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Protection by *Candida albicans* of *Staphylococcus aureus* in the Establishment of Dual Infection in Mice

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***Candida albicans* has been shown to stimulate infection in mice by a number of bacteria when both organisms are inoculated intraperitoneally (E. Carlson, Infect. Immun. 39:193–197, 1983). When subcutaneous and intraperitoneal inoculations were given with *Staphylococcus aureus* and *C. albicans* injected at opposite sites, mixed infection was established at the site of fungal inoculation but not at the site of the bacterial injection. Histopathologic evaluation of tissues for the presence of *C. albicans* and *S. aureus* after intraperitoneal inoculation of both showed fungal growth in the mesentery and omentum of the abdominal cavity. Cocci were numerous and always associated with the fungi, located within the fungal growth rather than at its periphery. It was concluded that this growth pattern in some way protected the bacteria and was the basis for the generalized fungal stimulation of the bacterial infections observed. In addition to *C. albicans*, *Candida stellatoidea*, *Candida tropicalis*, *Candida parapsilosis*, *Torulopsis glabrata*, and heat-inactivated *C. albicans* also demonstrated some ability to protect bacteria injected simultaneously, although *C. parapsilosis* and *T. glabrata* were less effective than the other yeasts in this respect.**

In clinical candidiasis, *Candida albicans* is frequently found with *Staphylococcus aureus* and *Streptococcus* species (2, 7, 10, 11, 14–20, 23). In addition, *S. aureus* has been found to be a frequent opportunist in experimentally induced candidiasis (19). In vitro, *C. albicans* has been found to enhance the growth of a number of bacteria, including *S. aureus* (18, 22).

In previous reports from this laboratory, we described enhancement by *C. albicans* of *S. aureus*, *Serratia marcescens*, and *Streptococcus faecalis* in the establishment of experimental infection in mice when fungi and bacteria were inoculated intraperitoneally (i.p.) (4). Moreover, a synergistic effect of *C. albicans* and *S. aureus* on mouse mortality (3, 5) has been reported. The present study was designed to examine further the nature of this synergistic interaction. The following questions were addressed. Would *Candida* stimulation of bacterial infection take place if the two pathogens were introduced at different sites? Would species other than *C. albicans* or heat-inactivated *C. albicans* exhibit a protective effect on bacteria injected at the same time? What is the physical relationship and growth pattern of *C. albicans* and *S. aureus* in the tissues when introduced together i.p.?

MATERIALS AND METHODS

Mice. Outbred CD-1 mice were obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass. Male mice weighing between 22 and 25 g were used and were caged in groups of four after inoculation.

Pathogens. Yeasts used in this study were selected for low virulence, similarity in i.p. dose causing 50% mortality in 5 days (LD_{50}) in mice, and ability to grow at 37°C. LD_{50}s of various yeasts did not differ significantly and ranged from 3.2×10^8 to 3.9×10^8 CFU. *C. albicans* L-1, the strain used in previous studies (3–5), had an LD_{50} of 3.2×10^8 CFU which agreed with the value previously reported (3). The LD_{50} of heat-inactivated (60°C, 60 min) *C. albicans* L-1 (1.4×10^9 CFU before inactivation) was four times that of the living yeast. *Candida stellatoidea* ATCC 11006 ($LD_{50} = 3.4 \times 10^8$), and *Torulopsis glabrata* ATCC 2001 ($LD_{50} = 3.9 \times 10^8$) were obtained from the American Type Culture Collection (Rockville, Md.). *Candida parapsilosis* CDC 909 ($LD_{50} = 3.5 \times 10^8$ CFU), and *Candida tropicalis* CDC 1072 ($LD_{50} = 3.4 \times 10^8$ CFU) were obtained from the Centers for Disease Control (Atlanta, Ga.) as part of a mycology training collection.

S. aureus FRI1169 was obtained from the collection of M. S. Bergdoll (University of Wisconsin, Madison). It was originally isolated from a patient with confirmed toxic shock syndrome according to the criteria of the Centers for Disease Control (6) and has a LD_{50} in mice of 1.2×10^{10} CFU which agreed with that previously reported (5).

Pathogen inoculations. Cultures used for inoculation were grown on 5% sheep blood agar (blood agar base [BBL Microbiology Systems, Cockeysville, Md.] plus whole blood) for 24 h at 37°C (for *S. aureus*) or on Sabouraud dextrose agar (BBL) for 48 h (for *C. albicans*), washed, vigorously mixed, and titered as described previously (3). Organisms were introduced subcutaneously (s.c.) under the back skin or i.p. with each desired dose suspended in 0.2 ml of nonpyrogenic saline (Abbott Laboratories, North Chicago, Ill.) and mixed immediately before inoculation. When only one agent was used, 0.2 ml of saline was substituted for the second agent.

Quantitation of viable organisms in animal tissues. Animals were sacrificed (cervical dislocation) at various times after inoculations as indicated by the experimental protocol, and organs or abscesses were removed aseptically, homogenized, and resuspended with vigorous mixing in 10 ml of saline. Uniform dispersion of individual bacterial cells by this procedure was demonstrated by Gram stain and reproducibility of CFU titer of the infected resuspended homogenized organ. Blood was drawn by cardiac puncture. The number of CFU of pathogens was determined by dilution onto selective media as described previously (4). Organs selected for enumeration of organisms were those which in

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TABLE 1. Recovery of *S. aureus* from mice 48 h after i.p. inoculation[a]

Inoculum type (6 animals/type)	Avg CFU of *S. aureus* recovered/organ (no. of animals in group with tissue positive for *S. aureus*)		
	Blood (per ml)	Pancreas	Spleen
Dual i.p.[b]			
C. albicans L-1 + *S. aureus*	3.1×10^3 (6)[c]	6.8×10^7 (6)[c]	2.0×10^7 (6)[c]
C. albicans[d] + *S. aureus*	8.5×10^3 (6)[c]	7.2×10^7 (6)[c]	1.5×10^7 (6)[c]
C. stellatoidea + *S. aureus*	6.0×10^3 (6)[c]	6.9×10^7 (6)[c]	2.0×10^7 (6)[c]
C. tropicalis + *S. aureus*	1.1×10^3 (6)[c]	9.8×10^6 (6)[c]	2.1×10^6 (6)[c]
C. parapsilosis + *S. aureus*	5.0×10^3 (3)	3.1×10^6 (6)[c]	4.3×10^5 (6)[c]
T. glabrata + *S. aureus*	<10[e] (0)	1.5×10^3 (3)	7.0×10^2 (3)
Single i.p.			
S. aureus	<10 (0)	$<10^2$ (0)	$<10^2$ (0)

[a] Yeasts were recovered in similar numbers whether inoculated alone or with *S. aureus*.
[b] *S. aureus* dose, 10^7 CFU; yeast dose, 10^8 CFU, except when heat-inactivated fungus was used.
[c] Significant difference from single i.p. group (*S. aureus* alone) with $P < 0.005$ by Wilcoxon-Mann-Whitney two-tailed test.
[d] Heat-inactivated (60°C for 1 h) *C. albicans* dose, 5×10^8 CFU (before inactivation).
[e] Denotes no CFU found in least dilute sample tested.

previous studies (3, 4) have been found to harbor the highest numbers of infecting bacteria.

LD_{50}. The i.p. LD_{50} for each pathogen in mice was determined by the moving average method (1) or probit analysis (9) as described previously (3).

Heat inactivation. Washed *C. albicans* organisms were resuspended in saline at dose concentrations and subjected to 60°C for 60 min in a constant-temperature water bath. Inactivation was confirmed by swabbing a sample onto Sabouraud dextrose agar (BBL).

Histopathology. Animals were sacrificed by cervical dislocation and necropsied. Tissues representing all major systems of the body were collected, fixed in 10% neutral buffered Formalin, and processed by routine histologic procedures for light microscopy. Alternating sections of tissue were subjected to one of three staining procedures: one section was stained with hematoxylin and eosin for routine microscopy; another was stained with periodic acid-Schiff, which selectively stains fungi; and another was stained with Brown and Hopp's Gram stain (B and H), which stains both *S. aureus* and *C. albicans*. This staining sequence was repeated throughout the tissue sample. The tissues were evaluated for the presence of organisms and lesions by using standard light-microscopic procedures.

Statistical analysis. Data were analyzed by using the Wilcoxon-Mann-Whitney (two-tailed) and chi-square tests.

Reproducibility. All experiments reported in this study were repeated at least once with good reproducibility. The data presented are representative.

RESULTS

C. albicans, three additional species of *Candida*, *T. glabrata*, and heat-inactivated *C. albicans* were each independently combined with *S. aureus* and inoculated i.p. into mice to determine whether fungi other than *C. albicans* or heat-inactivated *C. albicans* would affect the survival of bacteria. *S. aureus* (10^7 CFU) inoculated i.p. alone could not be recovered 48 h later (Table 1). When the same dose of *S. aureus* was inoculated with *C. albicans*, *C. stellatoidea*, or *C. tropicalis*, *S. aureus* could be recovered 48 h later in all samples of blood and abdominal organs. *C. parapsilosis* appeared less effective than the above group of yeasts in protecting the bacteria, as was indicated both by the number of tissues positive for bacteria and the number of CFU recovered from the infected tissue. *T. glabrata* was relatively ineffective in influencing the survival of the bacteria. When heat-inactivated *C. albicans* (5×10^8 CFU before inactivation) was substituted for living fungi, bacteria could be recovered 48 h later in the blood and organs sampled (Table 1). When *S. aureus* was introduced into mice with *C. albicans* (alive or heat inactivated) or *C. stellatoidea*, more *S. aureus* CFU were recovered than were present in the infecting dose, demonstrating the ability of the bacteria to multiply under these conditions (Table 1). Tissues (blood, pancreas, and spleen) from the mice infected with both organisms and from mice infected with the yeasts along (10^8 CFU) were also assayed for yeasts, and the numbers of CFU recovered from the two animal groups were compared. All yeasts were recovered in similar numbers whether inoculated alone or with *S. aureus* (data not shown) as shown

TABLE 2. Effect of site of inoculation on location of mixed infection 5 days after dual injection of 10^8 CFU of *C. albicans* L-1 and 10^7 CFU of *S. aureus* FRI1169 into mice

Inoculation group (dual infection)	Inoculation site	CFU of organisms recovered/organ or abscess[a]		No. of dead mice (total)[b]
		s.c. abscess	Pancreas	
C. albicans	s.c.	1.0×10^6	$<10^2$	0 (6)
S. aureus	i.p.	4.2×10^8	2.3×10^3	
C. albicans	i.p.	$<10^2$[c,d]	3.9×10^4	2 (6)
S. aureus	s.c.	2.0×10^6	5.1×10^2	
C. albicans	i.p.	ND[e]	1.8×10^5	13 (16)
S. aureus	i.p.	ND	7.0×10^7	
C. albicans	s.c.	4.8×10^5	$<10^2$	0 (6)
S. aureus	s.c.	3.6×10^8	$<10^2$	

[a] CFU determinations were carried out on all animals which survived 5 days; *n* ranged from 3 to 6; number given represents an average. In the group in which both pathogens were introduced i.p., all 6 animals initially inoculated were dead at 5 days, and an additional group of 10 animals was inoculated; the 3 animals surviving at 5 days were then sacrificed for this determination.
[b] Infections with either pathogen alone at either site resulted in no deaths.
[c] $<10^2$, No organisms recovered in sample diluted by 100.
[d] When this experiment (*C. albicans*, i.p.; *S. aureus*, s.c.) was repeated, the entire abscess was swabbed onto a medium selective for *C. albicans*; no fungi were recovered.
[e] ND, Not determined, since no abscess was present.

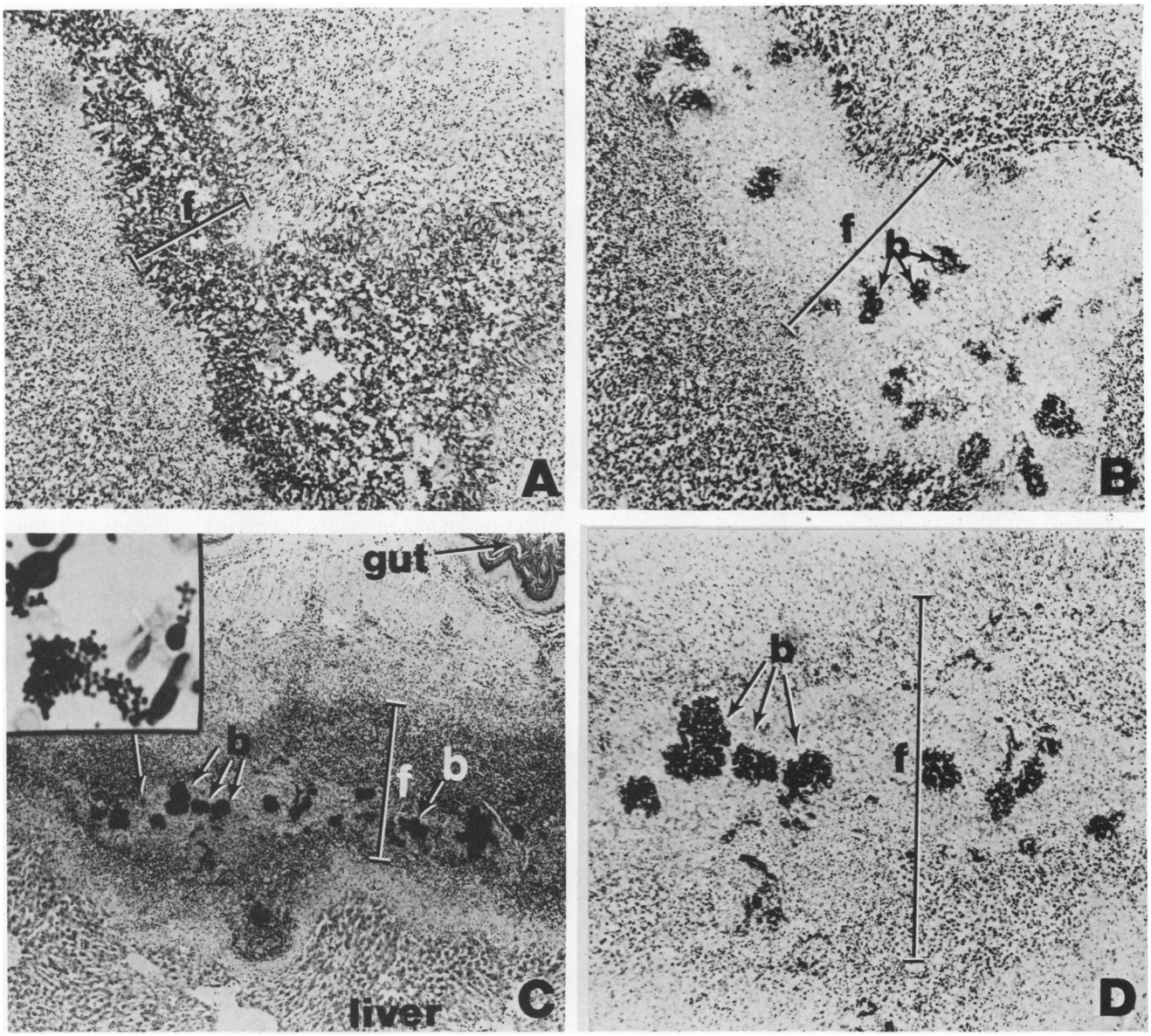

FIG. 1. Sections through tissue of mice 10 days after dual i.p. inoculation with *C. albicans* and *S. aureus* illustrating the relationship of these organisms to each other in infection. (A) Fungi (f) are clearly visible in the liver. Magnification, ×100 (periodic acid-Schiff stain). (B) In the same liver lesion, the location of the bacteria (b) can be seen. Magnification, ×100 (hematoxylin and eosin stain). (C) Adhesion of gut to liver by a lesion. Fungi (f) and bacteria (b) can both be seen. Magnification, ×40 (B and H stain). Inset shows individual pathogens within the fungal growth area. Magnification, ×1,000 (B and H stain). (D) Bacteria (b) in the same lesion as is shown in panel C. Magnification, ×100 (hematoxylin and eosin stain).

previously for *C. albicans* (4). In addition, heat-inactivated *S. aureus* (10^{10} CFU before inactivation) injected with 10^6, 10^7 or 10^8 CFU of *C. albicans* did not influence the number of yeast CFU recovered as compared with tissues from animals injected with *C. albicans* alone.

Experiments were conducted to establish whether both pathogens had to be inoculated at the same site to observe the *C. albicans* stimulation of mouse lethality and *S. aureus* survivability. In addition, we wished to determine whether a mixed bacterial-fungal infection could be established in locations other than the peritoneal cavity. Mouse mortality was observed only when both pathogens were introduced, with *C. albicans* injected i.p. (Table 2). Also, regardless of the site of injection, *S. aureus* infection was established at the site of *C. albicans* inoculation and not vice versa. In the case in which the fungus was introduced s.c. and the bacterium was introduced i.p., mixed infection was confined to a s.c. abscess yielding both pathogens in all six animals. No abscess developed when saline was introduced s.c. with *S. aureus* (introduced i.p.). When *C. albicans* was introduced i.p. and *S. aureus* was introduced s.c., a mixed infection of the pancreas in all four surviving animals ensued, with two of six animals dying. However, in the same mice, at the site of the *S. aureus* injection (s.c.), a purulent infection resulted, from which only *S. aureus* could be recovered (Table 2). When the two pathogens were introduced at different sites, mixed infection was significantly ($P < 0.025$) associated with site of *C. albicans* injection, as

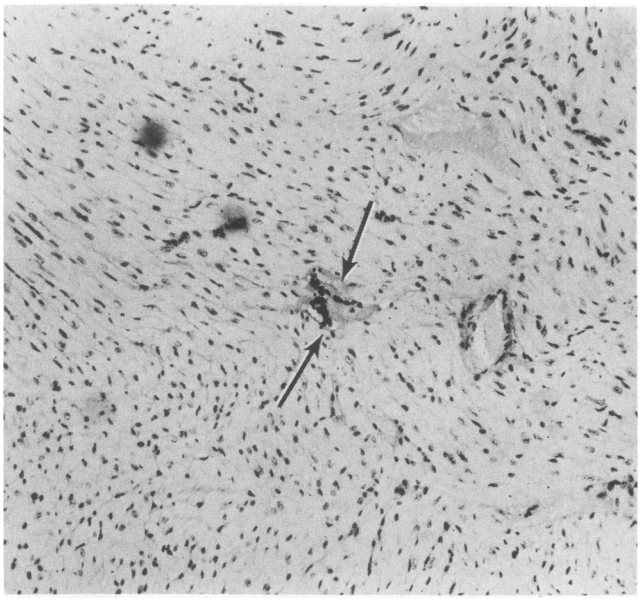

FIG. 2. Section through heart tissue of the mouse 48 h after dual inoculation i.p. with *C. albicans* and *S. aureus* illustrating lack of widespread infection; *C. albicans*, although present, is seen within a blood vessel (shown by arrow). Magnification, ×100 (periodic acid-Schiff stain).

determined by using the chi-square test. Repeated experiments resulted in the same patterns of infections.

To determine the physical relationship of the two organisms inoculated i.p., animals received an i.p. injection containing 10^8 CFU of *C. albicans* and 10^5 CFU of *S. aureus*. Groups of two animals were killed at 2 and 10 days after infection, and their tissues were removed, sectioned, and stained as described above.

At both 2 and 10 days, the largest and most numerous colonies of fungi (mainly in the hyphal form) were seen in the mesentery and omentum of the abdominal cavity. Cocci were also most numerous in the omentum and mesentery. The bacterial colonies were always associated with the fungi and were located within the fungal growth rather than at its periphery (Fig. 1). Three zones of lesion morphology were observed: an inner zone of degenerated tissue and unidentifiable debris among which were bacterial colonies, an intermediate zone of fungi, and an outer zone of the host inflammatory cells which included a moderate number of polymorphonuclear leukocytes.

Fungal colonies seen in organs not located in the peritoneal cavity were very small, usually consisting of a few hyphae, and there were no cocci present. No inflammatory cell infiltrate was observed except for an occasional perivascular macrophage. We examined heart tissue and found no widespread infection, although fungi were observed within a blood vessel (Fig. 2). The only case where large numbers of fungi were found in tissue other than the peritoneal cavity was seen on the keratinized surface of the esophagus, which was moderately colonized by fungi and cocci 48 h after infection. This site was heavily colonized with fungi 10 days postinfection (Fig. 3); however, small rods (not visible in Fig. 3), not cocci, were found in association with the fungi. The rods were gram positive when stained with B and H.

Infected animals were apathetic during the entire period of experimentation, and no animal-inflicted or other injuries were observed.

DISCUSSION

These findings confirm those of a previous report (4) and suggest that the stimulation of growth of *S. aureus* by *C. albicans* in lesions results from the growth pattern of the two infecting organisms. Fungal growth was seen in the mesentery and omentum of the abdominal cavity, with cocci always associated with the fungi and located within the fungal growth rather than at its periphery. It seems reasonable to assume that this growth pattern protected the bacteria in some way and was the basis for the fungal stimulation of growth of various genera of bacteria observed previously (4). The premise of the protective role of the fungi is further supported by the finding that when the bacteria and fungi were injected at different sites, mixed infection was established at the site of the fungal inoculation but not at the site of bacterial injection. This suggests that fungal growth was necessary for the growth of the bacteria.

Certain early papers report inconclusive and often contradictory results of studies carried out on animals inoculated i.p. simultaneously with *C. albicans* and various bacteria (for review, see reference 7). In some reports, a synergistic effect on animal mortality between *C. albicans* and gram-negative organisms (24) or *Mycobacterium tuberculosis* (13) was described, but this effect of *C. albicans* was not observed with the gram-positive bacteria *S. aureus*, *Streptococcus* spp., and *Bacillus subtilis* (24). Previous reports from our laboratory suggest that *Candida* stimulation of the growth of the infecting bacteria is a general effect (4) but that the degree of resulting mortality depends on the array of toxins produced in the host by the bacteria (5). *Staphylococcus epidermidis* and certain *S. aureus* strains showed little or no synergism with *C. albicans* in mouse mortality. In contrast, the mouse LD_{50}s of other *S. aureus* strains were reduced by >3 orders of magnitude when inoculated with sublethal amounts of *C. albicans* (5). Studies are presently in progress to determine whether the synergistic effect on

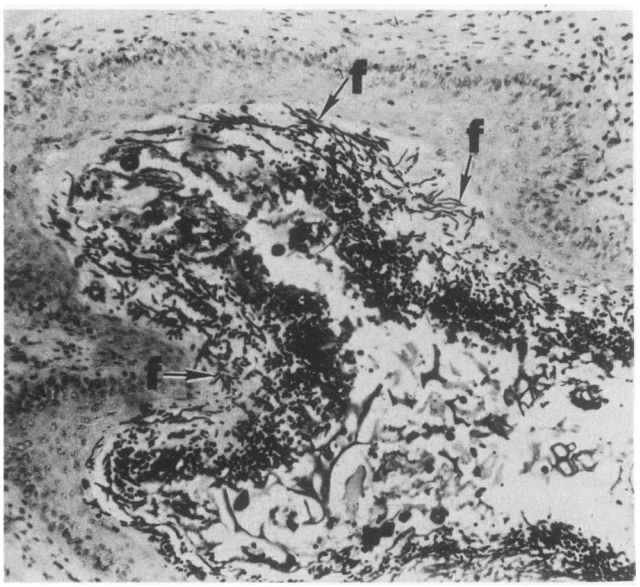

FIG. 3. Section through esophagus 10 days after infection with *C. albicans* and *S. aureus* illustrating heavy colonization of the keratinized surface by *C. albicans* (f). Magnification, ×100 (B and H stain).

mouse mortality (5) between *C. albicans* and selected strains of *S. aureus* is specific for *C. albicans* or if other yeasts will also exhibit this effect in accordance with their ability to protect or stimulate bacterial growth.

Others have observed a hierarchy among yeasts for adherence to endothelium in the order: *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *T. glabrata* (14). This ranking correlates with the ability of these yeasts to protect *S. aureus* when the two organisms are injected simultaneously. Heat-inactivated *C. albicans* has been reported to retain its ability to adhere to endothelium (12) and, when injected i.p. in large amounts (as compared to the living dose), to have a synergistic activity with certain bacteria on mouse morality (20; for a review, see reference 8). Accordingly, it is reasonable that it also protects bacteria which are located within accumulations of fungal cells. Our finding that heat-inactivated *C. albicans* stimulated the growth of *S. aureus* further supports the premise of the protective role of the heat-killed fungus. It is also noted that the two yeasts least able to protect *S. aureus* (*C. parapsilosis* and *T. glabrata*) are smaller than the other yeasts tested.

The only tissue outside the peritoneal cavity to become colonized as a result of the i.p. inoculation was the esophagus, which was moderately colonized by the two simultaneously injected pathogens 48 h after inoculation. Our observation that the esophagus is a preferential site of *Candida* infection agrees with a recent study on human gastrointestinal candidiasis, which reported that an esophageal involvement was most common (21). Further experimentation is necessary to determine the relationship between the heavy fungal colonization of the esophagus and the non-introduced bacterial rods seen 10 days after pathogen inoculation.

It must be emphasized that the study reported here was not designed to serve as a model for human disease, but for further study of the fungal-bacterial synergistic effect previously reported. However, *C. albicans* has been reported to be associated with *S. aureus* or *Streptococcus* spp. in a number of diverse disease conditions (2, 7, 10, 11, 14, 15, 20, 23).

Many factors associated with lesions must be considered in attempting to explain the protection of the bacteria by the fungus. However, note that in vitro, the proteolytic products resulting from the growth of *C. albicans* are able to convert a serum-protein medium unsuitable for the growth of *S. aureus* to one suitable for *S. aureus* as well as other bacterial genera (18). It has been suggested that this commensalism may also occur in vivo; antifungal agents alone have been reported to cure chronic combined *S. aureus* and *C. albicans* infections (17, 18). Thus, it is possible that the condition of chronic candidiasis may provide a situation in which bacterial growth may be protected and provided with a favorable growth medium, and thereby able to present a chronic problem.

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