Damage to Pseudohyphal Forms of *Candida albicans* by Neutrophils in the Absence of Serum In Vitro

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ABSTRACT Large forms of *Candida* are characteristically present in invasive lesions and are often cleared by host defenses. Therefore, an in vitro system was developed to study interactions between leukocytes and pseudohyphae. By light, phase contrast, and electron microscopic observations, in the absence of serum, neutrophils attached to and spread over the surfaces of partially ingested pseudohyphae, which then appeared damaged. Using a new assay which measured neutrophil-induced inhibition of uptake of [^{14}C]cytosine by *Candida*, damage to *Candida* in the absence of serum was 53.04±2.96% by neutrophils from 27 normal subjects. With serum, damage to *Candida* increased because of opsonization by low levels of anti-*Candida* immunoglobulin G in normal sera. Damage to *Candida* was inhibited by colchicine, cytochalasin B, and 2-deoxyglucose, which interfered with spreading of neutrophils over the surfaces of *Candida*. Dibutyryl cyclic AMP, theophylline, and isoproterenol also inhibited damage to *Candida*. Hydrocortisone was inhibitory in levels (10 μM) achievable with pharmacologic doses in man. Light, fluorescence, and electron microscopy indicated that neutrophils degranulated after contact with *Candida*. Quantitative studies revealed only a minimal increase in specific release of lysosomal enzymes from azurophil granules, but much greater release of lysozyme from specific granules. *Candida* activated neutrophil oxidative microbicidal mechanisms, as shown by iodination of *Candida* by neutrophils, and chemiluminescence from neutrophils interacting with *Candida*. Unlike live *Candida*, killed *Candida* did not induce chemiluminescence, were not iodinated, and did not attach to neutrophils by microscopy. Like *Candida* pseudohyphae, contact between neutrophils and hyphal forms of *Aspergillus* and *Rhizopus* occurred in the absence of serum. This did not occur with *Cryptococcus neoformans*, an encapsulated yeast, and was low with *Candida* yeasts. These findings indicate that neutrophils can recognize and attach to *Candida* pseudohyphae, then damage the *Candida*. This may represent a general reaction between neutrophils and large forms of fungi. Though the size of the organisms precludes complete ingestion, neutrophil oxidative microbicidal mechanisms are activated, and preferential release of contents of specific granules appears to occur.

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INTRODUCTION

Candidiasis is the most commonly observed systemic mycosis in the compromised host, constituting a significant percentage of all infections and of causes of death in this population (1–3). Within invasive lesions, pseudohyphal and hyphal forms of *Candida* are more prominent than yeasts (4, 5), and must be cleared if the host is to survive. Pseudohyphal and hyphal forms of *Candida* can be cleared in at least some systemic infections in experimental animals (6), and in man (1–3, 7, 8). Reproducible quantitative determinations of viability of hyphae and pseudohyphae have been difficult. Most in vitro studies have concentrated on the ability of leukocytes to ingest and kill yeasts. Such studies (9–13), as well as the observed clinical association of neutropenia with disseminated candidiasis (14), suggest that neutrophils are important in host defenses against systemic candidiasis.

The interactions of leukocytes with filamentous forms of fungi may not necessarily correlate directly with observations on phagocytosis and killing of yeasts (9–12, 15), especially because hyphae and pseudohyphae

are too large to be ingested completely. We describe a new leukocyte mechanism where, in the absence of serum opsonins (including antibody and complement), human neutrophils partially ingested and damaged *Candida albicans* pseudohyphae.

METHODS

Candida *and other fungi.* An isolate of *Candida albicans* orginally obtained from a patient with systemic candidiasis was maintained in yeast phase on Sabouraud's agar. For germination, yeasts were washed in phosphate-buffered saline and suspended in Eagle's minimal essential medium, which had been supplemented with nonessential amino acids (Grand Island Biological Co., Grand Island, N. Y.) (16, 17). After 4 h of incubation at 37°C, and at 20–30-min intervals thereafter, aliquots were removed and examined for germ tube formation by phase contrast microscopy. When ≥95% of cells had formed germ tubes ≥30 μM in length, test tubes were stored at 4°C. *Candida* used immediately or those stored at 4°C for 18 h gave comparable results. For some experiments, *Candida* were killed by heating at 100°C (in a boiling water bath) for 1 h or by exposure to ultraviolet light for 16 h. Samples were plated on Sabouraud's agar and observed for 72 h to verify that no live organisms remained. Before incubation with leukocytes, live or killed *Candida* were washed three times and resuspended in Hanks' balanced salt solution (HBSS,[1] Grand Island Biological Co.). Patient isolates of *Aspergillus fumigatus* and *Rhizopus oryzae* were supplied by Dr. John Rippon, University of Chicago, Chicago, Ill., and a small capsule isolate of *Cryptococcus neoformans* by Dr. John E. Bennett, National Institutes of Health, Bethesda, Md. *Aspergillus* and *Rhizopus* spores were germinated and washed as described above for *Candida*. Cryptococci were heat-killed and washed following the same procedure as with *Candida*.

Leukocytes. Human peripheral venous blood anticoagulated with preservative-free heparin (Connaught Laboratories, Toronto, Ontario, Canada) was obtained from normal volunteer subjects. Neutrophils were separated by sedimentation with 3% dextran (average molecular weight 250,000; Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) in saline. Remaining erythrocytes were lysed in an ammonium chloride-Tris buffer solution (18). Leukocytes were washed in modified Hanks' solution (19) which contained no calcium or magnesium, then suspended in HBSS. For some studies, more purified preparations of neutrophils were obtained by using Hypaque (sodium and meglumine diatrizoates; Winthrop Laboratories, New York) and Ficoll (Pharmacia Fine Chemicals) followed by dextran sedimentation as described by Böyum (20).

Serum. Serum was obtained from each normal volunteer donor at the same time as blood for leukocytes, and from two subjects with rheumatoid arthritis (rheumatoid factor titers 1:320 and 1:1,280 by latex fixation). Sera were used fresh, or after storage at −70°C or heating at 56°C for 60 min.

Immunoglobulins. Two commercial preparations of immunoglobulin G (IgG) were obtained, one >95% pure (Fraction II, Pentex Company, Kankakee, Ill.) and one >98% pure (Mann Research, Inc., New York). IgG was also purified to give a single line on immunoelectrophoresis using minor modifications in the method of Stanworth (21). For some experiments, IgG was aggregated by heating for 20 min at 63°C.

*Detection of anti-*Candida *immunoglobulins.* Tests for anti-*Candida* immunoglobulins were performed on sera and immunoglobulin preparations used in these studies. Agglutinins and precipitins were detected using slight modifications in the technique of Preisler et al. (22). For detection of antibodies by indirect fluorescence, live *Candida albicans* were incubated with sera, stained with goat anti-human IgG conjugated with fluorescein isothiocyanate (Microbiological Associates, Inc., Bethesda, Md.), washed and examined for fluorescence (graded from 0 to 4+).

Radioisotope assays for metabolic activity of Candida. Suspensions containing 1×10^6 *Candida albicans* pseudohyphae and 1×10^6–2×10^7 neutrophils in HBSS were placed in 15-ml plastic centrifuge tubes (Corning Glass Works, Corning, N. Y), and brought to 1 ml total volume with HBSS. Triplicate tubes were placed in a rotator (Scientific Industries, Inc., Springfield, Mass.) and incubated at 37°C for timed intervals. Additional tubes were added to permit removal of aliquots for microscopic observations. To end the incubation, 0.25 ml of 2.5% sodium deoxycholate was added to each tube. This induced lysis of leukocytes without damage to *Candida* cells (26). Control tubes contained only *Candida* pseudohyphae, but neutrophils were added just before deoxycholate. After lysis of neutrophils, remaining *Candida* were centrifuged, then washed twice in distilled water, and once in yeast nitrogen base broth which had been supplemented to contain 1% dextrose and 0.15% asparagine. Radioisotopically labeled compounds, including [^{14}C]cytosine (59–61 mCi/mmol), [^{3}H]mannose (2 Ci/mmol), and [^{3}H]-glucose (1.1 Ci/mmol) (Amersham/Searle Corp., Arlington Heights, Ill.) were diluted in supplemented yeast nitrogen base broth, but glucose supplementation was omitted in all studies were [^{3}H]glucose or [^{3}H]mannose were used. The optimum dose, time period, and temperature for incubation were determined for [^{14}C]cytosine (0.25 μCi and 1-h incubation at 30°C), as well as for [^{3}H]mannose and [^{3}H]-glucose (2.0 μCi and 2 h at 30°C). *Candida* were then separated and washed free of unbound radioisotope by using an automated, multiple-sample harvester (Otto Hiller Co., Madison, Wis.). Filters containing *Candida* were air-dried overnight, then placed in scintillation vials with 8 ml Aquasol, (New England Nuclear, Boston, Mass.) and counted in a liquid scintillation counter. The neutrophil-induced percentage of reduction in uptake of isotope by *Candida* was calculated from (mean counts per minute in control tubes − mean counts per minute in experimental tubes)/(mean counts per minute in control tubes) × 100. In some studies, instead of reduction of uptake in *Candida*, release of radioisotopes from prelabeled *Candida* was measured. For measurement of radioisotope release, *Candida* pseudohyphae in yeast nitrogen base broth supplemented as described above were incubated at 30°C for 1 h for labeling with 0.25 μCi [^{14}C]cytosine, and 2 h for labeling with [^{3}H]mannose. After washing three times in HBSS, *Candida* were resuspended in HBSS and incubated with neutrophils at 37°C as described above. After 2 h, *Candida* were pelleted by centrifugation at 600 *g*. The supernate and pellet were then removed separately for scintillation counting. The total counts per minute in samples were determined from the sums of counts per minute in supernates and pellets of triplicate tubes. The percentage of isotope released was then determined by the (counts per minute in the supernatant)/(total counts per minute in supernatant plus pellet) × 100.

Inhibitors. In some studies, inhibitors of neutrophil function were added during incubations. In others, neutrophils were preincubated with inhibitors for 20 min before the addition of *Candida*, and inhibitors remained in the media for the duration of the incubation with *Candida*. Unless

[1] *Abbreviation used in this paper:* HBSS, Hanks' balanced salt solution.

otherwise specified, inhibitors were obtained from Sigma Chemical Co. (St. Louis, Mo.). Cytochalasin B was dissolved in dimethyl sulfoxide. Controls contained dimethyl sulfoxide without cytochalasin B, diluted comparably in HBSS. Colchicine was dissolved and directly diluted in HBSS, as were cyclic AMP, theophylline, and isoproterenol. For experiments using 2-deoxyglucose, glucose was excluded from HBSS, and controls contained 2-deoxyglucose plus glucose at 10 times the concentration of the 2-deoxyglucose. For studies of the effects of steroid hormones, their precursors, and metabolites on neutrophil function, the following compounds were dissolved in absolute ethanol: hydrocortisone, testosterone, estriol, androsterone, progesterone, dehydroisoandrosterone, and 17α-hydroxyprogesterone. Control tubes included absolute ethanol diluted comparably to the above compounds which had been originally dissolved in ethanol.

Microscopic observations. At times intervals during the incubation of *Candida* with neutrophils, aliquots were removed for continuous observation under phase contrast microscopy using a stage incubator (Incustage, Lab-Line Instruments, Inc., Melrose Park, Ill.), and for staining using Wright-Giemsa. Trials of visual methods for determination of *Candida* viability were made after lysis of neutrophils using deoxycholate. Dye exclusion using methylene blue was performed on pseudohyphae exactly as described by Lehrer and Cline (23), and Giemsa staining as outlined by Lehrer (9). Viability of neutrophils was determined before and after incubation with *Candida* using exclusion of 0.1% trypan blue. In selected experiments, neutrophils were labeled with ^{51}Cr (24), and release of ^{51}Cr during incubations was measured. Degranulation of neutrophils was observed under phase contrast microscopy, and by fluorescence microscopy after staining of granules with a 1:100,000 dilution of acridine orange in HBSS according to the method of Allison (25) and D'Arcy Hart and Young (26). For electron microscopic studies of neutrophil degranulation, the method of Armstrong and D'Arcy Hart (27) was used with slight modifications. Neutrophil granules were labeled with 10 mg/ml ferritin (twice recrystallized cadmium free; Pentex Company) for 2.5 h at 37°C, washed three times in HBSS, and incubated with *Candida* in the usual way.

Four electron microscopy, pellets containing *Candida* and neutrophils were fixed in 2.5% glutaraldehyde solution overnight. After washing overnight in cacodylate-buffered solution, pellets were postfixed in 2% osmium tetroxide for 2 h. They were then dehydrated in increasing, graded concentrations of ethanol, and embedded in Epon. Thin sections were cut with diamond knives on an automatic Porter-Blum ultramicrotome (DuPont Instruments, Sorvall Operations, Newtown, Conn.). After staining with uranyl acetate and lead citrate, sections were mounted on carbon-coated copper grids, examined with an RCA 3H electron microscope (Special Products Div., RCA Corp., Cherry Hill, N. J.) and photographed.

Iodination of Candida *by neutrophils.* The fixation of iodide to *Candida* (trichloroacetic acid-precipitable iodide) by neutrophils attaching to the surface of pseudohyphae was measured by the method of Klebanoff and Clark (28), with the following modifications. In addition to pH 7.4 sodium phosphate buffer with added potassium, calcium, and magnesium salts, for optimum conditions, the reaction mixtures in triplicate or quadruplicate tubes contained 1×10^7 neutrophils, 5×10^6 *Candida*, or other fungi, but no serum unless otherwise stated. The optimum concentration of ^{125}I-Na was found to be 0.5 μM, containing 0.2 μCi ^{125}I, with a 1-h incubation time.

Chemiluminescence by neutrophils attached to Candida *pseudohyphae.* Chemiluminescence was measured in a Beckman Model LS 230 liquid scintillation counter (Beckman Instruments, Inc., Electronic Instruments Div., Schiller Park, Ill.) as described by Stjernholm et al. (29). Vials contained 1×10^7 *Candida* in HBSS, or HBSS alone. Unless otherwise noted, reaction mixtures contained no serum.

Release of enzymes from neutrophils. Enzyme assays were kindly performed by Dr. Philip Davies and his colleagues (Department of Inflammation and Arthritis, Merck Institute for Research, Rahway, N. J.). β-Glucuronidase was assayed by the method of Talalay et al. (30), using phenolphthalein glucuronate (Sigma Chemical Co.) as substrate. *N*-acetyl-β-D-glucosaminidase was assayed according to the method of Woolen et al. (31), using *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminidase as substrate. Lactate dehydrogenase was quantitated by assay of the change of optical density at 340 nm of NADH being oxidized to NAD by the conversion of pyruvate to lactate. Lysozyme was measured by using *Micrococcus lysodeikticus*, according to the method of Parry et al. (32).

Statistical methods. Means and SEM were compared using two sample T tests (33).

RESULTS

Microscopic observations of the interaction of Candida albicans *pseudohyphae with neutrophils.* Preliminary observations using light, phase contrast, and fluorescence microscopy suggested that neutrophils attached to pseudohyphae in the absence of serum, spread over pseudohyphal surfaces, and degranulated, resulting in altered *Candida* morphology. To extend these findings, electron micrographs were prepared from samples obtained at timed intervals during incubations of neutrophils with *Candida* pseudohyphae. Normal *Candida* morphology was apparent at the start of incubations (Fig. 1). By 20 min of incubation, neutrophils had spread over the surfaces and around *Candida*, and degranulation of neutrophils over the *Candida* surface was evident as shown in studies where neutrophil lysosomes were labeled by incubation with ferritin. Ferritin was specifically released by neutrophils over the surfaces of pseudohyphae (Fig. 2). By 1 h of incubation, damage to the *Candida* was apparent, as there were striking changes in the cell wall and internal morphology (Fig. 3), compared with normal *Candida* (Fig. 1), even though Candida were not completely ingested by neutrophils. *Candida* which were not in close contact with neutrophils had no apparent alterations in morphology.

To quantitate this apparent damage to *Candida* pseudohyphae by neutrophils, several staining techniques for light microscopy were used. These included methylene blue vital staining of *Candida*, and Giemsa staining of fixed specimens. As expected, these procedures were useful for *Candida* yeasts; unfortunately, they did not provide reproducible results with *Candida* pseudohyphae. Similarly, tube dilutions and plate counts were extremely variable and not reproducible, because of clumping of pseudohyphae.

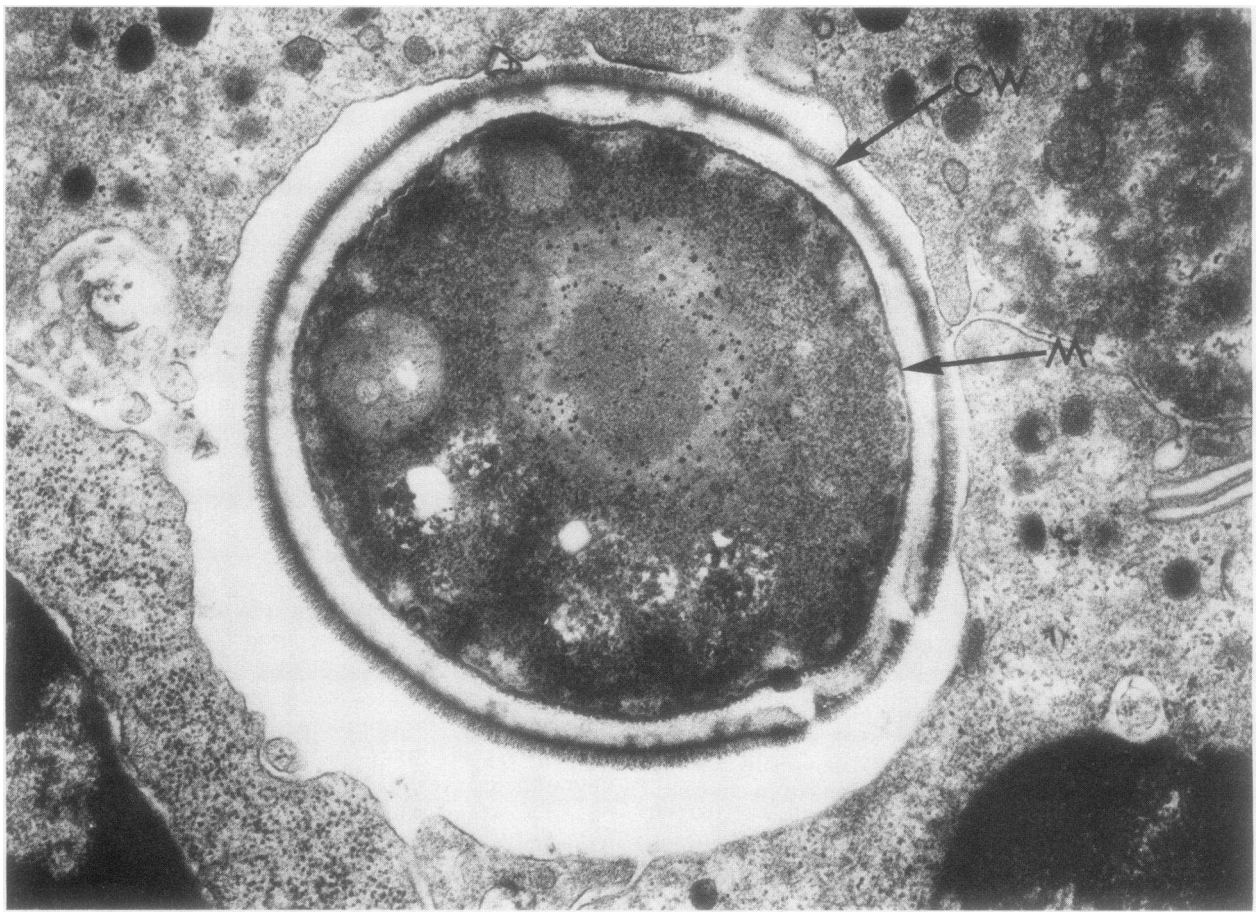

FIGURE 1 Electron micrograph showing normal *Candida albicans* pseudohyphal morphology seen in transverse section surrounded by neutrophils, at the start of an experiment. Cell wall (CW) and cell membrane (M) are shown by arrows, and internal structures are identifiable. Magnification ×35,910.

Radioisotope assays for metabolic activity of Candida. Because routine methods for quantitating viability of fungi gave inconsistent results, use of radiolabeled compounds was investigated as a method which might permit quantitation of viability of *Candida* pseudohyphae before and after incubation with leukocytes. Experiments included the effects of neutrophils upon both reduction of uptake of radioisotopes by *Candida*, as well as release of radioisotopes from *Candida* during incubations (Fig. 4). With a short incubation time (1–2 h), percentage release of [^{14}C]cytosine or [^{3}H]mannose was smaller than reduction in uptake. Prolongation of the incubation time resulted in growth of *Candida* and in consistent results. Reduction of uptake of [^{14}C]-cytosine proved a reproducible method, which correlated with reduction of uptake of [^{3}H]mannose and glucose, as well as microscopic observations. Uptake of [^{14}C]cytosine by *Candida* was linear over a 3-log range, from 1×10^4 to 1×10^7 live organisms. A ratio of 10 neutrophils: 1 *Candida* and a 1 h incubation time were optimum. In 40 experiments using neutrophils from 27 subjects under optimum conditions (Fig. 4), controls (*Candida*, which leukocytes added at the end of incubations) took up 56,654±2,806 cpm (mean ±SEM) of [^{14}C]cytosine. There was a 10.9% range of variation within triplicates. Results were comparable in experimental tubes where leukocytes and *Candida* were incubated together. To determine whether metabolic damage was reversible, [^{14}C]cytosine uptake was measured over 3 h immediately after lysis of leukocytes, and *Candida* were stored at 4°C for 4 and 18 h. In four experiments, uptake of [^{14}C]-cytosine did not increase at 4 and 18 h over baseline values (mean −2.8%, range −11.2 to +3.4%), but increased 20.3–38.7% ($P < 0.025$) for control *Candida* where leukocytes were added just before deoxycholate lysis.

Opsonization of live Candida albicans *pseudohyphae.* Because interactions between *Candida* and

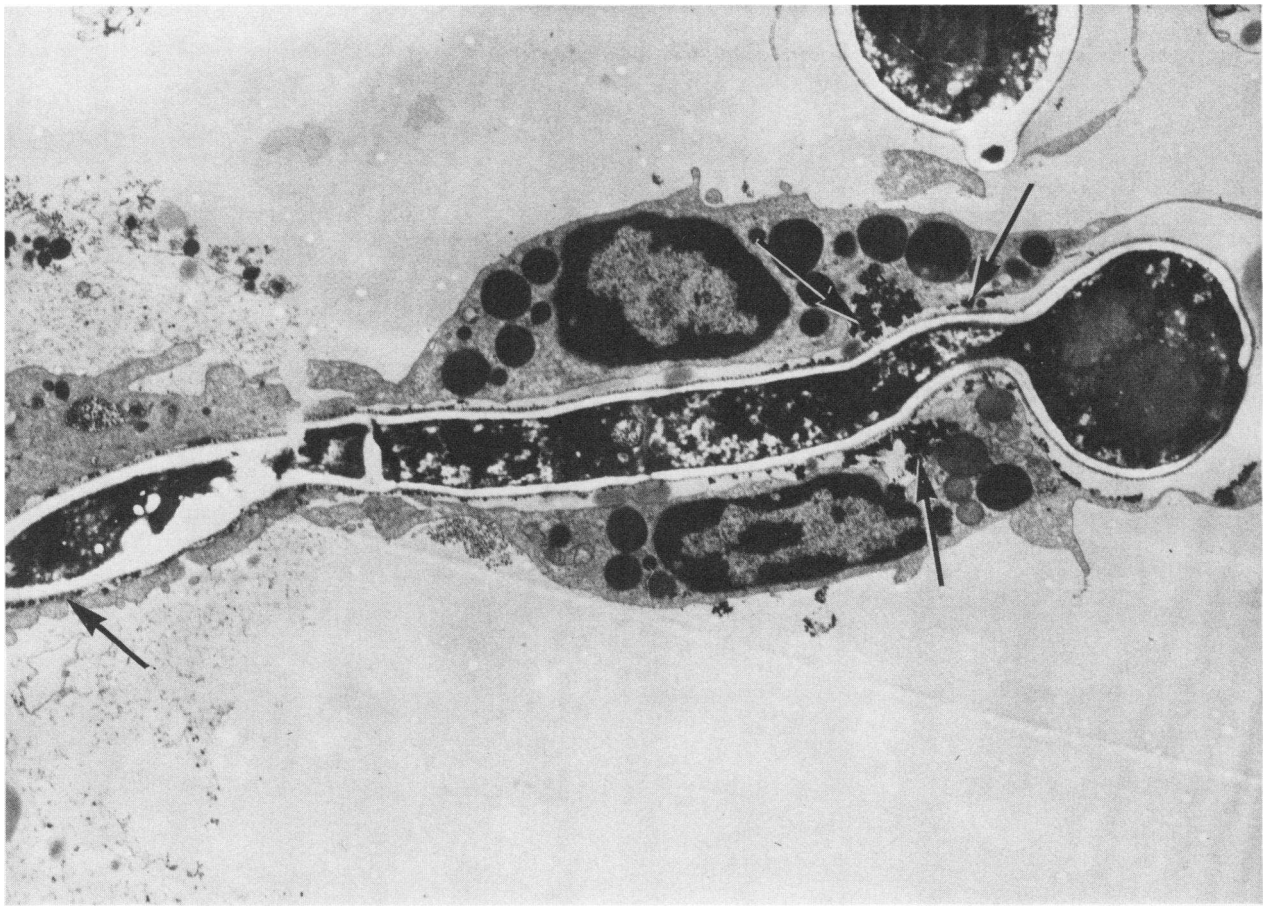

FIGURE 2 Electron micrograph taken after 60 min of incubation of neutrophils with *Candida albicans* pseudohyphae. In this study, neutrophil granules had been labeled with ferritin, and excess ferritin was washed away. Ferritin (arrows) is located specifically surrounding the partially ingested pseudohypha, and nowhere else. Magnification ×11,800.

neutrophils occurred in the absence of serum, it was possible that serum might not effect these interactions, or might increase or decrease damage to *Candida* by neutrophils. Using neutrophils from 17 normal subjects, autologous serum consistently increased the percentage reduction of [^{14}C]cytosine uptake by *Candida* ($P < 0.001$ by twotailed two-sample t test) (Fig. 5). This occurred whether serum was fresh or heated at 56°C for 60 min. When neutrophils were preincubated in serum and washed before exposure to *Candida*, there was no increment. However, with preincubation of *Candida* in serum followed by washing before incubation with neutrophils, there was augmentation of damage to *Candida*, comparable to that seen when serum was present throughout incubations (Fig. 5). Because opsonization of the organism by heated serum was as effective as fresh serum, it appeared that anti-*Candida* IgG might be involved rather than complement. Indirect fluorescent antibody determinations using fluorescein-conjugated anti-IgG indicated faint (1–2+/4+) fluorescence on *Candida* which had been incubated in normal sera diluted 1:1–1:16 in HBSS. Under the same conditions, there was no fluorescence on *Candida* surfaces using fluorescein-conjugated antialbumin or antibody to the third component of complement. However, when sera were tested for anti*Candida* antibodies using standard techniques for detection of agglutinins and precipitins, none were found. Purified preparations of human IgG were used to determine if low levels of anti-*Candida* activity might be present in immunoglobulins in normal sera. When three different preparations of normal human IgG were added to incubations containing *Candida* and neutrophils, an increment in opsonization was seen ($P < 0.05$), similar to that noted when whole serum was used (Fig. 5). To establish further that low levels of anti-*Candida* IgG were interacting with neutrophil Fc receptors, attempts were made to block activity of anti-*Candida* IgG. Heat aggregation of IgG ($P < 0.01$) or the

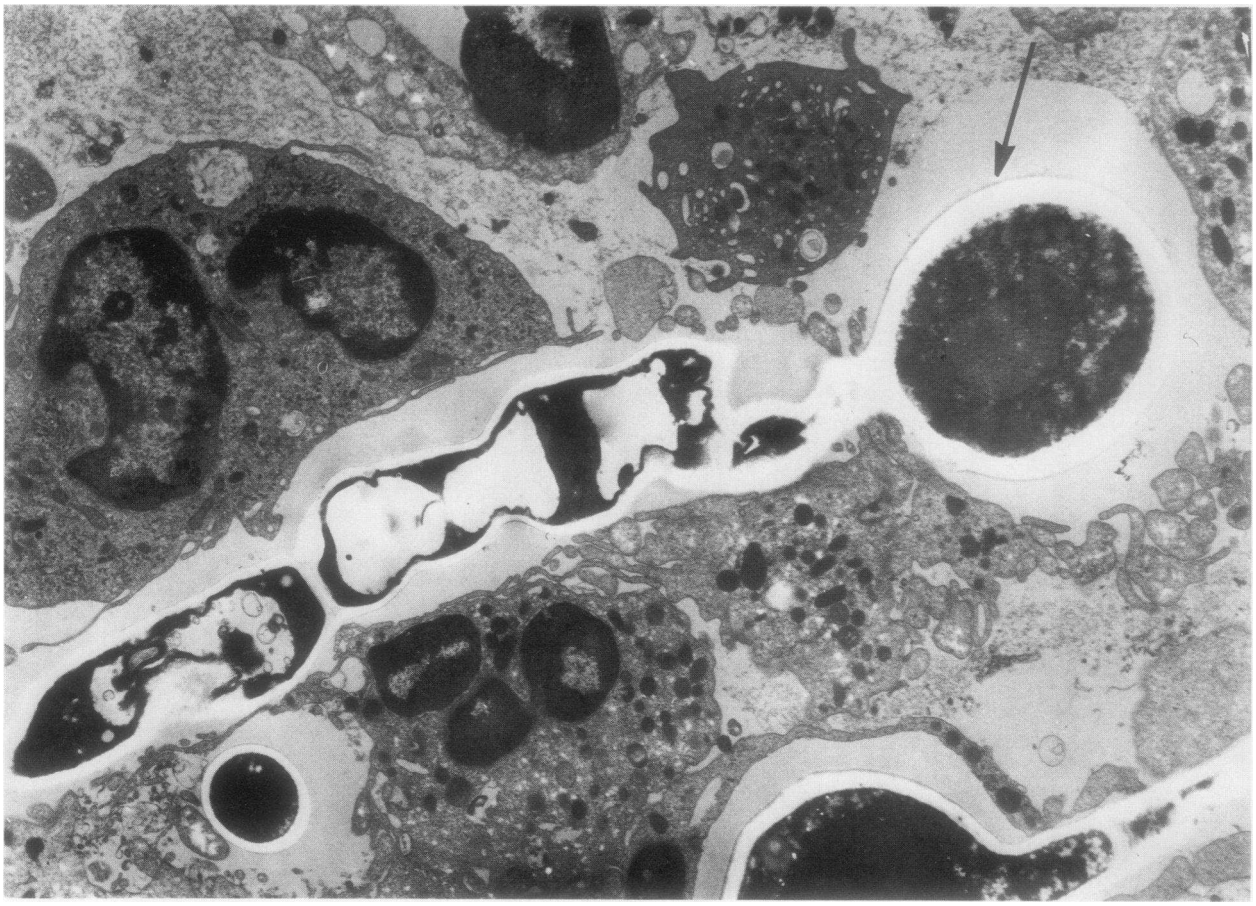

FIGURE 3 Electron micrograph taken after 60 min of incubation of neutrophils with *Candida albicans* pseudohyphae, showing changes in cell wall (arrows) and loss of discernable internal morphology of *Candida*, which is between several neutrophils. Magnification ×5,265.

presence of rheumatoid factor ($P < 0.025$) in serum (34) both eliminated the increments in opsonization normally seen with IgG or whole serum, as did absorption of IgG with *Candida*.

Unlike serum, removal of divalent cations from the medium completely inhibited interactions of neutrophils with *Candida albicans* pseudohyphae. In two experiments in HBSS, neutrophils reduced uptake of [^{14}C]-cytosine into *Candida* by 83%. However, when 10 mM sodium citrate was added to HBSS, or a calcium and magnesium-free buffer (modified Hanks' solution) was substituted for HBSS, there was no reduction in uptake of [^{14}C]cytosine into *Candida*.

Effect of inhibitors of neutrophil function on damage to Candida albicans *pseudohyphae.* Inhibitors were used which were known to be active in inhibiting the function of neutrophils with several microorganisms. The results were calculated as percent inhibition of the [^{14}C]cytosine assay. Several concentrations of each inhibitor were tested. Concentrations of inhibitors were eliminated from studies if they inhibited uptake of [^{14}C]cytosine in control incubations of *Candida* without neutrophils or if they caused ≥5% decrease in viability of neutrophils, as judged by phase contrast microscopy and by trypan blue exclusion, as well as ^{51}Cr release in selected studies. The most effective concentration of each inhibitor was then determined.

Colchicine, cytochalasin B, and 2-deoxyglucose inhibited the nonphagocytic interaction of neutrophils with *Candida* pseudohyphae (Table I). Dibutyryl cyclic AMP, and two compounds known to raise intracellular levels of cyclic AMP, theophylline and isoproterenol, all inhibited damage to *Candida albicans* pseudohyphae by neutrophils. With these compounds, there were no apparent morphologic changes which were attributable to the inhibitors on review of Giemsa-stained slides of interactions of neutrophils and *Candida*. Hydrocortisone, along with other steroid hormones and metabolites of hormones for comparison were also tested. Hydrocortisone and estriol had the highest inhibitory activity of all the

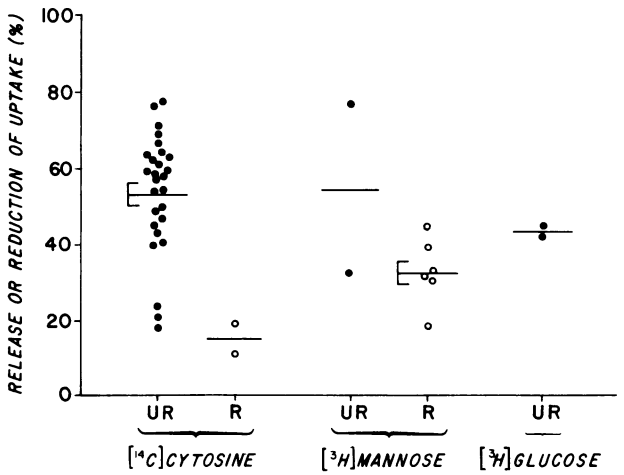

FIGURE 4 Effect of neutrophils on uptake and release of radioisotopes by *Candida albicans* pseudohyphae. Radiolabeled compounds are noted on the horizontal axis, with results for reduction in uptake of isotopes (UR), and isotope release (R). Percentage of uptake or release of isotopes is shown on the ordinate. Each point represents the mean of one or more experiments performed in triplicate on one normal individual. Horizontal lines denote means and brackets SEM for sets of observations. Experiments were performed using optimum numbers of neutrophils, and an optimum duration of incubations.

compounds which were tested (Table I). Testosterone had less inhibitory activity, whereas other steroids tested had little or no inhibitory activity.

Release of contents of neutrophil granules during contact with Candida *pseudohyphae.* Because degranulation was observed microscopically, quantitative studies were performed. A ratio of neutrophils to *Candida* of 1:1 gave optimum release of lysosomal enzymes β-glucuronidase and *N*-acetyl-β-D-glucosaminidase from neutrophils. There was only a slight but significant ($P < 0.05$ by two-sample t test) release of lysosomal enzymes by neutrophils (Table II), though microscopic observations indicated that over 90% of neutrophils were attached to *Candida*. Hydrocortisone, in the concentration which completely blocked damage to *Candida* as measured by the [^{14}C]cytosine assay, did not inhibit this small amount of release of granule enzymes from neutrophils. This was confirmed in fluorescence microscopic studies. In contrast, lysozyme was released in larger amounts during incubations with pseudohyphae ($P < 0.001$) (Table II). Hydrocortisone also did not inhibit lysozyme release.

Live vs. killed Candida albicans *pseudohyphae in iodination of* Candida *and stimulation of chemiluminescence by neutrophils.* In light microscopic studies, it was noted that neutrophils did not readily attach to killed pseudohyphae. Therefore, iodination of pseudohyphae and chemiluminescence by neutrophils in response to interaction with pseudohyphae were evaluated, to determine if these processes were activated by surface attachment of neutrophils to pseudohyphae in the absence of serum, and whether live fungi were required. In the absence of serum, neutrophils actively iodinated live *Candida albicans* (Table III). In contrast, iodination was markedly reduced when pseudohyphae were used which had been killed by heat or ultraviolet light. Comparable results were obtained in measurements of chemiluminescence. Live *Candida* stimulated chemiluminescence. by neutrophils even when serum was not present in incubations (Fig. 6). However, killed pseudohyphae did not stimulate chemiluminescence.

Interaction of fungi other than Candida *pseudohyphae with neutrophils.* In order to determine the specificity of the interaction between *Candida* pseudohyphae and neutrophils, other live fungi were tested. By phase contrast microscopic observation, in the absence of serum, only 5–8% of *Candida* yeasts were ingested by neutrophils, and only ≅5% of the yeasts were attached to the surfaces of neutrophils. The encapsulated yeast, *Cryptococcus neoformans*, was not ingested by neutrophils at all in the ab-

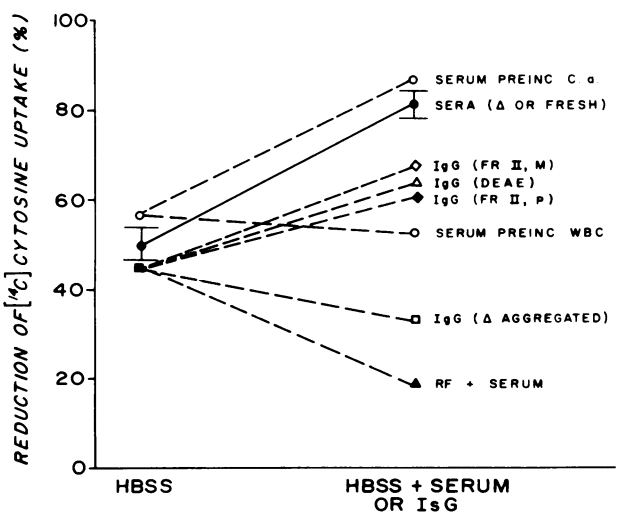

FIGURE 5 Effect of serum and IgG on opsonization of live *Candida albicans* pseudohyphae. Interaction of *Candida* with neutrophils is measured by neutrophil-induced reduction of uptake of [^{14}C]cytosine by *Candida*, shown on the ordinate. Each point represents the mean of at least two experiments, each performed in triplicate. Results are shown using buffer (HBSS) alone. The solid line connects mean and SEM values for experiments performed in HBSS with those performed with 10% heated (△) or fresh serum from 17 normal subjects. Shown separately, connected to results in controls by dotted lines, are results of preincubation of *Candida albicans* (PREINC C.a.) or neutrophils (PREINC WBC) in serum, which was washed away before incubations. Also shown separately are results using two commerical (FR II,M; FR II,P) and one freshly purified (DEAE) preparations of IgG, as well as heat (△) aggregated IgG, and sera containing rheumatoid factor (RF+).

TABLE I

Effect of Inhibitors of Neutrophil Function on Reduction of Cytosine Uptake by Candida albicans *Pseudohyphae*

Inhibitor (concentration added)	Inhibition*
	%
Colchicine (0.1 mM)	62.1
Cytochalasin B (2 μg/ml)‡	44.7
Cytochalasin B (20 μg/ml)‡	57.6
2-Deoxyglucose (0.2 mM)	100.0
2-Deoxyglucose (0.2 mM) + glucose (2 mM)	0.0
Dibutyryl cyclic AMP (1 mM)	42.8
Theophylline (1 mM)	48.1
Isoproterenol (1 mM)	65.2
Hydrocortisone (1 μM)§	34.6
Hydrocortisone (10 μM)	100.0
Estriol (1 μM)	69.9
Estriol (10 μM)	100.0
Testosterone (10 μM)	48.5
Androsterone (10 μM)	14.3
Progesterone (10 μM)	0.0
Dehydroisoandrosterone (10 μM)	2.3
17α-Hydroxyprogesterone (10 μM)	10.5

* Calculated from the results of [^{14}C]cytosine uptake in the presence and absence of inhibitors. Values represent means of at least two separate experiments, each performed in triplicate.

‡ Controls contained equivalent concentrations of dimethylsulfoxide.

§ Controls for all corticosteroids contained equivalent concentrations of ethanol.

sence of serum, nor was there close surface contact between neutrophils and yeasts. In contrast, *Rhizopus oryzae* and *Aspergillus fumigatus* became covered with neutrophils spreading over the surfaces of

TABLE II

Selective Release of Markers of Azurophil and Specific Granules of Neutrophils During Interaction with Candida *Pseudohyphae*

Present in incubation			Release*		
Neutrophils‡	*Candida*§	10 μM Hydrocortisone	β-Glucuronidase	*N*-Acetyl-β-D-glucosaminidase	Lysozyme
			%		
Yes	No	No	5.7±2.4	3.6±0.6	1.0±1.0
Yes	Yes	No	12.6±1.9	11.1±1.1	27.9±1.5
Yes	No	Yes	6.0±1.0	3.6±1.1	0.1±0.1
Yes	Yes	Yes	9.0±4.5	13.6±2.2	28.7±4.6

* Mean±SEM.

‡ Each tube contained 5 × 10^6 neutrophils.

§ Each tube contained 5 × 10^6 *Candida*.

TABLE III

Effect of Viability of Candida albicans *Pseudohyphae on Iodination of* Candida *by Neutrophils in the Absence of Serum*

Candida viability	Iodination*	Inhibition of iodination
	nmol ^{125}I fixed per 10^7 neutrophils/h	%
Live	39.8	—
Heat killed	9.0	76.8
Ultraviolet-light killed	13.2	66.0

* Mean of three experiments, each performed in triplicate.

hyphae. These observations were confirmed and better quantitated using iodination of these fungi by neutrophils in the presence and absence of serum (Table IV). Dose-response curves were performed for each organism, and the concentration which gave maximum iodination was used. Like *Candida* pseudohyphae, *Rhizopus* and *Aspergillus* were iodinated in the absence of serum, and showed an increment in iodination when serum was present. The encapsulated yeast, *Cryptococcus neoformans*, was iodinated significantly only when serum was present. *Candida* yeasts, even in increased numbers to account for differences in surface area, were not iodinated as much as *Candida* pseudohyphae.

DISCUSSION

These experiments have established that neutrophils can damage and probably kill *Candida* pseudohyphae. Pseudohyphae were too large to be completely ingested by neutrophils. Nevertheless, neutrophils at-

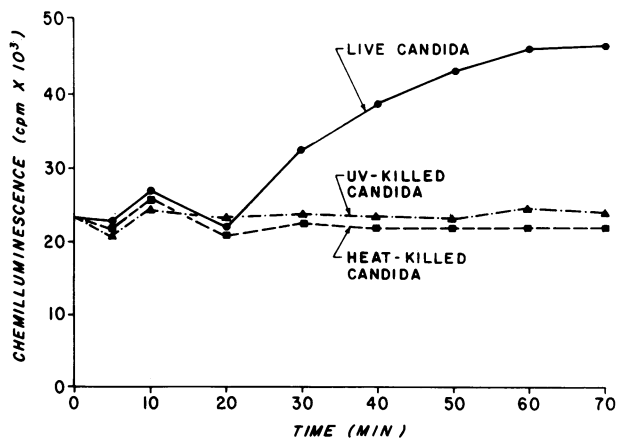

FIGURE 6 Effect of viability of *Candida albicans* pseudohyphae on chemiluminescence by neutrophils induced on contact with *Candida* in the absence of serum. Results using live *Candida* are compared with those using *Candida* killed by heat or ultraviolet (UV) light.

TABLE IV
Iodination of Fungi by Neutrophils in the Presence and Absence of Autologous Serum

Fungus* (growth phase)	Serum in incubations	Iodination
		nmol ^{125}I fixed per 10^7 neutrophils/h
Candida albicans (pseudohyphae)	No	50.6
	Yes	94.9
Candida albicans (yeasts)	No	10.0
	Yes	21.7
Cryptococcus neoformans (yeasts)	No	0.5
	Yes	14.2
Rhizopus oryzae (hyphae)	No	24.9
	Yes	86.6
Aspergillus fumigatus (hyphae)	No	65.4
	Yes	96.3

* Each tube contained 5×10^6 fungi with 1×10^7 neutrophils, except for *Candida* yeasts, where 5×10^7 tube were required for maximum iodination.

tached to and spread over the surfaces of pseudohyphae. Morphologic changes occurred in the *Candida*, as judged by light, phase contrast, and electron microscopic observations. Dramatic changes in the *Candida* cell wall by electron microscopy were similar to those described by others who observed organisms in tissues of infected mice or human patients (35) or in vitro with leukocytes (36). The significance of these morphologic changes was confirmed by parallel studies using radioisotopically labeled nutrients. These experiments indicated that metabolism of *Candida* was impaired after contact with neutrophils, and that *Candida* labeled with radioisotopes of cytosine or mannose released these compounds during incubations with neutrophils. Because conventional methods for quantitating viability of fungi could not be used with pseudohyphae, it remains possible that metabolic damage to *Candida* pseudohyphae was reversible. However, the concomitant striking morphologic changes suggested fungal cell death. In addition, even in the fungi were not killed, damage to hyphae which are too large to be ingested by phagocytic cells might still be an important host defense mechanism. This capability of neutrophils may be especially important because the large pseudohyphal and hyphal forms of *Candida* are predominant in lesions in tissues (4, 5) which contain both large and small forms of *Candida*. There have been many studies on interactions of *Candida* blastospores (or yeasts) with leukocytes (9–13, 23). However, in addition to morphologic differences between forms of *Candida*, antigens (37) and other properties may not be identical. Our experiments indicated several differences between *Candida* yeasts and pseudohyphae in interaction with neutrophils.

First, interactions between *Candida* pseudohyphae and neutrophils required no serum. In the presence of serum, there was an increment in contact between *Candida* and neutrophils, and damage to *Candida* increased. This effect was duplicated by normal human IgG, was blocked by heat aggregation of IgG or by sera which contained rheumatoid factor (34), and appeared to be attributable to opsonization of *Candida* by low levels of anti-*Candida* IgG which was present in normal human sera. This is consistent with the observations of Weiner and Yount (38), who found that antibodies to *Candida* mannan were universally present in sera from adult normal control subjects.

Despite the absence of serum, interactions of neutrophils with *Candida* pseudohyphae appeared to activate leukocyte microbicidal mechanisms, as evidenced by studies of iodination and chemiluminescence. Iodination of microorganisms generally occurs after phagocytosis, with interaction of myeloperoxidase and hydrogen peroxide within phagocytic vacuoles (39). The light emission in chemiluminescence of neutrophils has been correlated with the production of oxidizing agents by phagocytosing neutrophils (40). For microorganisms, this also requires either the presence of serum opsonins, or opsonization of organisms by incubation with serum prior to incubation with neutrophils (41). In contrast, *Candida* pseudohyphae required neither complete ingestion nor serum opsonins to be iodinated by neutrophils or induce neutrophils to chemiluminesce.

Iodination (28) and chemiluminescence (41) have both been used as sensitive screening techniques for abnormalities in the phagocytic process. Similarly, the occurrence of iodination and chemiluminescence during incubations of neutrophils with *Candida* pseudohyphae provided useful procedures for quantitation of surface interactions between fungi and leukocytes, which correlated well with light microscopic studies. These studies suggested that surface factors are present on live but not killed fungi which permit recognition by leukocytes. These factors were not limited to *Candida* pseudohyphae, as *Rhizopus oryzae* and *Aspergillus fumigatus* were iodinated in the absence of serum, and became attached to neutrophils on microscopic examination. In contrast, iodination of *Candida* yeasts was less than iodination of pseudohyphae, and *Cryptococcus neoformans*, where the yeast cell wall is covered by a capsule, was iodinated significantly only when serum was present. Consequently, it appears possible that the capacity of fungi to interact with leukocytes in the absence of serum may reflect a general property of cell walls of large forms of fungi.

Further insights were gained into interactions between *Candida* pseudohyphae through the use of substances which are known to inhibit neutrophil function. Several inhibitors of neutrophil phagocytic and other functions also inhibited nonphagocytic damage to *Candida* pseudohyphae. Damage to pseudohyphae by neutrophils was inhibited by colchicine, cytochalasin B, and 2-deoxyglucose. Colchicine is known to inhibit function of neutrophil microtubules, and may inhibit phagocytosis under some conditions as well (42). Cytochalasin B inhibits neutrophil microfilaments and blocks phagocytosis, but also causes exocytosis of lysosomal hydrolases from neutrophils, and inhibits hexose transport (43). The compound 2-deoxyglucose inhibits aerobic and anaerobic cellular metabolism as well as phagocytosis, though inhibition of phagocytosis may occur by a separate mechanism (44). Dibutyryl cyclic AMP, theophylline, and isoproterenol, all of which elevate intracellular levels of 3′5′ cyclic AMP in neutrophils, inhibited damage to *Candida* pseudohyphae without clearly interfering with surface contact or spreading over surfaces of *Candida*. Elevated levels of cyclic AMP have been shown to inhibit several functions of neutrophils, such as phagocytosis, release of lysosomal enzymes, and killing of microorganisms, including *Candida* yeasts (45). Because microscopic observations indicated that neutrophils degranulated during contact with pseudohyphae, the effects of hydrocortisone were then studied. Besides the well-known association between corticosteroid therapy and invasive candidiasis (4, 5), cortisone may inhibit release of lysosomal enzymes from neutrophils (46). Hydrocortisone inhibited damage to *Candida* pseudohyphae by neutrophils at levels (10–1 μM) which may be reached in vivo after pharmacologic doses. Nevertheless, there was no visible or quantitative inhibition of degranulation of neutrophils by hydrocortisone. Hydrocortisone appeared to inhibit damage to pseudohyphae by inhibiting a neutrophil mechanism other than degranulation.

Despite the visual evidence for degranulation of neutrophils during contact with pseudohyphae, there was only minimal quantitative release of two lysosomal enzymes from neutrophils. In contrast, lysozyme was released in much larger amounts. Within neutrophils, lysosomal enzymes are located in the azurophil granules, while lysozyme is present in the specific granules, which appear to discharge first (47). Contact between neutrophils and pseudohyphae may provide a less complete stimulus for degranulation than phagocytosis, so that contents of specific granules are released preferentially.

Further experiments will be required to delineate which mechanism is primarily responsible for metabolic and morphologic damage to *Candida* pseudohyphae by neutrophils. In addition, neutrophils directly recognize *Candida* pseudohyphae and other fungi and activate cellular microbicidal mechanisms in the absence of serum opsonins by a mechanism which must be characterized, although the interaction is enhanced by serum. Although the biologic importance of this new neutrophil mechanism remains to be determined, these experiments have established the utility of new methodology which permits quantitative studies of the interactions between neutrophils and large forms of pathogenic fungi.

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