, Milwaukee, WI 53295, USA.

Neutrophilic infiltrates in the epidermis represent a major part of the early inflammatory response to acute cutaneous *Candida albicans* infections in humans or experimental animals (1–6); however, in chronic mucocutaneous candidiasis, these infiltrates are rarely found (7). Because neutrophils are generally thought to provide host defense by killing invading pathogens (8, 9), clearance of the infections by these cells might be expected to result in non-viability of the organisms. Proliferation of the epidermis is another local cutaneous response which occurs in both cutaneous candidiasis (10, 11) and dermatophytosis (12–14). Epidermal proliferation could promote removal of organisms from the infected skin when the superficial layers are shed from the surface at an increased rate, and such organisms could still be viable. Each of these two cutaneous responses has been postulated to be important for the defense against superficial fungal infections (10, 12–16).

Delayed hypersensitivity reactions to the infecting organisms seem to relate to the clearance of superficial fungal infections (14, 15, 17). There is some evidence to suggest that this type of immune response may promote clearance through a local enhancement of epidermal proliferation (10, 13, 14).

The present studies were undertaken using a mouse model of cutaneous candidiasis in order to assess the viability of individual organisms at the surface of the infected skin as a way of determining whether the epidermal neutrophilic infiltrates or epidermal proliferation might be most important for the clearance of this type of infection.

MATERIALS AND METHODS

Organisms

The strain of *C. albicans* used in these studies was obtained originally as an isolate from a patient at the Milwaukee VA Medical Center (Milwaukee, WI, USA) and identified by standard laboratory criteria. Organisms for inoculation of the animals were cultured in Sabouraud's dextrose broth at room temperature for 70 h, harvested by centrifugation, and washed with saline before being used. It should be noted that in addition to the strain used in these studies, we have earlier tested a variety of other *C. albicans* strains in this model system and found that different ones produce a similar type of infection, although there are some minor quantitative differences between strains in invasiveness and in the number of infective foci produced (18, 19).

Animals

Inbred C57BL/6 mice obtained from a commercial vendor (Sasco King Laboratories, Omaha, NE, USA) were used in these experiments. The animals were of either sex and were used at 8–13 weeks of age. They were housed in the Milwaukee VA Medical Center Veterinary Medical Unit (which is fully approved by the American Association for Accreditation of Laboratory Animal Care). Groups of 5 mice were studied for each experiment, with the experiments being repeated 4–12 times for each point.

Cutaneous infections

A shaved area of flank skin (approximately 2×3 cm) was inoculated directly with approximately 2.5×10^8 washed *C. albicans* yeast cells that were gently rubbed into the skin with a cotton-tipped swab until the paste of organisms was no longer grossly visible. No dressings were used over the site of inoculation. The sites of infection involved primarily telogen skin. These experiments were all conducted during the same period of time, from the spring to the fall of 1990.

Histologic evaluation

Groups of mice were killed on days 1–5 after inoculation, and 1 cm skin samples were removed, processed for histologic examination, and stained with periodic acid – Schiff stain. In this model system, the infections produce small numbers of *C. albicans* pseudohyphae, invading into the epidermis in localized, isolated foci that are evident microscopically, but usually not grossly. These foci were quantitated as previously described (19); in brief, a focus of infection was defined as a group of *C. albicans* pseudohyphae (containing at least one readily identifiable organism) invading the epidermis at the same site and separated from any other pseudohyphae by at least 50 µm.

To evaluate the skin specimens, the slides were coded and read in a

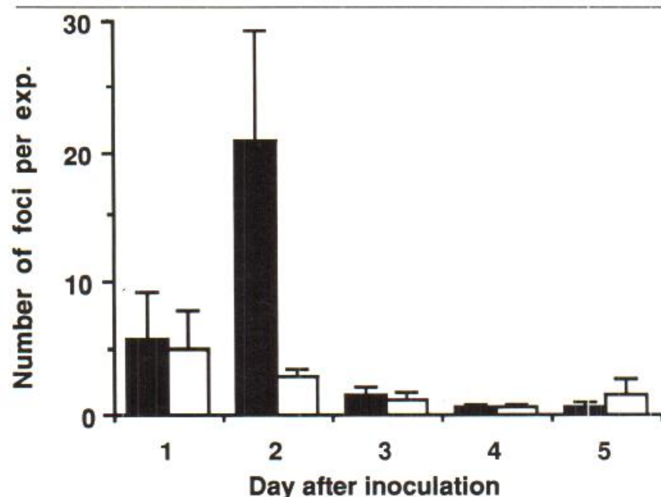

Fig. 1. Numbers of *C. albicans* foci associated with neutrophils (filled bars) or not associated with neutrophils (open bars) in infected skin on days 1 to 5 after inoculation. Note that neutrophils had infiltrated into most of the foci by the second day, and that the total number of foci declined thereafter.

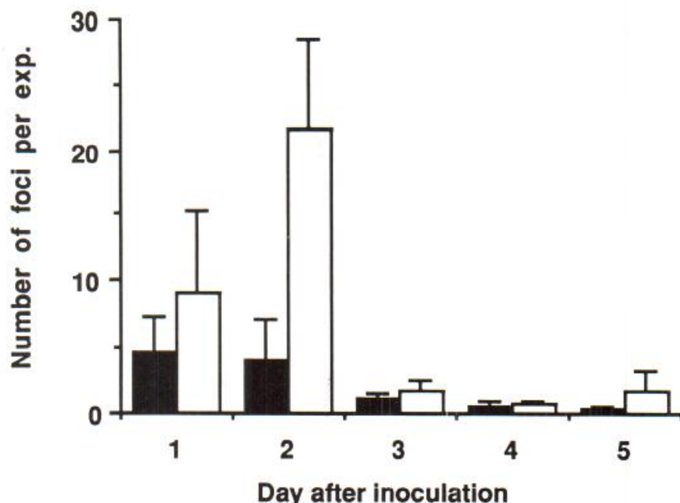

Fig. 2. Numbers of *C. albicans* foci in the cellular layers of the epidermis (filled bars) and in the stratum corneum (open bars) of infected skin on days 1 to 5 after inoculation. Note that by the second day most of the foci were located in the stratum corneum, and that their numbers declined afterwards.

blind manner with the following characteristics recorded for each focus: (a) the number of pseudohyphae present; (b) the location of the organisms in the skin – i.e., in the stratum corneum or in the stratum Malpighi (consisting of the stratum germinativum, the stratum spinosum, and the stratum granulosum); and (c) the presence or absence of neutrophils in the focus. The data were expressed as the mean (± SE) of results of experiments of 5 animals each, with at least 3 experiments being carried out for each time point.

Retrieval of organisms

To obtain organisms from the skin surface for viability studies, the infected skin of anesthetized animals was rubbed gently with a wooden applicator stick, with the material removed being collected on a glass slide. The slide was washed with a solution of 0.1% Triton X 100 in phosphate buffered saline (PBS; 0.02 M, pH 7.4), which was collected in a 6 ml test tube and held at room temperature for 1 h. The organisms were then centrifuged to the bottom of the tube and the supernatant discarded. The pellet was resuspended as discussed below for determination of viability. This retrieval technique yielded small numbers of superficial pseudohyphae which were usually fairly well dispersed.

Viability assay

The acridine orange staining method was used to determine fungal viability. Acridine orange is a fluorochrome that when examined by fluorescence microscopy has a green emission if in contact with double stranded DNA, but an orange-red emission if in contact with denatured or depolymerized DNA (20). In this assay, the organisms from the pellets obtained as discussed above were exposed to 0.14% acridine orange (Sigma Chemical Co., St. Louis, MO) in PBS for 1 min at room temperature; the organisms were then centrifuged to remove most of the solution, with the pellet being resuspended in 100 μl of the remaining fluid. Fifty μl of this material was placed on a microscope slide and examined with a Leitz fluorescence microscope equipped with a blue filter. The number of pseudohyphae on the entire slide was determined, and each one was evaluated for its viability. Organisms which appeared green were considered to be alive, whereas those which appeared orange-red were considered to be dead.

Studies on the effects of drying

In these experiments, 10^7 *C. albicans* pseudohyphae germinated for 3 h in medium 199 were applied to the shaved flank skin of mice, and then retrieved 24 h later using the method described above. In addition, similar quantities were also applied to plastic Petri dishes in distilled water, allowed to dry, and retrieved after being held for 24 h at room temperature. Viability was determined using the acridine orange method.

RESULTS

Histologic examination of the infected skin demonstrated that on the first day after inoculation some of the *C. albicans* foci had not yet been contacted by neutrophils; however, after the second day neutrophils appeared to have infiltrated into most of the foci (Fig. 1). Also, by this time most of the foci were located in the stratum corneum, and their numbers were observed to decrease rapidly between the second and third day after inoculation (Fig. 2). Similarly, values for the total numbers of organisms observed in the infected skin, as determined histologically from the individual counts of organisms in each

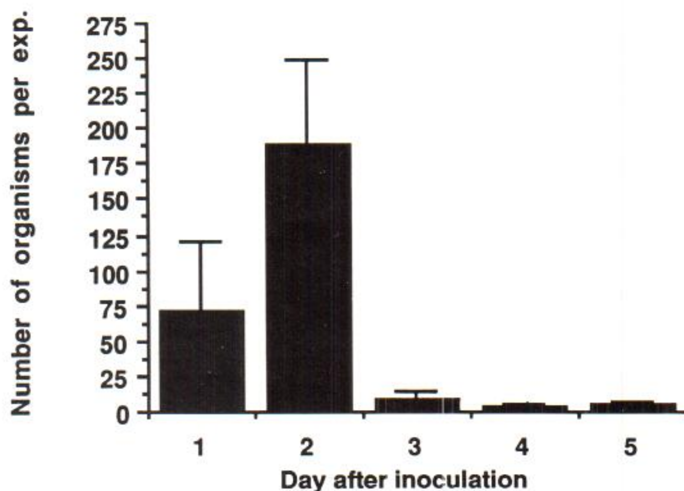

Fig. 3. Total numbers of *C. albicans* pseudohyphae observed histologically in infected skin on days 1 to 5 after inoculation. Note that the number of organisms declined markedly after the second day.

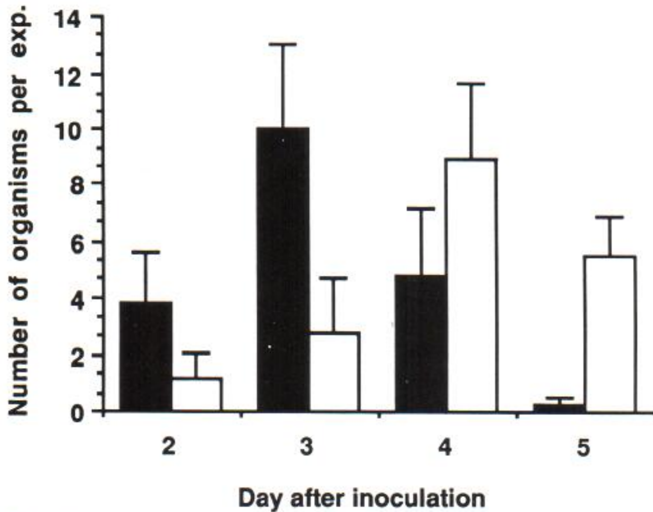

Fig. 4. Viability of organisms retrieved from the infected skin on days 1 to 5 after inoculation. Note that the total numbers of organisms retrieved (filled bars) were greatest on the third and fourth day, and also that the number of dead organisms (open bars) remained low until the fourth day, at which time it appeared to increase.

focus, also appeared to decrease most rapidly during this period (Fig. 3). Therefore, these data indicate that clearance of these infections, as evaluated histologically, begins some time after the second day following inoculation.

Numbers of *C. albicans* pseudohyphae retrieved from the surface of the infected skin appeared to be greatest on the third or fourth day after inoculation, and to decrease thereafter (Fig. 4). In preliminary studies, very few organisms were obtained on the first day after inoculation (data not shown), presumably because they had not yet become superficial enough to be obtained by the retrieval method used. Also, a majority of the organisms retrieved on the fourth and fifth day were found to be non-viable by the acridine orange method, whereas those obtained at earlier time points were most often shown to be viable by this method.

When *C. albicans* yeast cells were germinated in vitro in medium 199 and then applied to shaved flank skin of the mice, most were found to be non-viable when removed 24 h later (in 4 experiments involving 2 animals each, a total of 34 organisms were obtained, and all were non-viable by the acridine orange method). Similarly, when pseudohyphae were applied to a plastic petri dish and allowed to dry, virtually all of them were found to be non-viable when examined 24 h later. In addition, we tested the acridine orange assay to determine the viability of *C. albicans* yeast germinated for 3 h in medium 199 and then exposed to a variety of treatments, including 10% formaldehyde for 60 min, heating to 100°C for 30 min, or 10 μg/ml of amphotericin B for 60 min; in triplicate experiments, there was very good agreement between the percent dead organisms determined by the acridine orange method and that obtained by colony counts on trypticase soy agar (96.7 ± 2.9 vs 100.0 ± 0.0 for formaldehyde, 99.4 ± 0.3 vs 100.0 ± 0.0 for heating, and 97.0 ± 3.0 vs 99.3 ± 0.7 for amphotericin B). The number of dead organisms in the control preparations was usually less than 10% using the acridine orange method.

DISCUSSION

Our previous studies of these experimental cutaneous *C. albicans* infections in mice have demonstrated that yeast applied to the skin surface initially invades into the stratum Malpighi, but then are extruded into stratum corneum between 1 and 2 days after inoculation (11, 15). Also, neutrophils appear to accumulate in most of the foci by the time they have reached the stratum corneum (15). We have previously examined serial sections of infected skin in this system and determined that single sections give fairly accurate estimates of the presence or absence of neutrophils and the location of the organisms in the skin for a particular focus (11, 19). On the other hand, one would expect a single section to give less accurate information on the number of organisms present because it might not transect the center of the focus; however, considering that the sections should contact the foci in a random manner, we feel that single sections give reasonably accurate estimates of numbers of organisms in the foci for the purpose of comparing specimens prepared in a similar manner.

The techniques used in these studies for retrieval and assessing the viability of the superficial infecting organisms appeared to be superior to a number of others which were evaluated. For example, in preparing the specimens, neither tape stripping nor the use of frozen sections after incubation with vital stains was satisfactory because the quantity of material removed made the identification of individual organisms difficult. Digestion of the material removed from the skin surface with trypsin did not appear to aid in dispersion of the organisms. A number of other viability assays were evaluated, including methylene blue (21), the MTT (3[4,5-dimethyl-thiazoyl-2-yl]2,5-diphenyltetrazolium bromide) assay (22), and cultivation of the organisms on microscope slides coated with Sabouraud's dextrose agar to show the development of yeast microcolonies from filamentous organisms. None of these methods was satisfactory, either because they did not distinguish both viable and non-viable organisms, or because the organisms were difficult to find among the scales of keratin removed from the skin surface.

The present study shows that shedding of individual organisms, as indicated by organisms retrievable by gentle rubbing of the skin surface, peaks approximately 1–2 days after the foci have become relocated to the stratum corneum. Viability of the superficial organisms is apparently maintained for at least 1–2 days after they have been contacted by neutrophils, although many of the remaining organisms subsequently become non-viable. These findings suggest that many of the infecting organisms, perhaps a majority, are still alive when they are about to be lost from the surface of the infected skin.

Our data give some information about possible involvement of the neutrophilic infiltrates and the epidermal proliferative response in the clearance phase of these infections. Because neutrophils generally kill microorganisms they ingest or contact within minutes or hours, it seems unlikely that the late loss of viability of the superficial organisms in these infections is directly due to the microbicidal processes of these cells. Although some kind of delayed effect might be possible, it would seem more likely that this loss of viability was due to other

factors at the skin surface. For example, death of the organisms could be due to activity of certain fungicidal proteins which have been identified in the stratum corneum (23). Also, extrusion of the organisms to a site outside of the barrier to water loss which exists in the interior of the stratum corneum (24) could expose them to conditions with insufficient moisture for their survival. As demonstrated above, *C. albicans* pseudohyphae do appear to be quite susceptible to drying. Other physical or chemical factors could perhaps also be lethal to the organisms when they are no longer protected from the environment by being buried within the the stratum corneum.

The timing of the death of the organisms being shed, along with the fact that many of them are apparently alive when lost from the skin, suggests that neutrophil microbicidal processes are not required for the clearance of these infections. However, it is possible that these cells might function in some other way to promote clearance of the infections. For example, neutrophils have been found to contain a protein in their cytoplasm which can inhibit the growth of a variety of microorganisms (24, 25). This antimicrobial protein is capable of preventing the growth of *C. albicans* pseudohyphae without killing the organisms (26); hence, it appears to be distinct from the usual microbicidal processes of these cells. Perhaps release of this substance from dying neutrophils in the epidermal infiltrates prevents growth of the organisms toward the deeper layers of the skin, thereby allowing them to be carried up into the stratum corneum by the epidermal proliferative response. This type of neutrophil function is consistent with the observations reported above. It might also be postulated that the neutrophil infiltrates in these infections actually cause the epidermal proliferative response; however, we have previously investigated this possibility and found the two processes to be apparently unrelated (11).

Based on the data from the present study as well as that from our previous work and that of other investigators on this type of infection, the major elements of host defense against acute cutaneous candidiasis in this model system can be described: (a) the defense against this type of infection consists of two major components – containment of the infection to the epidermis and eventual clearance of the infecting organisms; (b) preventing dermal invasion does not appear to be related to epidermal proliferation (19), whereas this containment process does seem to be due to the epidermal neutrophilic infiltrates and perhaps some kind of epidermal barrier (17, 18, 19); (c) eventual clearance of the infections does not appear to involve microbial killing by the epidermal neutrophilic infiltrates, but rather, extrusion of the organisms to the most superficial layers of the stratum corneum where they are either lost from the skin in a viable state, or killed by a hostile environment at that site.

REFERENCES

1. Smith EC. Prickly heat – Its aetiology and pathology. Trans R Soc Trop Med Hyg 1927; 20: 344–351.
2. Maibach HI, Klingman AM. The biology of experimental human cutaneous moniliasis (*Candida albicans*). Arch Dermatol 1962; 85: 233–257.
3. Schwartzman RM, Deubler J, Dice PF II. Experimentally induced cutaneous moniliasis (*Candida albicans*) in the dog. J Small Anim Prac 1965; 6: 327–332.
4. Sohnle PG, Frank MM, Kirkpatrick CH. Mechanisms involved in elimination of organisms from experimental cutaneous *Candida albicans* infections in guinea pigs. J Immunol 1976; 117: 523–530.
5. Maestrone G, Semar R. Establishment and treatment of cutaneous *Candida albicans* infection in the rabbit. Naturwissenschaften 1968; 55: 87–88.
6. Ray TL, Wuepper KD. Experimental cutaneous candidiasis in rodents. J Invest Dermatol 1976; 66: 29–33.
7. Kirkpatrick CH, Rich RR, Bennett JE. Chronic mucocutaneous candidiasis: model-building in cellular immunity. Ann Int Med 1971; 74: 955–977.
8. Thomas EL, Lehrer RI, Rest RF. Human neutrophil antimicrobial activity. Rev Infect Dis 1988; 10 (Suppl 2): S450–S456.
9. Gallin JI. The neutrophil, *in* Samter M, Talmage DW, Frank MM, Austen KF, Claman HN (Eds), Immunological Diseases, 4th Ed. Boston: Little, Brown and Co., 1988; 737–788.
10. Sohnle PG, Kirkpatrick CH. Epidermal proliferation in the defense against experimental cutaneous candidiasis. J Invest Dermatol 1978; 70: 130–133.
11. Sohnle PG, Hahn BL. Epidermal proliferation and the neutrophilic infiltrates of experimental cutaneous candidiasis in mice. Arch Dermatol Res 1989; 281: 279–283.
12. Berk SH, Penneys NS, Weinstein GD. Epidermal activity in annular dermatophytosis. Arch Dermatol 1976; 112: 485–488.
13. Tagami H. Epidermal cell proliferation in guinea pigs with experimental dermatophytosis. J Invest Dermatol 1985; 85: 153–155.
14. Lepper AWD. Experimental bovine *Trichophyton verrucosum* infection. The cellular responses in primary lesions of the skin resulting from surface or intradermal inoculation. Res Vet Sci 1974; 16: 287–298.
15. Wilson BD, Sohnle PG. Participation of neutrophils and delayed hypersensitivity in the defense against experimental cutaneous candidiasis. Am J Pathol 1986; 123: 241–249.
16. Ray TL, Wuepper KD. Experimental cutaneous candidiasis in rodents. II. Role of the stratum corneum barrier and serum complement as a mediator of a protective inflammatory response. Arch Dermatol 1978; 114: 539–543.
17. Kirkpatrick CH, Smith TK. Chronic mucocutaneous candidiasis. Immunologic and antibiotic therapy. Ann Int Med 1974; 80: 310–320.
18. Hahn BL, Sohnle PG. Characteristics of dermal invasion in experimental cutaneous candidiasis of leucopenic mice. J Invest Dermatol 1988; 91: 233–237.
19. Sohnle PG, Hahn BL. Effect of immunosuppression on epidermal defenses in a murine model of cutaneous candidiasis. J Lab Clin Med 1989; 113: 700–707.
20. Smith DL, Rommel F. A rapid micro method for the simultaneous determination of phagocytic-microbicidal activity of human peripheral blood leukocytes in vitro. J Immunol Methods 1977; 17: 241–247.
21. Lehrer RI, Cline MJ. Interaction of *Candida albicans* with human leukocytes and serum. J Bacteriol 1969; 98: 996–1004.
22. Levitz SM, Diamond RD. A rapid colorimetric assay of fungal viability with the tetrazolium salt MTT. J Infect Dis 1985; 152: 938–945.
23. Kashima M, Takahashi H, Shimozuma M, Epstein WL, Fukuyama K. Candidacidal activities of proteins partially purified from rat epidermis. Infect Immun 1989; 57: 186–190.
24. Montagna W, Parakkal PE. Structure and Function of Skin, 3rd Ed. New York: Academic Press, 1974; 62–64.
25. Sohnle PG, Collins-Lech C, Wiessner JH. Antimicrobial activity of an abundant calcium-binding protein in the cytoplasm of human neutrophils. J Infect Dis 1991; 163: 187–192.
26. Steinbakk M, Naess-Andresen CF, Lingaas E, Dale I, Brandtzaeg P, Fagerhol MK. Antimicrobial actions of calcium binding leucocyte L1 protein, calprotectin. Lancet 1990; ii: 763–765.
27. McNamara MP, Wiessner JH, Collins-Lech C, Hahn BL, Sohnle PG. Neutrophil death as a defence mechanism against *Candida albicans* infections. Lancet 1988; ii: 1163–1165.