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Comparison of Candidacidal and Candidastatic Activities of Human Neutrophils

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Disruption of neutrophils causes the release of a cytoplasmic protein which can inhibit the growth of *Candida albicans* without killing the organisms. The present study was undertaken with a combined system in which candidacidal and candidastatic activities of human neutrophils could be compared. In this system it was found that disruption of either half or all of the neutrophils in the samples markedly improved the ability of the cells to handle an inoculum of *Candida* yeast cells over a 48-h period, even though the disrupted cells had primarily candidastatic activity, with very little candidacidal activity.

Although the microbicidal activities of neutrophils appear to be an established mechanism of defense against *Candida* infections, we have recently found that these cells contain an abundant protein which can control the growth of *C. albicans* and several other human pathogens without killing them. The mechanism involved does not appear to depend upon iron binding (3). This protein is located in the cell cytoplasm, rather than the granules, and is released only upon lysis of the cell. Because of the short life span of neutrophils in tissues (2), we have postulated that release of this protein in dense inflammatory infiltrates or in actual abscesses could prevent the proliferation of invading pathogens which have not yet been killed by the inflammatory cell infiltrate. In order to investigate the potential role of this proposed alternative mechanism in the overall ability of neutrophils to handle *C. albicans*, we developed a combined system in which to examine the effect of disruption of a portion of the cell population on the ability of neutrophils to kill and/or suppress the growth of an inoculum of *Candida* yeast cells.

These studies were carried out with human neutrophils obtained from the peripheral blood of normal volunteers by Hypaque-Ficoll centrifugation, as described previously (6). The cells were washed three times in normal saline before being suspended in phosphate-buffered saline (supplemented phosphate-buffered saline, 0.01 M at pH 7.4, and containing 1.0 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 2.4 mM KCl, and 5.5 mM glucose). The neutrophils were used as either intact, viable cells (approximately 98% viable by trypan blue staining), as whole cell lysates prepared by five cycles of freezing and thawing in a slurry of dry ice and ethanol, or as a mixture of half of each. The freezing and thawing procedure produced disruption of virtually all of the cells. *C. albicans*, originally obtained as a clinical isolate at the Milwaukee VA Medical Center, was grown on Mycosel agar (BBL Microbiology Systems, Cockeysville, Md.), and the yeast cells obtained were washed three times in saline before being suspended in phosphate-buffered saline for the experiments.

A combined assay system was used to evaluate killing and growth inhibition concurrently by using 4×10^6 neutrophils and 10^4 *Candida* yeast cells in 0.2 ml of phosphate-buffered saline (supplemented as described above with calcium, magnesium, potassium, and glucose) plus 10% human serum that had been frozen shortly after being obtained and then maintained at −70°C. Killing of the yeast was evaluated by lysing the neutrophils with 0.1% Triton X-100 and plating various dilutions of the lysates on tryptic soy agar (Difco Laboratories, Detroit, Mich.) to determine the numbers of colony-forming units remaining. Under these conditions, growth inhibition by the neutrophil lysates did not affect the numbers of colonies produced (presumably because of dilution during the plating procedure). A time course of killing was carried out initially, and thereafter killing was evaluated after 3 h of incubation at 37°C because little additional candidacidal activity occurred after this time. Growth of *C. albicans* as yeast cells was evaluated by adding an equal volume of 2× neopeptone-glucose broth at 3 h (when killing by the neutrophils had apparently reached a plateau) and microscopically counting the yeast cells which were present in the samples after either 24 or 48 h of continued incubation at 37°C. The data were expressed as either percent killing of the initial inoculum, percent inhibition of growth, or the $\log_{10}$ of the number of yeast cells growing in the samples.

The effect of disruption of the neutrophils on their ability to kill *Candida* yeast cells was studied by adding the organisms to cells which were either all intact, all disrupted, or half of each. As shown in Fig. 1, killing of the inoculum of *Candida* yeast cells reached approximately 85 to 90% in the samples with either all or half of the neutrophils intact but was markedly reduced in those samples in which all of the cells had been disrupted. Therefore, although intact neutrophils killed much better than did disrupted ones in this system, lysis of half of the cells apparently did not significantly interfere with the killing function of the other half.

To assess the combined effect of killing and growth inhibition for intact and disrupted neutrophils, *Candida* yeast cells were added to neutrophils which were all intact, all disrupted, or half of each. The organisms and cells were incubated for 3 h at 37°C to allow for killing to take place, and then an equal volume of twice-concentrated neopeptone-glucose broth was added. As can be seen in Fig. 2, after a further incubation of 48 h, growth was greatest in those tubes with either no neutrophils at all or in those containing only intact cells. In contrast, if either all or half of the cells had been disrupted, the number of yeast cells present after these incubations generally did not exceed the original

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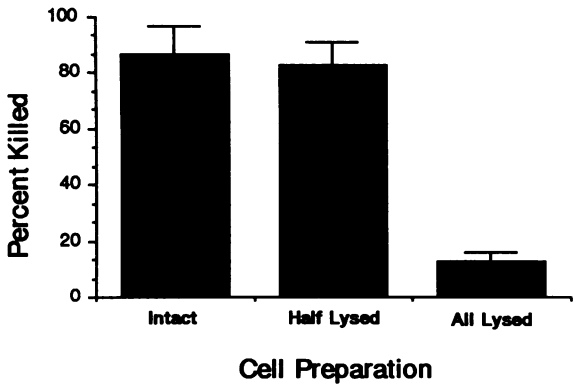

FIG. 1. Effect of disruption of half or all of the neutrophils on the killing of *C. albicans* yeast cells. Note that lysis of all the neutrophils markedly decreased the degree of killing, whereas lysis of only half did not. The data represent the means ± the standard error of results from four experiments.

inoculum. A similar pattern of results was obtained for 24-h cultures (data not shown).

These results indicate that although neutrophil lysates can inhibit the growth of *Candida* yeast cells under the conditions used in these experiments, they apparently do not interfere with the ability of the remaining intact cells to kill the organisms. Also, it is apparent that in a system in which both killing and growth of the organisms can occur, killing by itself during one 3-h period may not be sufficient to control an inoculum of the organisms because the small numbers remaining may be able to outgrow the effect of killing alone. However, disruption of part of the neutrophil population may control this growth, as long as the final concentration of the lysates exceeds that required for growth inhibition (approximately 10^7 disrupted cells per ml). In addition, under the conditions used in these experiments, the growth-inhibiting effect of the lysates appears to last for at least 48 h of incubation at 37°C.

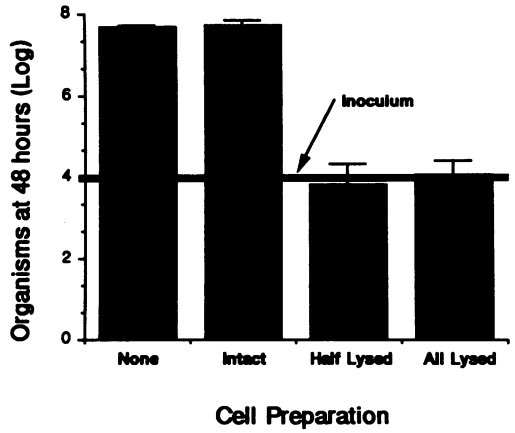

FIG. 2. *Candida* growth after 48 h in the presence of intact or lysed neutrophils. For these experiments *Candida* yeast cells were added to neutrophils which were all intact, all disrupted (by freezing and thawing), or half of each; after killing of the yeast cells had proceeded for 3 h, an equal volume of twice-concentrated neopeptone-glucose broth was added to promote growth of the organisms as yeast cells, with the samples then incubated 48 h longer at 37°C. Note that an increase in the number of organisms over the inoculum was found to occur only in those samples which did not contain disrupted neutrophils. The data represent the means ± the standard error of results from four experiments.

Although the above results suggest a potential role for neutrophil lysis in the control of infective foci, it is possible that this kind of result might not be seen under other conditions. Thus, at in vivo sites the conditions might be different enough that the microbicidal activities of neutrophils could far outweigh their growth-inhibiting potential. However, the vast majority of in vitro systems used in the past to study neutrophil-mediated defenses against *C. albicans* have analyzed only microbicidal mechanisms (1), so that they probably reflect in vivo conditions even less well than does the system used in the present studies. Therefore, although growth inhibition by proteins released from disrupted neutrophils in vivo may not occur to the extent shown by the present study, it still could have a role in the control of those infective foci in which sterilization of the site does not occur rapidly enough to preclude regrowth of the infecting organisms.

The results discussed above may be particularly relevant to our understanding of what occurs when an infected tissue progresses to the stage of abscess formation. From studies of experimental intra-abdominal infections in animals, there appears to be an initial phase of acute infection with a high mortality rate from sepsis, followed by a prolonged period in which the major pathology consists of localized abscesses (4, 7). The abscesses in this model system can sometimes persist for months (7). In clinical practice, intra-abdominal abscesses generally require drainage for resolution to occur (5). Within the interior of an abscess there may be a number of competing processes occurring concurrently, including killing of the organisms, continuing regrowth of those not killed, influx of new phagocytic cells, death and lysis of those cells already present, exchange of soluble components with the surrounding interstitial fluids, and finally, organization of the site with eventual healing of involved tissues. Growth inhibition of the remaining pathogens by neutrophil cytoplasmic proteins could have the potential to shift the course of these events to a direction more favorable to the host.

In summary, the studies discussed above indicate that within the parameters of the system used in these experiments, disruption of a proportion of the neutrophils used may actually improve the function of these cells in controlling an inoculum of *C. albicans* yeast cells. This effect is presumably due to growth inhibition by cytoplasmic contents of the disrupted cells. Because neutrophil lysis does readily occur at infected sites in vivo, this mechanism could play a role in helping inflammatory cells to control infective foci.

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