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Fungicidal Properties of a Chymotrypsin-Like Cationic Protein from Human Neutrophils: Adsorption to *Candida parapsilosis*

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Human neutrophils contain a chymotrypsin-like cationic protein (CLCP) that binds to the surface of *Candida parapsilosis* and is preferentially adsorbed by yeasts mixed with unfractionated extracts of neutrophil granules. Adsorption of CLCP to opsonized or nonopsonized yeasts was rapid at pH 4 through 8. Irreversible inhibition of the enzymatic site of CLCP by phenylmethylsulfonylfluoride or *N*-acetyl-Ala-Ala-Phe-chloromethyl ketone did not affect its adsorption by yeast. Adsorption, highly sensitive to ionic strength, was abrogated by 0.15 M KCl. The number of CLCP molecules adsorbed per yeast cell and the loss of colony-forming units are described by an exponential relationship.

Human neutrophils possess several ways to kill ingested microorganisms. Oxygen-dependent systems involving myeloperoxidase, hydrogen peroxide (11, 15), superoxide (12), and reactive radicals that may include singlet oxygen (1, 10) have been extensively studied.

Nonoxidative systems are currently under investigation by this and other laboratories. Prominent among the microbicidal components of cell granules is a group of chymotrypsin-like cationic proteins (CLCP) that kill certain bacteria (19, 25) and fungi (16) in vitro.

The exact microbicidal mechanisms of these proteins are undefined. Odeberg and Olsson (20) recently reported that these proteins inhibit macromolecular syntheses by *Escherichia coli* and *Staphylococcus aureus*. We are investigating the fungicidal properties of this group of proteins. Since previous work demonstrated that *Candida parapsilosis* is highly susceptible to nonoxidative systems (14), we chose this species to examine.

We studied the initial contact between CLCP and the yeast surface. Adsorption was dependent on ionic strength but independent of enzymatic activity, pH, and prior opsonization of the fungi. An exponential relationship describing the number of molecules adsorbed per yeast cell and the loss of colony-forming units was derived.

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MATERIALS AND METHODS

Leukocyte preparation. (i) Method I. Granulocytes were prepared from peripheral blood of normal subjects by previously described methods (14). These included removal of mononuclear cells by Hypaque-Ficoll gradients, dextran sedimentation, and hypotonic lysis of contaminating erythrocytes. Most granulocyte preparations contained 95 to 97% granulocytes, 3 to 5% eosinophils, and 0 to 1% basophils in addition to neutrophils. The other cells were small lymphocytes and less than 0.5% monocytes. Platelets were absent.

The granule-rich fraction was prepared as previously described (16). Briefly, this involved homogenization in 0.34 M sucrose, low-speed centrifugation to remove cell debris, and subsequent centrifugation at 27,000 × *g*. The resulting light green granule-rich fraction was carefully separated from the supernatant and stored at −20°C.

(ii) Method II. Granulocytes were collected by continuous-flow leukapheresis from an untreated patient (informed consent) with chronic myelogenous leukemia. Approximately 4×10^{10} cells were obtained, of which 37.5% were mature (segmented and band cells). These leukocytes were diluted in 2 liters of phosphate-buffered saline containing 5 U of heparin per ml and were subjected to the same conditions in method I to free them from the more immature granulocytes, mononuclear cells, erythrocytes, and platelets.The final preparation (1.25×10^{10} cells) contained 94% segmented and band neutrophils, 4% myelocytes and metamyelocytes, 1% eosinophils, and 1% lymphocytes. The granule-rich fraction was prepared as in method I.

Extraction of granule-rich fraction. The granule-rich fraction from 10^8 neutrophils (ca. 0.5 mg of protein) was mixed with 0.30 ml of 0.01 M citric acid

for 60 min at 0°C. One-tenth volume of 0.21 M Na_2HPO_4 was added, raising the pH to ca. 5.0, and the mixture was centrifuged at 27,000 × *g* for 20 min.

Electrophoresis. Polyacrylamide electrophoresis was performed by modifications of the method of Reisfeld et al. (24). Running gels contained 15 or 10.8% acrylamide. All gels were photopolymerized, and persulfate was not used. Electrophoresis was conducted (4 mA/tube) with the gel tubes immersed in ice-cold buffer. After electrophoresis, the gels were removed from the tubes, the dye front was marked with India ink, and the gels were stained for protein in 0.2% amido black (Sigma Chemical Co., St. Louis, Mo.) for 30 min. Destaining was performed in methanol-water-glacial acetic acid (5:5:1). Occasionally, prior to staining, the gels were hemisected longitudinally; one half was stained for protein, and the other was stained for esterase activity. The esterase stain procedure was performed as previously described (16), with naphthol AS D acetate (Sigma) as the substrate.

Purification of CLCP. CLCP was purified from neutrophils by a modification of the elastase method of Baugh and Travis (2). Aprotinin (Trasylol, Sigma) was coupled to Sepharose 4B (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) by the procedure of March et al. (18) as modified by Baugh and Travis (2). For 30 ml of Sepharose 4B, 300,000 Kallikrein-inactivating units of aprotinin (30 ml) was used. A column (1.8 by 20 cm) was packed with the coupled product and equilibrated with 50 mM tris(hydroxymethyl)aminomethane in 50 mM NaCl at pH 8.0. A 0.01 M citric acid extract (80 mg of protein in 10 ml) of the granule-rich fraction obtained from CML granulocytes was adjusted to pH 8.0 and applied to the column. After washing through nonbound components, elastase and chymotrypsin (CLCP) were eluted with 50 mM sodium acetate–1 M NaCl at pH 4.5. This mixture was exhaustively dialyzed against 20 mM sodium acetate–0.15 M NaCl (pH 5.5), and 35 ml was applied to a column (16 by 2.5 cm) of *O*-(carboxymethyl)-cellulose (Whatman, Kent, England) equilibrated with the same buffer. The elastase eluted at 0.3 M NaCl, but the CLCP eluted only when the NaCl concentration was raised to 1 M. The fractions containing chymotrypsin activity and lacking elastase activity were pooled, concentrated by sucrose, and dialyzed against 50 mM sodium phosphate–0.1 M NaCl (pH 7.0).

Purified CLCP was composed of four isozymes on acid discontinuous gels (Fig. 1). These were used without further separation. The purified CLCP catalyzed the hydrolysis of 53 μmol of substrate per min per mg of protein under our assay conditions, employing $\epsilon_{347.5} = 5.5 \times 10^3\ M^{-1}\ cm^{-1}$ for *p*-nitrophenol (26). The preparation hydrolyzed *t*-*N*-*tert*-butoxycarbonyl (boc)-L-alanine-*p*-nitrophenol ester (Fox-Vega, Tucson, Ariz.) at 1/130 the rate that it hydrolyzed the chymotrypsin substrate (vide infra). Based on the specific activity of purified granulocyte elastase, this could have represented contamination by no more than 1.6% elastase. No lysozyme activity was detected. A total of 1.5 mg of protein was recovered.

Enzyme assays. Spectrophotometric assays for granulocyte myeloperoxidase and for lysozyme activities were performed by standard techniques (27). Granulocyte elastase was assayed with a 0.2 mM solution of *t*-boc-L-alanine-*p*-nitrophenyl ester as the substrate, by the method of Visser and Blout (26) as modified by Janoff (8). Granulocyte chymotrypsin activity was initially measured with *N*-benzoyl-L-tyrosine ethyl ester (Sigma) as the substrate (7). The assay was usually performed kinetically in 5 min, but, for low levels of activity, samples were

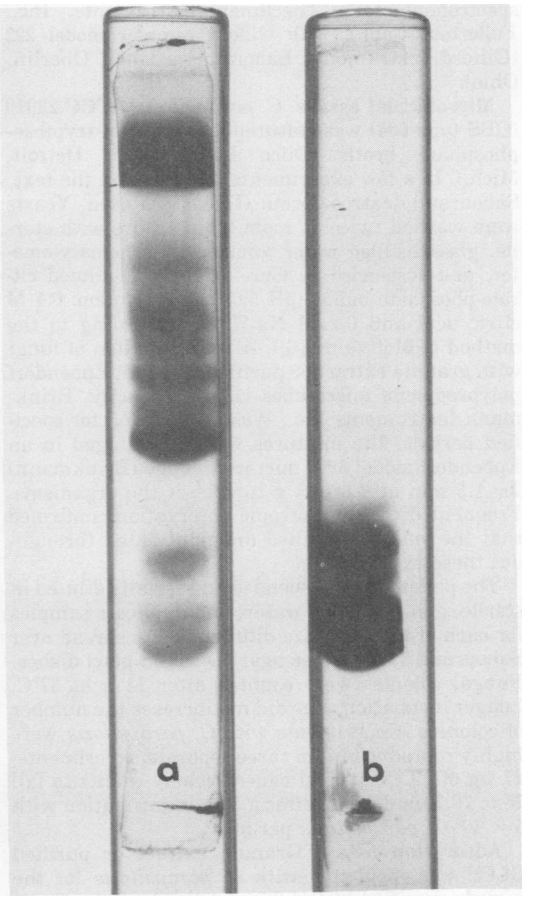

FIG. 1. *Purification of CLCP from neutrophil granule extract. O-(carboxymethyl)-cellulose column fractions that contained chymotrypsin but lacked elastase activity were pooled, concentrated by sucrose, and dialyzed against 50 mM sodium phosphate–0.1 M NaCl (pH 7.0). Electrophoresis of (a) unfractionated extract (300 μg of protein) and (b) purified material (120 μg of protein) was performed in 10.8% polyacrylamide gels and stained for protein as described in the text. Gel (b), the purified CLCP preparation, contained four CLCP isozymes without other evident proteins. Cathodal end of gel at bottom.*

read at 60-min intervals. We later adopted the method of Frank Powers, of the Georgia Institute of Technology, which used *t*-boc-L-tyrosine-*p*-nitrophenyl ester (Fox-Vega) as the substrate. The working solution was prepared by adding 50 μl of stock substrate (10 mg/ml in dioxane) to 4.25 ml of methanol and then diluting it to 25 ml with 50 mM citrate buffer in 1 M NaCl (pH 6.5). The reaction rate was measured at 347.5 nm. This method had far greater sensitivity than the *N*-benzoyl-L-tyrosine ethyl ester assay.

Protein was measured by the method of Lowry et al., with bovine serum albumin as the standard (17). All assays were performed in a Beckman model DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.), with Gilford recorder model 222 (Gilford Instruments Laboratories, Inc., Oberlin, Ohio).

Microbicidal assays. *C. parapsilosis* ATCC 22019 (CBS type 604) was cultured overnight in tryptose-phosphate broth (Difco Laboratories, Detroit, Mich.). In a few experiments, indicated in the text, Sabouraud dextrose broth (Difco) was used. Yeasts were washed twice at room temperature with sterile, glass-distilled water, counted in a hemacytometer, and suspended in four- or eightfold-diluted citrate-phosphate buffer (pH 5.0) (stock solution: 0.1 M citric acid and 0.2 M Na_2HPO_4, according to the method of McIlvaine [6]). After incubation of fungi with granule extract or purified CLCP in Eppendorf polypropylene microtubes (1.5-ml capacity, Brinkmann Instruments Inc., Westbury, N.Y.) for specified periods, the mixtures were centrifuged in an Eppendorf model 3200 microcentrifuge (Brinkmann) for 1.5 min at 7,680 × *g* to deposit the organisms. Frequent direct microscopic observation confirmed that the yeasts remained unagglutinated throughout these experiments.

The pellets were suspended and serially diluted in sterile, glass-distilled water, and duplicate samples for each of two separate dilutions were spread over Sabouraud 2% dextrose agar (Difco) in petri dishes. Fungal colonies were counted after 72 h at 37°C. Longer incubation time did not increase the number of colonies. Experiments with *C. parapsilosis* were highly reproducible. In three separate experiments, 27 μg of CLCP per ml caused colony counts to fall 78.6, 79.7, and 85.6% after a 30-min incubation with 5×10^7 *C. parapsilosis* per ml.

Adsorption assay. Granule extract or purified CLCP was incubated with *C. parapsilosis* for the periods specified in the figure legends. Subsequent centrifugation in the microfuge pelleted the yeasts. The supernatant solution was assayed for enzyme activities by spectrophotometry or by esterase staining on polyacrylamide gels. Tubes containing leukocyte proteins without yeasts provided the controls for total enzyme activity. Examination of the supernatants on gels allowed qualitative evaluation of adsorption, whereas the spectrophotometric system provided a quantitative assay. Percent adsorption, as determined by spectrophotometric assay, was calculated as [1 − (supernatant activity/control supernatant activity)] × 100. The mean number of molecules adsorbed per organism was calculated by assuming the molecular weight of the purified CLCP to be 25,000 (21), using Avogadro's number, and specific activity of our protein preparation.

When adsorption was measured with less than 100 μg of CLCP per ml, dilutions were made in 0.5 mg of bovine serum albumin per ml to prevent nonspecific adherence of the protein to the surface of the test tube. In the absence of albumin, CLCP (100 μg/ml) solutions lost 6.2% of their activity by nonspecific adherence.

RESULTS

Preferential adsorption of cationic proteins to *C. parapsilosis*. When *C. parapsilosis*, at 10^7/ml, was exposed to relatively high concentrations of unfractionated granule extract (0.6 to 0.8 mg/ml), CLCP was preferentially and rapidly removed from the supernatant solution (Fig. 2). This preferential adsorption occurred independently of the pH of the incubation mixture (pH 4 through 8) in constant-ionic-strength buffers (4).

If the yeast concentration was increased 10-fold, preferential CLCP adsorption was replaced by more generalized adsorption of extract components, including elastase, lysozyme, and myeloperoxidase (Fig. 3).

Kinetics of adsorption. Adsorption by *C. parapsilosis* of CLCP from unfractionated granule extract occurred within minutes (Fig. 2). In contrast, when purified CLCP was used. its adsorption to yeast was virtually instantaneous, as demonstrated in the following experiment. We added 27 μg of CLCP per ml to *C. parapsilosis* (1.5×10^7/ml). After mixing and immediately centrifuging this in a microcentrifuge for 1.0 min, we found that greater than 90% of enzyme activity had already been removed from the supernatant. Identical results were obtained when yeasts, preopsonized by incubation in normal serum for 15 min at 37°C and then washed, were added. In these experiments, albumin prevented nonspecific adherence of CLCP to tubes containing buffer but no yeast.

KCl inhibition of adsorption. When *C. parapsilosis* was incubated with unfractionated granule extract or purified CLCP in the presence of increasing concentrations of KCl, adsorption of CLCP to yeasts was inhibited. Adsorption of purified CLCP to yeasts was two-thirds inhibited at 0.1 M KCl and was abolished at 0.15 M KCl (Table 1).

Reasonable correlation existed between adsorption of CLCP and subsequent killing of the yeasts (Table 1). The percentage of *C. parapsilosis* killed, as measured by colony counts, substantially decreased when incubation mixtures contained as little as 0.1 M KCl. At higher KCl concentrations, a low level of killing occurred.

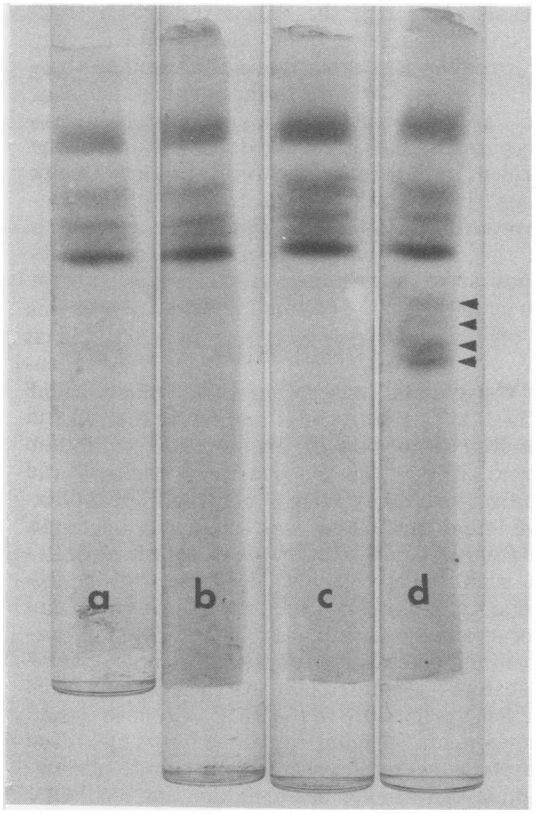

FIG. 2. *Removal of CLCP from unfractionated granule extract by C. parapsilosis. Unfractionated granule extract (final concentration, 0.8 mg/ml) was added to C. parapsilosis (final concentration, 1.5 × 10⁷ cells per ml) in dilute citrate-phosphate buffer (pH 5.0). After 3 min (a), 5 min (b), and 10 min (c), a portion of the mixture was removed and centrifuged for 1 min. The supernatant solution (160 μg of protein) was subjected to electrophoresis on 15% polyacrylamide gels and stained for esterase activity as described in the text. Gel (d), on the far right, represents the control granule extract. Arrows indicate the CLCP activity. Protein stains (not shown) confirmed the selective nature of CLCP adsorption.*

Equimolar concentrations of sodium chloride exhibited similar effects.

Effect of chymotrypsin inhibitors on adsorption. We examined the effects of phenylmethylsulfonyl fluoride (PMSF, Sigma) and *N*-acetyl-Ala-Ala-Phe-chloromethyl ketone (Fox-Vega), substances that irreversibly inhibit the enzymatic activity of CLCP. The former, a serine esterase inhibitor (5), inhibits other granulocyte proteins as well as CLCP, whereas the latter, a substrate analog, is specific for chymotrypsin-like proteases (23). When either the unfractionated granule extract or the purified CLCP was reacted with millimolar concentrations of these inhibitors, chymotryptic activity was abolished, as evidenced by lack of esterase staining on polyacrylamide gels and greatly diminished activity by spectrophotometric assay. With PMSF, enzymatic inhibition was complete; with *N*-acetyl-Ala-Ala-Phe-chloromethyl ketone, inhibition varied from 85 to 100%.

After preincubation with either inhibitor for 40 min, unfractionated granule extract or purified CLCP was added to tubes containing *C. parapsilosis* for 5 or 30 min. After centrifugation, the supernatants were examined on polyacrylamide gels. There was equal and complete adsorption of CLCP from supernatants of inhibitor-treated preparations and control preparations, suggesting that the enzymatic-binding site of CLCP is distinct from its yeast-binding site. *C. parapsilosis* was killed with substantially equal effectiveness by inhibitor-treated and untreated CLCP (Table 2).

Desorption of CLCP from *C. parapsilosis*. Adsorbed CLCP remained bound to the surface of *C. parapsilosis* after repeated washings with

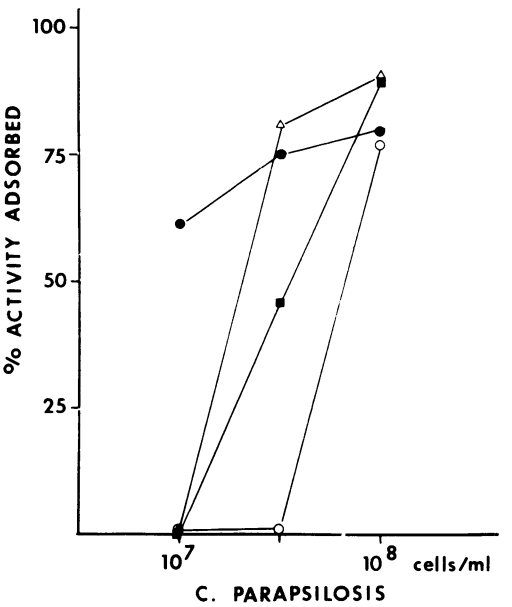

FIG. 3. *Adsorption of granule enzymes to various concentrations of C. parapsilosis. Various concentrations of C. parapsilosis in citrate-phosphate buffer (pH 5.0) were incubated with unfractionated granule extract (final concentration, 0.6 mg/ml) in the same buffer for 30 min. After centrifugation in a microcentrifuge for 1.5 min, supernatants were assayed for residual enzyme activity as described in the text. Symbols: ○, myeloperoxidase; ■, lysozyme; △, elastase; and ●, CLCP. CLCP activity was measured with N-benzoyl-L-tyrosine ethyl ester as the substrate. Note that 10⁷ C. parapsilosis per ml caused extensive CLCP adsorption without adsorption of the other neutrophil granule enzymes.*

TABLE 1. *Effect of KCl on adsorption to and killing of C. parapsilosis by CLCP*

KCl concn (M)[a]	Supernatant enzyme activity[b]	Enzyme adsorption (%)	CFU[c]/ml	% Killed
0	0.001	98.7	1.44×10^7	85.6
0.05	0.004	94.7	2.70×10^7	73.0
0.10	0.050	33.3	7.80×10^7	21.8
0.15	0.075	0	8.70×10^7	13.3
0.2	0.077	0	8.40×10^7	15.7
Control, CLCP in 0.2 M KCl[d]	0.073	0	0	0
Control, *C. parapsilosis*[e]	0	0	1.0×10^8	0

[a] KCl solutions prepared in dilute citrate-phosphate buffer (pH 5.0).

[b] Expressed as change in optical density (347.5 nm) per minute per microgram of CLCP, measured after incubation for 30 min with *C. parapsilosis* with *t*-boc-L-tyrosine-*p*-nitrophenol ester as substrate.

[c] CFU, Colony-forming units.

[d] 27 μg/ml in a 0.5-mg/ml bovine serum albumin solution without *C. parapsilosis*.

[e] 10^8/ml in citrate-phosphate buffer (pH 5.0) without CLCP.

distilled water. However, it was removed by incubating the yeasts with KCl. Concentrations of KCl between 0.15 and 0.5 M released up to 60% of adsorbed enzymatic activity after 1 h. CLCP incubated with PMSF or *N*-acetyl-Ala-Ala-Phe-chloromethyl ketone prior to reaction with *Candida* was also eluted from the yeasts (data not shown).

Relationship of adsorption to killing. Various dilutions of purified CLCP were incubated with *C. parapsilosis* to correlate the amount of CLCP adsorbed per yeast cell with subsequent killing of the yeasts. Colony counting was used to assess yeast viability; spectrophotometric assays of supernatants were performed to measure CLCP adsorption. The percentage of *C. parapsilosis* killed by purified CLCP was related to the mean number of CLCP molecules adsorbed per cell $\times 10^6$ by a power function: $y = 70x^{-0.7482}$ (Fig. 4). Fifty percent of the yeast cells were killed (i.e., unable to form colonies on nutrient agar) when a mean of ca. 10^6 CLCP molecules had been adsorbed per yeast cell. Adsorption of 10^7 molecules per cell led to 90% cell death.

We observed that CLCP at high concentrations was less effectively adsorbed by *C. parapsilosis* grown in Sabouraud broth than by yeasts grown in tryptose-phosphate broth. At 54 μg of CLCP per ml, 6×10^6 mean molecules per organism were absorbed by Sabouraud-grown *Candida*, whereas 2.3×10^7 molecules were adsorbed to tryptose-phosphate-grown yeasts. Although the Sabouraud-grown organisms bound fewer molecules of CLCP, they were at least as sensitive as the tryptose-phosphate-grown *Candida* to the fungicidal actions of CLCP per molecule bound. Direct microscopic examination revealed that the organisms grown in either broth were similarly sized; thus, it is unlikely that tryptose-phosphate-grown *Candida* had four times the surface area of the Sabouraud-grown yeast. This suggests qualitative alterations in the yeast surface structures serving as binding sites.

DISCUSSION

We studied adsorption of a microbicidal CLCP to *C. parapsilosis* and found that CLCP derived from human granulocytes exhibited great affinity for the yeast. In studies with unfractionated extracts of neutrophil granules, we found that CLCP was selectively adsorbed to the yeast surface at low yeast concentrations. If such selectivity also occurred within the phagocytic vacuole of neutrophils, it would favor a primary antifungal role for CLCP relative to other potentially fungicidal components of neutrophil granules (16). As the concentration of yeast cells relative to CLCP increased, other components (myeloperoxidase, lysozyme, elastase) also were adsorbed. We attribute adsorption of these molecules to exhaustion of CLCP, which allowed components with lower binding affinities to occupy unsaturated binding sites.

Adsorption of CLCP to yeasts occurred rapidly. CLCP was also adsorbed by *Candida* exposed to opsonically active normal human serum. Thus, opsonization, with its attendant surface binding of immunoglobulin and complement molecules, does not block CLCP-binding

TABLE 2. *Effect of pretreatment of CLCP with PMSF on C. parapsilosis killing*

Treatment[a]	CFU/ml[b]	% Killed
CLCP + PMSF[c]	6.5×10^6	82.9
CLCP alone	2.5×10^6	93.4
Control[d]	3.8×10^7	

[a] 36 μg of CLCP was incubated with PMSF (final concentration, 1 mM in 10% MeOH) or with 10% MeOH in 0.1 M sodium phosphate buffer (pH 7), for 40 min. *C. parapsilosis* (final concentration, 5×10^7/ml) was added, and the mixture was allowed to incubate for 30 more min. Supernatants were removed, and the pellets were serially diluted in water and plated as described in the text.

[b] CFU, Colony-forming units.

[c] After the first 40-min incubation, a portion were assayed for CLCP activity (with *t*-boc-l-tyr-*p*-matic activity as assayed spectrophotometrically.

[d] *C. parapsilosis* alone.

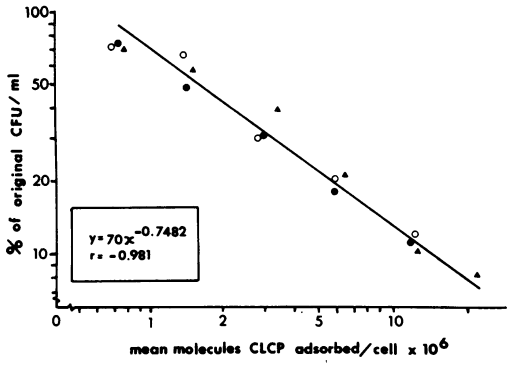

FIG. 4. *Relationship between survival of C. parapsilosis and mean number of CLCP molecules bound per organism. Various concentrations of purified CLCP prepared in a 0.5-mg/ml bovine serum albumin solution were incubated with C. parapsilosis (final concentration, 5 × 10⁷ or 1.0 × 10⁸ cells per ml in dilute citrate-phosphate buffer, pH 5.0) for 30 min at 37°C and then centrifuged. Supernatants were assayed for CLCP activity (with t-boc-L-tyr-p-nitrophenol ester as substrate). The yeasts were resuspended, serially diluted, plated on Sabouraud agar, and counted after 72 h. Percentage of CLCP adsorbed and mean number of molecules adsorbed per organism were calculated as described in the text. Symbols: ▲, ●, and ○ indicate different experiments.*

sites. Consequently, opsonization per se should not interfere with binding of CLCP to yeast cells within phagocytic vesicles of neutrophils.

CLCP is both enzymatically and microbicidally active. Are these two attributes related? Our evidence with the inhibitors PMSF and *N*-acetyl-Ala-Ala-Phe-chloromethyl ketone suggests that enzymatic activity is unrelated to *Candida* binding capacity or candidacidal activity. These data corroborate recent work by Thorne et al. (25), who showed that Cathepsin G (a chymotrypsin-like protease from human spleen, immunologically identical to leukocyte CLCP) retained its bactericidal activity to *Acinetobacter* 199A after inhibition by PMSF. It also substantiates previous reports that heat-inactivated CLCP retains its microbicidal properties (16, 20).

We found adsorption to be optimal in solutions of low ionic strength. It diminished as the salt concentration increased and was abolished by 0.15 M KCl. CLCP was desorbed from the yeast surface by high salt concentrations (0.2 to 0.5 M KCl or NaCl). At an ionic strength approximating that of serum, adsorption and killing were minimal. Thus, CLCP is unlikely to exert significant antifungal activity extracellularly.

Our data point to an ionic interaction between CLCP and the yeast surface. The isoelectric point of CLCP approximates that of granulocyte elastase, whose isoelectric point is 10.8 in a sucrose-supported gradient system (9). This provides it with a net positive charge at all conceivable pH values within the phagocytic vacuole. The cell wall of *Candida* species is composed predominantly of glucan and mannan. The amount of mannan that is phosphorylated varies greatly between species and strains of the same species (i.e., *C. albicans* B-792 possesses 18 mol of mannose per mol of phosphorus; strain AB-311 possesses 35 mol [22]). Negatively charged phosphate groups on the yeast surface could effectively bind CLCP.

It is known that growth conditions can control mannose/phosphorus ratios; e.g., *Histoplasma capsulatum*, grown in high-phosphate medium, incorporates more phosphorus into mannan (22). Our data with Sabouraud- and tryptose-phosphate-grown *Candida* indicated that the yeast binding site(s) for CLCP showed phenotypic modulation consequent to growth in different media. It is possible that differences in phosphate content of the media were responsible for this binding-site modulation. It would be interesting to learn how *Candida* cultured in vivo binds CLCP and whether binding or its modulation serves as a virulence factor.

A logarithmic plot of mean number of CLCP molecules adsorbed per *Candida* cell versus yeast survival resulted in a straight line (Fig. 4), reproducible in three such experiments. Approximately 90% of the *Candida* were killed after adsorption of 10^7 CLCP molecules per organism. In the range of 10^6 to 10^7 molecules adsorbed per cell, the curve is linear. At lower concentrations, a shoulder is apparent. The curve differs from those described as showing "single- or multiple-hit" killing kinetics when plotted in a semilogarithmic manner as well (3, 13).

Does CLCP exert fungicidal activity within intact human neutrophils? To date, evidence that it does is circumstantial. The enzyme is present in the lysosomal granules of human neutrophils, where, on a weight basis, it is approximately one-third as abundant as myeloperoxidase, elastase, and lysozyme (H. Odeburg et al., Blood Cells, in press). Its localization should assure it entry to the phagosome, but its concentration therein is not known. For the reasons discussed, its binding to yeast cells should be independent of any pH changes within the phagosome. Unfortunately, the ionic strength of the phagocytic vacuole is unknown, and the possibility that it is high enough to prevent binding of CLCP cannot be excluded.

Our attempts to demonstrate adsorption of CLCP to the surface of yeasts within phagosomes have been, thus far, technically unsuccessful, but we are currently investigating other approaches. It may be, however, that assessment of the microbicidal significance of CLCP in intact cells must await the detection of a deficiency state in humans.

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