Mechanisms of Attachment of Neutrophils to *Candida albicans* Pseudohyphae in the Absence of Serum, and of Subsequent Damage to Pseudohyphae by Microbicidal Processes of Neutrophils In Vitro

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ABSTRACT Mechanisms were studied that might explain the attachment and damage to *Candida albicans* pseudohyphae by neutrophils in the absence of serum. Attachment of neutrophils to pseudo hyphae was inhibited by *Candida* mannans (1–10 mg/ml), but not by mannose, dextran, chitin, conconavalin A, or highly charged polyamino acids. Contact was also inhibited by pretreatment of *Candida* before incubation with neutrophils with chymotrypsin, but not trypsin or several inhibitors of proteases. Similar results were obtained with pretreatment of neutrophils, except that trypsin was inhibitory. When pseudohyphae were killed with ultraviolet light, protein-polysaccharide complexes of mol wt <10,000 were released which appeared to bind to the surfaces of neutrophils and inhibit contact between neutrophils and *Candida*, as well as other fungi.

Damage to *Candida* by neutrophils was inhibited by agents known to act on neutrophil oxidative microbicidal mechanisms, including sodium cyanide, sodium azide, catalase, superoxide dismutase, and 1, 4 diazobicyclo (2, 2, 2) octane, a singlet oxygen quencher. Neutrophils from a patient with chronic granulomatous disease did not damage *Candida* at all. However, the hydroxyl radical scavengers mannitol and benzoate were not inhibitory. Cationic proteins and lactoferrin also did not appear to play a major role in this system. Low concentrations of lysozyme which did not damage *Candida* in isotonic buffer solutions damaged pseudohyphae in distilled water. Isolated neutrophil granules damaged pseudohyphae only with added hydrogen peroxide and halide, and damage occurred only with granule fractions known to contain myeloperoxidase. These findings suggest that neutrophils recognized a molecule on the *Candida* surface which has a chymotrypsin sensitive protein component, and which may be liberated from the cell surface upon death of organism. The neutrophil receptors for *Candida* appear to be sensitive to trypsin and chymotrypsin. Damage to *Candida* by neutrophils occurred primarily by oxidative mechanisms, including the production of superoxide and hydrogen peroxide interacting with myeloperoxidase and halide, as well as singlet oxygen, but did not appear to involve hydroxyl radical. Lysozyme might have an accessory role, under some conditions.

INTRODUCTION

In earlier studies, we demonstrated that human peripheral blood neutrophils could completely ingest, and probably kill *Candida albicans* pseudohyphae in vitro (2). There appeared to be receptors on the neutrophil surface which directly recognized *Candida* pseudohyphae, without opsonization of *Candida* by specific antibody or by complement components and triggered leukocyte microbicidal mechanisms. This might represent neutrophil receptors for immunoglobulin or complement, but a stereospecific receptor for a molecule on the surface of fungi may be involved as well (3). Neutrophil receptors may also have enzymatic activity which can be detected (4). Another possibility is that of a cell receptor for mannose (5), because the *Candida* cell wall has a high

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content of mannose, primarily in the form of mannans (6, 7). The *Candida* cell wall also contains glucans, glucomannans, perhaps chitin, and mannoproteins (7–10), which might be involved in reactions with surface receptors of neutrophils. Our previous studies indicated that only live and not killed *Candida* interacted with neutrophils (1), suggesting that the material which is recognized by neutrophils is either labile or loosely attached to the *Candida* surface.

Once neutrophils are in close surface contact with *Candida*, several potential neutrophil microbicidal mechanisms may be operative. Yeast forms of *Candida* are killed by oxidative mechanisms of neutrophils (11–14). Our earlier studies indicated that *Candida* pseudohyphae were iodinated by neutrophils (1), suggesting that incomplete ingestion of pseudohyphae activates oxidative microbicidal mechanisms in the neutrophil (14–16). In some circumstances, other mechanisms of candidacidal activity may be involved (18), including granulocyte cationic proteins (19). Other constituents of neutrophil granules, including lactoferrin (20) and lysozyme (21), may also inhibit *Candida* yeasts. There are morphologic and antigenic (22) differences between *Candida* yeasts and pseudohyphae and differences in their interactions with neutrophils, so that neutrophil microbicidal mechanisms for yeasts may not be identical to those for pseudohyphae.

In these studies, we have defined some of the surface factors of *Candida* pseudohyphae and neutrophils which are required for attachment and subsequent activation of neutrophil microbicidal mechanisms. We then examined potential neutrophil microbicidal mechanisms and found that damage to *Candida* pseudohyphae was largely if not totally attributable to oxidative processes.

METHODS

Fungi. An isolate of *Candida albicans* was obtained from a patient with systemic candidiasis and germinated in Eagle's minimal essential medium which had been supplemented with nonessential amino acids (Grant Island Biological Co., Grand Island, N. Y.). *Candida* were incubated at 37°C until ≥95% of cells had formed germ tubes ≥30 μM in length. Spores from patient isolates of *Aspergillus fumigatus* and *Rhizopus oryzae* (supplied by Dr. John Rippon, University of Chicago, Chicago, Ill.) were germinated in the same way. A small capsule isolate of *Cryptococcus neoformans* (supplied by Dr. John E. Bennett, National Institutes of Health, Bethesda, Md.) was also used in some studies. When required, fungi were killed by heating at 100°C (in a boiling water bath) for 1 h, or by exposure to ultraviolet light for 16 h. Before incubation with neutrophils, fungi were washed in Hanks' balance salt solution (HBSS).[1]

[1] *Abbreviations used in this paper*: DFP, diisopropylphosphorofluoridate; HBSS, Hank's balanced salt solution; TLCK, N-α-p-tosyl-L-lysine; TPCK (1-tosylamide-2-phenyl-ethyl-chloromethyl ketone).

Neutrophils. Human neutrophils were obtained by sedimenting peripheral blood erythrocytes with 3% dextran (average mol wt 250,000; Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) in saline for 20–30 min. Remaining erythrocytes were lysed in an ammonium chloride-Tris buffer solution (23). Where greater purity of neutrophils was required, a Ficoll-(Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) Hypaque (sodium and meglumine diatrizoates; Winthrop Laboratories, New York) separation was used, followed by dextran sedimentation, as described by Böyum (24). Autologous serum was obtained from normal volunteer subjects for selected studies at the same time as neutrophils.

Neutrophils were obtained from one patient with X-linked chronic granulomatous disease (supplied through the courtesy of Dr. Lauren Pachman, Children's Memorial Hospital, Chicago, Ill.).

Separation of neutrophil granules. Neutrophil granules were separated and purified into three components on sucrose gradients using the method of West et al. (25). After separation of neutrophils by Hypaque-Ficoll followed by dextran sedimentation, neutrophils were suspended in 0.34 M sucrose containing 100 U/ml preservative-free heparin (Connaught Laboratories, Toronto, Ontario, Canada). After lysis by multiple passages through a long needle, nuclei and nongranular debris were removed by sequential passage through 5 μm and 2 μm pore size polycarbonate filters (Nucleopore Corp., Pleasanton, Calif.). Continuous sucrose gradients were prepared by mixing light and heavy sucrose, and layering on a 3.01-ml cushion of heavy sucrose. Gradients extended from sp gr 1.10–1.287 gm/ml, or 1.4–1.24 gm/ml. After centrifugation at 95,000 *g* for 4 h at 4°C, fractions were collected. Bands containing the three granule fractions were either used directly, or were washed to remove sucrose and brought up in HBSS.

Granule preparations were then incubated with 1×10^6 *Candida albicans* pseudohyphae for 1 h at 37°C with and without added graded concentrations of sodium chloride or iodide and(or) hydrogen peroxide. In selected experiments, the effect of an individual granule constituent was studied using lysozyme. Egg white lysozyme (18,000 U/ml, three times recrystallized, Schwarz/Mann, Div., Becton, Dickinson & Co., Orangeburg, N. Y.) was dissolved in distilled water, diluted in distilled water or HBSS, and incubated with 1×10^6 *Candida albicans* as described above.

Effect of neutrophils on uptake of [^{14}C]*cytosine by* Candida *pseudohyphae.* Suspensions containing 1×10^6 *Candida albicans* pseudohyphae and 1×10^7 neutrophils in 1 ml HBSS were placed in 15 ml plastic centrifuge tubes (Corning Glass Works, Science Products Div., Corning, N. Y.). Instead of neutrophils, some studies contained graded concentrations of lysozyme or purified lysed neutrophil granules with or without hydrogen peroxide and(or) sodium iodide or chloride. Control tubes contained 1×10^6 *Candida* pseudohyphae in HBSS, without neutrophils, lysozyme or granule fractions. Triplicate tubes were incubated for 1 h at 37°C in a rotator (Scientific Industries, Inc., Springfield, Mass.). Additional tubes were added to provide aliquots for microscope observations. After the incubations, where experimental tubes contained neutrophils, 1×10^7 neutrophils were added to each control tube and neutrophils in all tubes were lysed by the addition of 0.25 ml of 2.5% sodium deoxycholate. Remaining *Candida* were washed twice in distilled water and once in yeast nitrogen base broth which had been supplemented to include 1% dextrose and 0.15% asparagine. To each tube was then added 0.25 μCi of [^{14}C]cytosine (59–61 mCi/mmol; Amersham/Searle Corp., Arlington Heights, Ill.) dissolved in yeast nitrogen

base broth which had been supplemented as noted above. After incubation at 30°C for 1 h, *Candida* were washed-free of unbound [^{14}C]cytosine using an automated multiple sample harvester (Otto Hiller Co., Madison, Wis.). Filters containing *Candida* were dried in air overnight, placed in vials with Aquasol (New England Nuclear, Boston, Mass.), and counted in a liquid scintillation counter. The neutrophil induced percentage of reduction in uptake of [^{14}C]cytosine by *Candida* was calculated from (mean counts per minute in control tubes)-(mean counts per min in experimental tubes)/(mean in counts per minute in control tubes) $\times 100$.

Iodination of Candida *and other fungi by neutrophils.* The fixation of iodide to *Candida* by neutrophils was measured by the method of Klebanoff and Clark (26), with the following modifications. Serum was excluded unless otherwise stated. Optimum concentrations of neutrophils, buffer constituents, ^{125}I, and each of the organisms were determined. Unless otherwise noted, this included 1×10^7 neutrophils and 5×10^6 *Candida* or other fungi, and 0.5 μM (0.2 μCi) Na ^{125}I (Amersham/Searle Corp.) and tumbled at 37°C for 1 h.

Chemiluminescence by neutrophils attached to Candida *pseudohyphae.* Chemiluminescence was measured in a Beckman model LS 230 liquid scintillation counter (Beckman Instruments, Inc., Republic Operations, Chicago, Ill.) by the method of Stjernholm et al. (27). Vials contained 1×10^7 *Candida* in HBSS, or HBSS alone. After base-line counts, 1×10^7 neutrophils were added, and counts were obtained at timed intervals.

Inhibitors. In some studies, potential inhibitors of contact between *Candida* pseudohyphae and neutrophils or inhibitors of neutrophil microbicidal function were added to incubations. Unless otherwise noted, compounds were obtained from Sigma Chemical Co., St. Louis, Mo. Purified types A and B *Candida albicans* mannans (5) were kindly supplied by Dr. Herbert Hasenclaver (Rocky Mountain Laboratory, Hamilton, Mont.). Concentrations of polymerized amino acids (polyaspartic acid, polyglutamic acid, and polyarginine) were calculated on the basis of moles of the single amino acid. A purified preparation of aprotinin (Trasylol; FBA Pharmaceuticals, Inc., New York) and α-1-anti-trypsin were gifts from Dr. Kenneth Robbins (Michael Reese Hospital, Chicago, Ill.). Diisopropylphosphorofluoridate (DFP) was dissolved in isopropyl alcohol and used with controls which contained equivalent concentrations of isopropyl alcohol but no DFP. Heparin was preservative-free (Connaught Laboratories, Toronto, Ontario, Canada). Triethylenediamine, also known as 1,4 diazobicyclo (2,2,2) octane, was supplied by Eastman Organic Chemicals Div., Eastman Kodak Co. (Rochester, N. Y.). Chitin was used as a particulate suspension, or dissolved in concentrated hydrochloric suspension, or dissolved in concentrated hydrochloric acid and buffered to pH 7.4 just before use. Ferric chloride and ferric ammonium sulfate were dissolved in HBSS, and pH adjusted to 7.4. Superoxide dismutase and catalase were used with controls which contained enzymes that had been inactivated by autoclaving. Other inhibitors used included conconavalin A, mannose, dextran (average mol wt 10,000), trypsin, chymotrypsin, L-1 tosylamide-2-phenylethyl-chlormethyl ketone (TPCK), *N*-α-p-tosyl-L-lysine (TLCK), sodium cyanide, sodium azide, mannitol, sodium benzoate.

For preincubation studies, either *Candida* pseudohyphae or neutrophils were incubated with inhibitors for 10–60 min at 37°C, then washed-free of inhibitor with HBSS. For other studies, inhibitors remained in incubations together with neutrophils and *Candida* for 1 h at 37°C.

Preparation of extract from Candida albicans. Cultures of *Candida albicans* pseudohyphae were suspended in saline, placed in flat petri dishes, and killed by exposure to ultraviolet light. *Candida* were removed by centrifugation. Supernates were then either dialyzed against 20 vol of normal saline for 48–72 h at 4°C, or ultrafiltered to exclude substances with mol wt $\geq$10,000, using an Amicon model 12 with a UM10 Filter (Amicon Corp., Lexington, Mass.) Filtrates were lyophilized or stored at −20°C until used. For further purification, gel chromatography was done using Sephadex G-25 (Pharmacia, Piscataway, N. J.) at 4°C on a column (40 cm long and 16 mm in diameter) at 15 ml/h flow rate. Protein concentration was estimated by absorbance at 280 nm and verified by Lowry's technique (28), whereas carbohydrates were quantitated by Morris' method for the anthrone test (29) as modified by Koehler (30, 31).

Statistical methods. Means and standard errors of means were compared using two sample *t* tests (32).

RESULTS

Effects of inhibitors upon contact between neutrophils and Candida *pseudohyphae.* Contact between neutrophils and *Candida* was observed under phase-contrast microscopy and quantitated by neutrophil-induced reduction of [^{14}C]cytosine uptake by *Candida*. All inhibitors were tested in a range of concentrations. Concentrations were excluded which inhibited uptake of [^{14}C]cytosine by *Candida* in control incubations without neutrophils. For each inhibitor, the concentration was noted which gave greatest inhibition, or which was the highest concentration tested where no inhibition occurred.

Purified *Candida albicans* mannans, the major constituents of the outer cell wall of *Candida*, inhibited contact, but only in high concentrations (Table I). However, similarly high concentrations of mannose, dextran, and chitin did not. Conconavalin A, a lectin which is known to bind to neutrophils (33) and poly-

TABLE I

Inhibition of Contact Between Neutrophils and Candida albicans *Pseudohyphae Determined by Changes in Uptake of* [^{14}C]*Cytosine by* Candida

Inhibitor (concentration added)	Inhibition*
	%
Type A *Candida albicans* mannan (1 mg/ml)	34.1
Type B *Candida albicans* mannan (10 mg/ml)	100.0
Mannose (25 mg/ml, 0.14 M)	13.4
Dextran (10 mg/ml)	4.9
Chitin (1 mg/ml)	0.0
Conconavalin A (100 μg/ml)	0.0
Polyaspartic acid (10 μM)	12.1
Polyglutamic acid (10 μM)	8.2
Polyarginine (10 μM)	11.3

* 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.

saccharides of fungal cell walls (5), was not inhibitory in concentrations ranging from 1 to 100 μg/ml. In fact, 10 μg/ml conconavalin A stimulated association between *Candida* and neutrophils 29.3%, as judged by the [^{14}C]cytosine assay. To determine whether cell surface charge was an important factor in contact between neutrophils and *Candida*, polymerized charged amino acids were used in concentrations known to inhibit crystallization of calcium oxalate (34), but not uptake of [^{14}C]cytosine in control incubations with *Candida*. Polyaspartic acid, polyglutamic acid, and polyarginine did not significantly alter contact between neutrophils and *Candida* (Table I).

Enzymes and enzyme inhibitors were then tested for their effects on the surfaces of neutrophils and *Candida*. Before incubation with neutrophils, *Candida* were preincubated for 10–60 min with trypsin and chymotrypsin, as well as the protease inhibitors Trasylol (aprotinin), TPCK, TLCK, and DFP. After these compounds were washed away and neutrophils were added, chymotrypsin strongly inhibited contact between neutrophils and *Candida*, but the other compounds had no effect (Table II). This correlated with observations using phase-contrast microscopy where chymotrypsin reduced the number of pseudohyphae attached to neutrophils by 97.8%. When these compounds were preincubated with neutrophils before the addition of *Candida*, both trypsin and chymotrypsin were inhibitory, whereas the enzyme inhibitors were not (Table II). Comparable inhibition of contact between neutrophils and pseudohyphae were observed by phase-contrast microscopy. Even when Trasylol (aprotinin) was present in incubations of *Candida* and neutrophils for 1 h, there was no inhibition of contact between *Candida* and neutrophils. This appeared to be true for DFP as well, but results were indefinite because both DFP and its isopropyl alcohol solvent inhibited *Candida* in control tubes after the 1 h incubation.

TABLE II

Changes in Uptake of [^{14}C]*Cytosine by* Candida albicans *Pseudohyphae After Preincubation of* Candida *or Neutrophils with Potential Inhibitors of Surface Contact*

Preincubation inhibitor*	Cell preincubated	Inhibition‡
		%
Trypsin (0.25%)	*Candida*	0.0
Chymotrypsin (0.25%)	*Candida*	100.0
Trasylol (aprotinin) (0.1 mM)	*Candida*	0.0
TPCK (1 mM)	*Candida*	2.9
TLCK (1 mM)	*Candida*	0.0
DFP (5 mM)§	*Candida*	0.0
Trypsin (0.25%)	Neutrophil	78.5
Chymotrypsin (0.25%)	Neutrophil	100.0
Trasylol (aprotinin) (0.1 mM)	Neutrophil	3.2
TPCK (1 mM)	Neutrophil	0.0
TLCK (1 mM)	Neutrophil	0.0
DFP (5 mM)	Neutrophil	0.0

* Either *Candida* (or neutrophils) were incubated with the potential inhibitor for 10–16 min., then washed-free of inhibitor before incubation with neutrophils (or *Candida*).

‡ Calculated from the results of [^{14}C]cytosine uptake in the presence and absence of inhibitors.

§ Controls contained equivalent concentrations of isopropyl alcohol.

Inhibition of contact between neutrophils and Candida *or other fungi by a product of* Candida albicans *pseudohyphae.* Previous studies showed that killing of *Candida albicans* pseudohyphae eliminated their ability to attach to neutrophils in the absence of serum (1). Therefore, cultures of *Candida* pseudohyphae were killed with ultraviolet light, and supernates were examined to determine whether some material released upon death of the organism might be related to contact with neutrophils. When added to incubations of live pseudohyphae with neutrophils, supernates from cultures of killed *Candida* inhibited contact between neutrophils and *Candida*, as judged by 100% inhibition of contact under phase-contrast microscopy, 100% inhibition of the [^{14}C]cytosine assay, and 80.2% inhibition of iodination of *Candida* by neutrophils. Upon dialysis of these supernates against 20 vol of saline, 79.6% of the inhibitory activity for iodination was recovered in the dialysate, when compared with the original whole supernate. Similarly, ultrafiltration of supernates to exclude substances with mol wt >10,000 resulted in recovery of 70.7% of inhibitory activity for iodination and 79.5% of inhibitory activity for the cytosine assay in the filtrate. Preliminary studies involving further purification of inhibitory substances on a Sephadex G-25 column revealed two major peaks with inhibitory activity, one in the void volume (>5,000 mol wt) and one of <5,000 mol wt. The latter had the greatest specific activity for inhibition of iodination of *Candida* by neutrophils. It was composed of 23.4% carbohydrate and 76.6% protein calculated using a glucose standard for the anthrone test, vs. 30.4% carbohydrate and 69.9% protein using a mannose standard.

Because earlier studies indicated that other fungi besides *Candida* interacted with neutrophils in the absence of serum (1), it appeared possible that inhibitory substances from *Candida* might affect other fungi as well. Therefore, the inhibitor from supernates of killed *Candida* was tested with neutrophils and several species of fungi, in the presence and absence of serum. *Cryptococcus neoformans* was included as an encapsulated organism which was known, from our previous studies, not to interact with neutrophils in

the absence of serum. Serum augmented iodination of all organisms tested, as in previous studies (1). Iodination of *Candida* pseudohyphae, *Rhizopus*, and *Aspergillus* was inhibited by *Candida* supernate in the presence and absence of serum (Table III). In contrast, iodination of *Cryptococcus* was inhibited by Candida supernate when serum was present, but was stimulated by the "inhibitor" in the absence of serum. There was no increment in phagocytosis or attachment to neutrophils when cryptococci were preincubated with the supernates from killed *Candida* for 1 h, washed three times by vortexing for 1 min in large volumes of either distilled water or saline, and then incubated with neutrophils. However, there were significant increments in phagocytosis of cryptococci when neutrophils were preincubated with the *Candida* supernates for 5–15 min, washed, and then incubated with cryptococci. Incubation of neutrophils in *Candida* supernates for 1 h in the absence of fungi resulted in complete agglutination of neutrophils.

Effects of inhibitors of neutrophil microbicidal function on damage to Candida *pseudohyphae by neutrophils.* To determine the relative importance of potential mechanisms for damage to *Candida* by neutrophils, known inhibitors of neutrophil mechanisms were used as in the previous work (1), concentrations of inhibitors were eliminated which impaired variability of *Candida* or leukocytes. Damage to *Candida* pseudohyphae, determined by the [^{14}C]-cytosine assay, was inhibited by several agents known to act on neutrophil oxidative microbicidal mechanisms, including sodium cyanide, sodium azide, catalase, superoxide dismutase, and 1,4 diazobicyclo (2,2,2) octone, a singlet oxygen quencher (35) (Table IV). However, mannitol and benzoate, scavengers of hydroxyl radical (16), were not inhibitory. To further test the role of oxidative microbicidal mechanisms, neutrophils from a patient with chronic granulomatous disease were used, and found completely lacking in capacity to damage pseudohyphae. By phase-contrast microscopy, these neutrophils attached to pseudohyphae and spread over the surfaces of *Candida* as well as neutrophils from normal subjects. Other potential microbicidal mechanisms were then evaluated. The amount of iron required for total binding of lactoferrin was calculated from the molecular weight and known content of lactoferrin in neutrophils (36, 37). 10-fold excess ferric chloride or ferric ammonium sulfate was added to incubations of neutrophils and *Candida*, without effect on damage to *Candida* (Table IV). Inhibitors of leukocyte cationic proteins were tested,

TABLE III

Iodination of Candida *and other Fungi by Neutrophils and its Inhibition by a Product of* Candida *Pseudohyphae*

Fungus incubated with neutrophils	Serum in incubations	*Candida* product* in incubations	Iodination (nmol ^{125}I fixed/10^7 neutrophils/h)	Change in iodination
				%
Candida albicans	No	No	42.8	—
(pseudohyphae)	No	Yes	12.4	−71.0
	Yes	No	97.9	—
	Yes	Yes	3.0	−96.9
Rhizopus oryzae	No	No	39.4	—
(hyphae)	No	Yes	0.8	−97.8
	Yes	No	92.0	—
	Yes	Yes	0.4	−99.0
Aspergillus	No	No	70.0	—
fumigatus	No	Yes	1.2	−98.3
(hyphae)	Yes	No	110.0	—
	Yes	Yes	0.5	−99.3
Cryptococcus	No	No	1.9	—
neoformans	No	Yes	19.3	+915.8
(encapsulated	Yes	No	45.1	—
yeasts)	Yes	Yes	6.8	−84.9

* Material purified from supernate of cultures of *Candida albicans* pseudohyphae which had been killed by ultraviolet light.

TABLE IV

Effect of Inhibitors of Neutrophil Microbicidal Mechanisms on Reduction of Cytosine Uptake by Candida albicans *pseudohyphae*

Inhibitor (concentration added)	Inhibition*
	%
Sodium cyanide (1 mM)	100.0
Sodium azide (0.1 mM)	54.9
Catalase (5,000 U)‡	20.5
Superoxide dismutase (100 U)‡	23.3
DABCO§ (0.1 mM)	30.0
Mannitol (0.4 M)	2.9
Sodium benzoate (20 mM)	0.0
Chronic granulomatous disease patient's neutrophils	100.0
Ferric chloride, ferric Ammonium sulfate (10 μM)	0.0
Heparin (10 U/ml)	17.8
Heparin (100 U/ml)	73.9
α1-Antitrypsin (0.1 mM)	61.0
Polyaspartic acid (10 M)	0.8
Polyglutamic acid (10 μM)	6.7

* Calculated from 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 heat inactivated enzymes.

§ DABCO, 1,4 diazobicyclo (2,2,2) octane.

including α1-antitrypsin, (38), heparin (39), and other polyanions (polyaspartic and polyglutamic acids). Heparin and α1-antitrypsin inhibited damage to *Candida*, but the polyamino acids did not (Table IV). If heparin and α1-antitrypsin act specifically on cationic proteins, they should have no effect on neutrophil oxidative mechanisms. Therefore, iodination of Candida by neutrophils and chemiluminescence by neutrophils interacting with *Candida* were tested. Direct and indirect inhibitors of neutrophil oxidative mechanisms inhibited iodination (Table V) and chemiluminescence (Fig. 1) as expected, but so did α1-antitrypsin and heparin. Lysozyme, another constituent of neutrophils which may damage fungi (21), damaged *Candida* pseudohyphae only in high concentrations in HBSS, but damaged the fungi in low concentrations when incubations were performed in distilled water (Table VI).

Effect of isolated neutrophil granules on Candida *pseudohyphae.* To further delineate the mechanisms of damage to *Candida* by neutrophils, neutrophils were lysed, and granules were separated from other cell constituents by double filtration. Granules were prepared with and without heparin. Hydrogen peroxide, with or without chloride or iodide was added to some preparations to assess the role of myeloperoxidase. With inhibition of uptake of [^{14}C]cytosine by *Candida*, granule preparations damaged *Candida* (87.2–97.0%) only if heparin was included, and only if hydrogen peroxide ($1.0–2.5 \times 10^{-4}$M) and excess chloride (in HBSS), or sodium iodide (0.6 μg/ml) was added. No damage to *Candida* occurred if heparin was present without peroxide or halide. Granules were then further separated into three fractions on sucrose gradients. As in the above findings with unseparated granules, there was no damage to *Candida* by any of these granule fractions alone. Similarly, the two denser fractions, which contain 86.7% of total granule myeloperoxidase (25), damaged *Candida* with added hydrogen peroxide and halide, whereas the lightest fraction remained inactive.

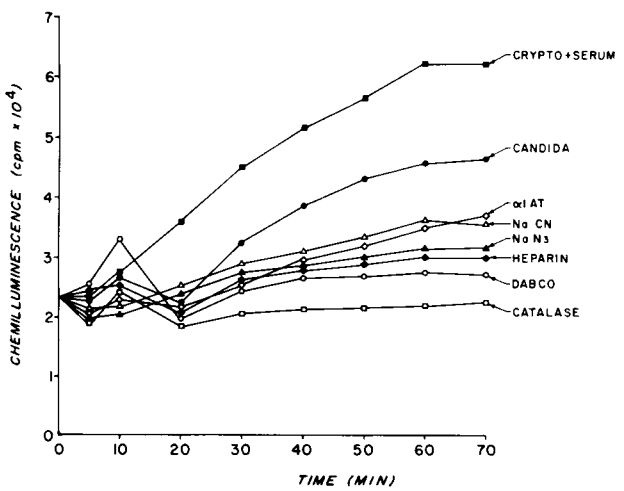

FIGURE 1 Effect of inhibitors of neutrophil microbicidal function on chemiluminescence of neutrophils stimulated by live *Candida albicans* pseudohyphae in the absence of serum. Results are shown for *Candida albicans* (CANDIDA) alone, and with inhibitors: α1-antitrypsin (α1-AT), sodium cyanide (NaCN), sodium azide (NaN_3), heparin, 1,4 diazobicyclo (2,2,2) octane (DABCO), and catalase. For comparison, results using *Cryptococcus neoformans* (CRYPTO) yeasts with serum are shown.

TABLE V

Effect of Inhibitors of Neutrophil Microbicidal Function on Iodination of Live Candida albicans *pseudohyphae by Neutrophils in the Absence of Serum*

Inhibitor (concentration added)	Iodination (nmol ^{125}I fixed to *Candida*/10 neutrophils/h)	Inhibition of iodination
		%
None	35.6	0.0
Sodium azide (0.1 mM)	4.0	88.9
Sodium cyanide (1 mM)	2.5	93.1
Catalase (5,000 U)	20.8	41.7
DABCO‡ (0.1 mM)	58.5	0.0*
Dibutyryl cyclic AMP (1 mM)	28.8	19.2
Theophylline	17.6	50.5
Heparin (100 U)	31.5	21.3
α1-Antitrypsin	7.2	79.7

* Represents 64.4% stimulation of iodination.
‡ DABCO, 1,4 diazobicyclo (2,2,2) octane.

DISCUSSION

For contact to occur between neutrophils and *Candida* pseudohyphae, the outer layer of pseudohyphae must be recognized by receptors on the surface of neu-

TABLE VI

Damage to Candida albicans *Pseudohyphae by Lysozyme as Shown by Reduction in Uptake of* [^{14}C]*Cytosine*

Concentration of lysozyme	Diluent of lysozyme	Reduction in [^{14}C]Cytosine uptake by *Candida*
μg/ml		%
10	HBSS	0.0
100	HBSS	11.9
1,000	HBSS	21.5
10,000	HBSS	43.0
10	Distilled water	76.6
100	Distilled water	99.7
1,000	Distilled water	99.6
10,000	Distilled water	89.6

trophils. The process resembles incomplete phagocytosis, and probably occurs by a similar mechanism, involving sequential interactions of receptors on organism and neutrophil (40). Through the use of inhibitors, we defined some features that affect attachment of neutrophils to *Candida*. Both trypsin and chymotrypsin treatment of neutrophils inhibited contact between neutrophils and pseudohyphae, suggesting a protein-containing structure on the neutrophil surface is required for attachment to pseudohyphae. The effects of serine esterases were also studied because phagocytosis (4, 41) has been related to the presence of serine esterases. The activity of such a molecule could not be confirmed in serum-independent interaction of neutrophils and pseudohyphae in our studies. Several protease inhibitors were used, including TPCK, which is most active against trypsinlike esterases, TLCK, which is most active against chymotrypsin-like esterases (42), Trasylol (43), and DFP.

Inhibitors were also used in an attempt to determine the structure on the cell wall of pseudohyphae which is recognized by neutrophils. Mannans in the *Candida* cell wall appeared to be strong possibilities for molecules which might be recognized by leukocytes, because of the known existence of mammalian cell receptors for mannose (5) and lectins (33). Purified Types A and B *Candida* mannans did specifically inhibit contact between neutrophils and pseudohyphae, but only at extremely high concentrations (1.0–10.0 mg/ml), making a role for them in vivo extremely doubtful. Highly charged polymers of amino acids, at concentrations high enough to cause charge-dependent inhibition of crystallization of calcium oxalate, did not inhibit contact between neutrophils and *Candida*, suggesting that highly charged molecules on the *Candida* (or neutrophil) surface are not involved. Chymotrypsin, but not trypsin treatment of *Candida* blocked contact with neutrophils. Like neutrophils, protease inhibitors (TPCK, TLCK, Trasylol, DFP) had no effect.

Because killing of pseudohyphae eliminated their affinity for neutrophils (1), it appeared possible that surface substances responsible for attachment to neutrophils, if not altered, might be released from pseudohyphae. In fact, supernates from killed pseudohyphae strongly inhibited contact between neutrophils and live pseudohyphae. This activity was attributable to a material or materials with a mol wt <10,000, composed of ≅70–75% protein and 25–30% carbohydrate. This material had the capacity to bind to neutrophils and inhibit the attachment of hyphal forms of *Rhizopus* and *Aspergillus* in addition to *Candida*, but augment the attachment of the encapsulated yeast, *Cryptococcus neoformans*. The protein composition of this material is consistent with our finding that chymotrypsin-treatment of *Candida* eliminates attachment to neutrophils. The *Candida* cell wall is known to contain mannoproteins and glycoproteins, some of which have enzymatic activity (44). Therefore, it is possible that a stereospecific receptor on the neutrophil surface (3) may recognize this material which is released from *Candida* pseudohyphae. It is known that neutrophils have receptors for oligopeptides which can result in triggering of phagocytic and other mechanisms in neutrophils (45). In addition, our previous work suggests that the surface material which binds to neutrophils appear to be present in greater amounts of pseudohyphae than on yeasts (2). Release of this material from the surfaces of pseudohyphae during systemic candidiasis might well occur, in much the same way as mannans are released during infections (46). If so, this might have significantly detrimental effects on host defenses against remaining live *Candida*. Release of this material might also provide an antigenic marker which could be useful in distinguishing local from disseminated candidiasis (22).

Once contact occurred between neutrophils and pseudohyphae, microbicidal mechanisms of neutrophils were activated. From our previous studies, this included activation of the myeloperoxidase-hydrogen peroxide-halide system (2), as evidenced by iodination of pseudohyphae by neutrophils, and chemiluminescence by neutrophils (16). However, the process was accompanied by only minimal release of lysosomal enzymes from the azurophil granules, which also contain myeloperoxidase (2). Nevertheless, the present experiments indicated that oxidative mechanisms of neutrophils were of central importance in damage to *Candida* pseudohyphae by neutrophils. Damage to pseudohyphae was inhibited by several agents known to act on neutrophil oxidative microbicidal mechanisms, including sodium cyanide, sodium azide, catalase, superoxide dismutase, and 1,4 diazobicyclo (2,2,2) octane, a singlet oxygen quencher (35). The inhibitory effects of azide and cyanide suggest a role for myeloperoxidase (47), though these compounds may have some inhibitory effects on cell respiration as well. It is known that myeloperoxidase, hydrogen peroxide, and iodide can kill *Candida albicans* yeasts (13), and that myeloperoxidase-deficient neutrophils cannot kill *Candida albicans* yeasts normally (14). A role for hydrogen peroxide in damage to pseudohyphae is supported by inhibition by catalase, as well as the defect in damage to pseudohyphae by neutrophils from a patient with chronic granulomatous disease. This defect is comparable to that described for such neutrophils with *Candida* yeasts (11, 12). During phagocytosis, myeloperoxidase is released into phagocytic vesicles, where hydrogen peroxide appears to be collected (15). Hydrogen perox-

ide also appears to be released into extracellular media during phagocytosis (48), results in iodination of particles, and accounts for the majority of stimulation of the hexose monophosphate shunt in neutrophils (50). Production of superoxide anion, another potentially microbicidal substance, accounts for ≅10% of the respiratory burst which occurs with phagocytosis by neutrophils (49). Like hydrogen peroxide production, superoxide production is defective in neutrophils from patients with chronic granulomatous disease (51). Superoxide dismutase may be present in phagocytic vacuoles, because it has been localized to the cytoplasm of neutrophils (52), and may then play a significant role in catalyzing the formation of hydrogen peroxide from superoxide (49). However, in addition to the phagocytic vacuole, superoxide production appears to take place on the outer surface of the cell membrane (52, 53). In fact, superoxide may be produced by neutrophils after exposure to certain stimuli in the absence of phagocytosis (54). Interactions of superoxide anion and hydrogen peroxide then may result in generation of other potentially microbicidal substances, including singlet oxygen, and hydroxyl radical (16), on the cell surface. Therefore, several agents that might be produced at the cell surface could have contributed to the damage to *Candida* pseudohyphae by neutrophils. In our experiments, the complete absence of damage to pseudohyphae by neutrophils from the patient with chronic granulomatous disease indicate a definite requirement for oxidative pathways of neutrophils. However, the experiments with various inhibitors cannot be used to single out one substance that is critically important in damage to pseudohyphae. Hydrogen peroxide, with and without myeloperoxidase, superoxide anion, and singlet oxygen may all contribute to damage. On the other hand, we could not find evidence for a role for hydroxyl-free radicals, as scavengers of hydroxyl radical, sodium benzoate, and mannitol (16), failed to inhibit damage to pseudohyphae by neutrophils.

In addition to oxidative mechanisms, alternative mechanisms have been described for killing of some types of *Candida* yeasts by neutrophils (18). This may be attributable to lysosomal cationic proteins of neutrophils (19). In our experiments, inhibitors of cationic proteins, including α1-antitrypsin and the polyanion heparin, inhibited damage to *Candida* pseudohyphae by neutrophils. However, polyanions other than heparin were not inhibitory. In addition, neither heparin nor α1-antitrypsin were specific inhibitors of cationic proteins, as they inhibited iodination and chemiluminescence, which reflect activity of neutrophil oxidative microbicidal mechanisms (16). Nevertheless, these experiments cannot definitely rule out a direct role for cationic proteins in damage to pseudohyphae. In addition, cationic proteins, by their activity on the cell surfaces and metabolism of microorganisms (55), may have synergistic effects with other microbicidal mechanisms (56). Because cationic proteins are released from neutrophils, they may also result in activation of the complement system and generation of chemotactic factors, and thereby play an important role in neutrophil anti-*Candida* function (57).

Lactoferrin is an iron-binding substance which inhibits *Candida* (20) and is probably (58), though not definitely (59) present in specific granules of neutrophils. In our experiments, damage to pseudohyphae was not inhibited by concentrations of iron which were 10-fold higher than that calculated as required to saturate all neutrophil lactoferrin (36, 37).

Lysozyme is also present in specific granules of neutrophils and was released in significant amounts from neutrophils during damage to *Candida* pseudohyphae (2). The results of our current experiments are comparable to those of other workers who used yeasts (21), i.e. damage to *Candida* in water but not in buffer solutions. It is unlikely that lysozyme alone is a major mechanism for damage to pseudohyphae from these results, as well as the complete absence of damage to pseudohyphae by neutrophils from the patient with chronic granulomatous disease. Chronic granulomatous disease neutrophils do not have defects in exocytosis of granular enzymes (60). In addition, our experiments with isolated neutrophil granules failed to detect any activity for damage to pseudohyphae unless hydrogen peroxide and a halide were added, and this occurred only in granule fractions which contained large amounts of myeloperoxidase. Nevertheless, lysozyme clearly alters the surfaces of pseudohyphae, as evidenced by its damage to pseudohyphae in distilled water. Furthermore, lysozyme is known to enhance killing of *Candida* yeasts by other agents (21), so that it may well play an important secondary role together with oxidative mechanisms in damage to pseudohyphae by neutrophils.

It is apparent that a defect in damage to pseudohyphae might occur due to a variety of factors, including inhibitory factors in serum, changes in the surface properties of neutrophils or pseudohyphae, defects in microtubules or microfilaments of neutrophils (2), and defects in neutrophil oxidative metabolism. The relative biological importance of these factors will have to be determined in future studies of patients with systemic candidiasis.

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