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# Nonoxidative Fungicidal Mechanisms of Mammalian Granulocytes: Demonstration of Components with Candidacidal Activity in Human, Rabbit, and Guinea Pig Leukocytes

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Granulocytes from the peripheral blood of normal subjects and a patient with hereditary myeloperoxidase deficiency were homogenized in 0.34 M sucrose. A granule-rich fraction, prepared by sedimentation at 27,000 × *g* for 20 min, contained components that killed *C. parapsilosis* in vitro. These were extractable with 0.01 M citric acid and were shown by micropreparative polyacrylamide electrophoresis to be multiple. The candidacidal activity of these neutrophil components was heat stable and they were somewhat more active at pH 5.0 than at pH 7.0. When rabbit or guinea pig heterophils were obtained from sterile peritoneal exudates and similarly fractionated, they also were found to contain components that killed *C. parapsilosis* in vitro. These were primarily associated with a group of lysosomal cationic proteins lacking direct counterpart in human neutrophils. Among the candidacidal components of the human neutrophil was a protein, more cationic than lysozyme, that exhibited naphthol-ASD acetate esterase activity.

By virtue of their ability to ingest and kill fungi, neutrophils contribute to host defenses against mycotic infections (10). The principal fungicidal mechanism of human neutrophils effective against *C. albicans* involves myeloperoxidase (MPO) and hydrogen peroxide (8, 14) or superoxide anion (9). Defective operation of this pathway has been described in chronic granulomatous disease (CGD) (13) and in MPO-deficient states (14, 15).

Considerable evidence points to the existence of alternative mechanisms of fungicidal activity in the granulocytes of man and other vertebrate species. Chicken heterophils, which lack peroxidase, can kill *C. albicans* in vitro (1), and the antibacterial lysosomal cationic proteins of guinea pig heterophils are effective against this organism (24). Human neutrophils deficient in MPO or in the capacity to generate hydrogen peroxide and superoxide anion can nevertheless kill certain fungi with normal or only moderately diminished efficacy (11).

We have attempted to ascertain the mechanism of such alternative fungicidal pathways in human, rabbit, and guinea pig granulocytes. Our data show that human neutrophils possess multiple components that exert candidacidal activity in vitro, and reveal differences between these and the candidacidal components of rabbit and guinea pig heterophils.

## MATERIALS AND METHODS

**Leukocyte preparation.** Granulocytes were prepared from peripheral blood of normal subjects and a previously described patient with hereditary MPO deficiency (14) by previously described methods (11). Most preparations contained 95 to 97% granulocytes. These included 3 to 5% eosinophils and 0 to 1% basophils in addition to neutrophils. The other cells present were small lymphocytes and less than 0.5% monocytes. Erythrocytes, removed by hypotonic lysis, and platelets were absent.

Granulocytes from 450 ml of blood ($0.7 \times 10^9$ to $2.0 \times 10^9$) were suspended in 1 ml of cold 0.34 M sucrose and homogenized with a Potter-Elvehjem homogenizer until more than 95% of the cells had been disrupted. The homogenate was centrifuged at 250 × *g* for 10 min, and the sediment was washed with 0.34 M sucrose and then recentrifuged 1 to 3 additional times. Supernatants were pooled, distributed into small cellulose nitrate tubes, each containing approximately $10^8$ granulocyte equivalents, and centrifuged at 27,000 × *g* for 20 min. The resulting light-green pellets and clear supernatants were carefully separated and stored at −20 C until used.

The 27,000 × *g* pellets were heterogeneous by electron microscopy. The predominant components were membrane-bound, electron-dense structures resembling the cytoplasmic granules of intact neutrophils. Most of the granules were well preserved, but some showed partial loss of internal content. Occasional mitochondria, vesicular structures lacking distinct internal content, and finely fibrillar interstitial material were also present. The last two were most

prominent at the upper surface of the pellet. We will refer to this leukocyte preparation as the granule-rich fraction (GRF).

Sterile peritoneal exudates were raised in guinea pigs and rabbits by intraperitoneal instillation of 0.1% glycogen in 0.9% sodium chloride containing *Escherichia coli* lipopolysaccharide (Difco). Rabbits received 750 ml of solution containing 5 μg of lipopolysaccharide, and guinea pigs received 75 ml with 2 μg of lipopolysaccharide. Exudates were collected after 16 h, washed several times with Hanks balanced salt solution containing 10% (vol/vol) fetal calf serum and 5 U of heparin per ml, resuspended in cold 0.34 M sucrose, and thereafter homogenized and processed as were human neutrophils.

**Extraction of granule-rich fractions.** The GRF from approximately $10^8$ neutrophils was mixed with 0.4 ml of 0.01 M citric acid for 60 min at 0 C and then centrifuged at 27,000 × *g* for 20 min. If supernatant extracts were used for microbicidal testing, centrifugation was preceded by addition of one-tenth volume of 0.21 M $Na_2HPO_4$ to raise the pH to approximately 5.0. This step was omitted when extracts were used for electrophoresis.

**Electrophoresis.** Polyacrylamide electrophoresis was performed by slight modifications of the method of Reisfeld et al. (20). Gels and buffers were as described, except that solution C contained 60 g of acrylamide and 1.8 g of *N,N'*-methylene bisacrylamide (Bio-Rad Laboratories, Richmond, Calif.). Small pore gels contained one part A, two parts C, one part E, and four parts distilled water. Sample gels contained 400 μg of leukocyte protein diluted with equal parts of a solution comprised of one part B, two parts D, and one part E, with 50 μg of methyl green per ml. All gels were photopolymerized; persulfate was not used.

Electrophoresis was conducted at room temperature by applying a current flow of 4 mA per gel until the dye front had migrated 40 to 45 mm (approximately 60 min). Gels were removed and the dye front was marked with india ink. The gels were frozen by placing them on dry ice and then hemisected longitudinally. Half of the gel was stained for protein with 0.2% amido black 10B (Sigma, St. Louis) in methanol-water-glacial acetic acid (5:5:1) for 30 min, and destained overnight with the same solvents. The other half of the gel was rinsed with sterile-distilled water for 15 min and then sectioned into 1-mm segments with a gel slicer (Hoefer Scientific Instruments. San Francisco, Calif.). Individual slices were placed in wells of a Microtest II plate (Falcon Plastics, Oxnard, Calif.), covered with 200 μl of sterile distilled water, and stored overnight at 4 C to elute their components.

**Other assays.** Protein was measured by the method of Lowry et al. with hen egg white lysozyme or bovine serum albumin as standards (17). Lysozyme activity of eluates was measured by the method of Schill and Schumacher (22) and correlated with the stained portion of the gel. In some experiments we estimated protein concentrations of eluates by a micromodification of the method of Lowry et al. that used an Aminco microfluorocolorimeter with fiberoptic accessories (American Instrument Co., Silver Spring, Md.). Although low but variable degrees of color development in eluates of gels lacking protein interfered in these assays, the results suggested that most active fractions were tested at protein concentrations between 10 to 30 μg/ml. Esterase staining of gels was performed at room temperature with substrate prepared by dissolving 5 mg of naphthol-ASD acetate (Sigma, St. Louis, Mo.) in 2 ml of dimethylformamide, adding 8 ml of 0.2 M potassium phosphate buffer, pH 7.0, and adjusting the final pH to 7.0 with 1 N HCl. To this solution was added 20 mg of fast blue RR salt (diazotized 4-benzylamino-2,5-dimethoxyaniline · $ZnCl_2$; Sigma, St. Louis, Mo.), and then the mixture was filtered. After gels were immersed in the stain for 30 min, the pH was restored to 7.0 by addition of 0.1 N NaOH. Staining continued for 3 h and was terminated by placing the gels in 7% acetic acid.

**Microbicidal assays.** *C. albicans*, strain 820, was cultured in Sabouraud dextrose broth (Difco) for 36 to 72 h at 33 C. *C. parapsilosis*, strain 12-10 or ATCC 22019, was cultivated for 36 h at 33 C in tryptose phosphate broth (Difco). Fungi were washed twice at room temperature with sterile glass-distilled water, counted in a hemocytometer, and suspended in water at the desired concentration.

Fungi ($7.5 \times 10^5$ cells), buffer, and the subcellular extract to be tested were combined in sterile plastic tubes (12 by 75 mm) or in Microtest II plates and incubated for 60 min at 37 C. Most incubations were conducted in a final volume of 100 μl. Buffers were prepared from 0.1 M citric acid and 0.2 M $Na_2HPO_4$ after McIlvaine (5) and were present in eightfold dilution in the final incubation mixtures. Fungal viability was assessed by colony counting and dye exclusion. For colony counting, replicate samples were removed, serially diluted in sterile glass-distilled water containing 0.01% gelatin, and spread over Sabouraud dextrose agar or Trypticase soy agar in petri dishes. Fungal colonies were counted after 48 h (*C. albicans*) or 72 h (*C. parapsilosis*). Longer incubation times did not increase the number of colonies.

In preliminary experiments, we found that 0.4% solutions of trypan blue or eosin Y stained heat-killed *C. parapsilosis* cells without staining viable ones. Solutions containing 0.3% trypan blue and 0.1% eosin Y afforded the best contrast between stained yeast cells and the background, and were nontoxic to viable yeast. Stock solutions of 0.3% trypan blue and 0.1% eosin Y, prepared and sterilized by filtration, were stable for months at room temperature. A drop of this mixture was mixed with approximately 2 drops of solutions containing *C. parapsilosis*, and after 10 min the percentage of stained organisms was determined by examining at least 100 yeast cells by direct microscopy. The percentage of stained organisms was stable for at least 12 h, when organisms were suspended in pH 5.0 to 7.0 buffers at room temperature. In more acidic solutions, *C. parapsilosis* gradually lost viability as indicated by a fall in colony count and an increase in the percentage of stained cells.

## RESULTS

**Preliminary studies.** When normal human neutrophils were homogenized in 0.34 M su-

crose, and the 250 × *g* sediment, 27,000 × *g* GRF, and 27,000 × *g* supernatant was tested against *C. parapsilosis* at pH 5.0, candidacidal activity was predominantly localized to the GRF. It could be extracted from the GRF into a 27,000 × *g* supernatant by repeated freezing and thawing in a 0.2% Triton X-100 or by exposing the GRF to cold 0.01 M citric acid or 0.02 M acetic acid. Extraction was most complete when citric acid was used. Such treatment solubilized 50 to 60% of the granule protein and left the residual material devoid of candidacidal activity even when it was tested at a concentration of 1 mg/ml.

*C. parapsilosis* was more susceptible to citric acid extracts from normal or MPO-deficient human neutrophils than was *C. albicans* (Fig. 1). Candidacidal effects were achieved rapidly and were not reversed if, after 2 to 5 min, the extracts were diluted to concentrations that would otherwise have been sublethal.

The citric acid extracts were effective at pH 5.0, 6.0, and 7.0 and exerted comparable effects by colony counting or dye exclusion at each of these pH levels (Fig. 2). The experiment shown demonstrates that colony counting was a more sensitive indicator of candidacidal activity than was dye exclusion. Note that the number of colonies was reduced to half the control levels after exposure to GRF extracts whose protein concentration approximated 20 μg/ml. In contrast, protein concentrations approximating

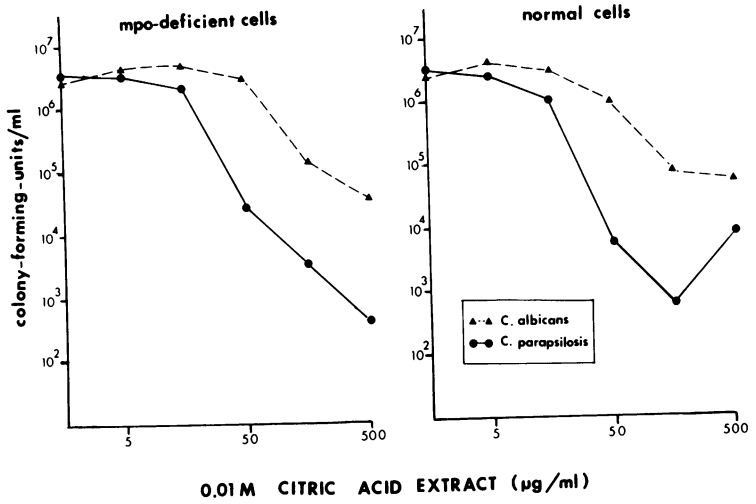


FIG. 1. *Candidacidal activity of human neutrophil granules. Effect of a citric acid extract of normal and MPO-deficient granule-rich fractions on Candida species. The assays were conducted at pH 5.0 as described in the text.*

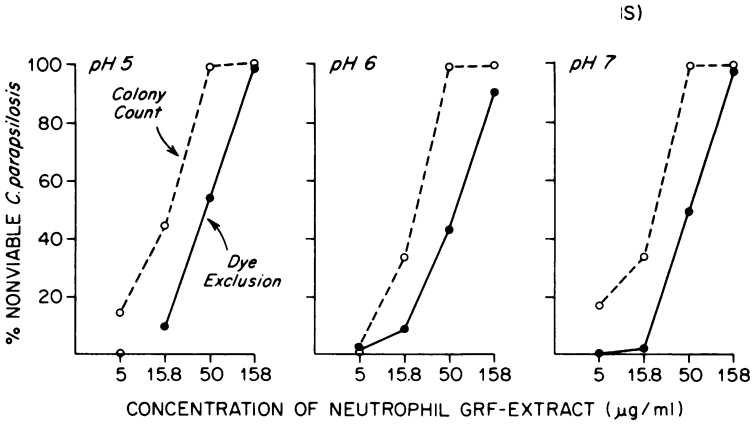


FIG. 2. *Comparison of dye exclusion and colony count assays (C. parapsilosis). Effect of pH on the candidacidal activity of a citric acid extract of normal neutrophil granule-rich fraction. The organisms were incubated for 60 min with C. parapsilosis and viability was assessed by colony counting and dye exclusion.*

50 μg/ml were required to render half of the yeast cells stainable by trypan blue and eosin Y.

**Polyacrylamide electrophoresis.** Material extracted from normal and MPO-deficient granules was heterogeneous and yielded up to 20 distinct protein bands on polyacrylamide (Fig. 3). MPO was apparent as a single sharp band, green on unstained gels, that displayed peroxidase activity when appropriately stained. Gels prepared from extracts of MPO-deficient GRF lacked both the green band and peroxidase activity. They contained two bands, also present in normal extracts, whose mobility approximated that of MPO; these showed acid phosphatase activity (naphthol AS-BI phosphate) when suitably stained.

To discern which of the leukocyte constituents was responsible for the candidacidal activity of the citric acid extract, we tested eluates from the polyacrylamide gels against *C. parapsilosis* (Fig. 4). Although eluates from gels containing 27,000 × *g* cytosol protein were relatively ineffective, multiple peaks of candidacidal activity were present among the materials eluted from gels containing the extracts of normal GRF. Eluates from gels that lacked protein were not candidacidal. *C. albicans* colony counts were not reduced by GRF gel eluates that were effective against *C. parapsilosis*. We attribute this to the fairly low concentrations of leukocyte protein used in these tests and to the lesser susceptibility of *C. albicans*.

We examined the effect of KI and $H_2O_2$ on the ability of eluates from gels of normal and MPO-deficient neutrophil GRF extracts to kill *C. parapsilosis* (Fig. 5). In the absence of any additions, fairly similar patterns of candidacidal activity were obtained with the two sets of eluates. Normal eluates developed an additional killing peak when KI and $H_2O_2$ were added. This peak corresponded to the location of MPO in the fractions and was not observed with MPO-deficient eluates. In a few experiments, such as the one illustrated, KI and $H_2O_2$ appeared to potentiate the candidacidal activity of other eluate fractions, but this was not observed in all of the studies.

The components eluted from the gels retained essentially complete candidacidal activity after being heated at 93 to 100 C for 15 or 30 min. Their activity against *C. parapsilosis* was also influenced by the pH of the incubation mixtures. Extracts were most effective at pH 5, somewhat less active at pH 6, and least active at pH 7. The differences were more marked when dye exclusion rather than colony counting was used to quantitate candidacidal activity, but less effective killing at pH 7 than at pH 5 was also observed when colony counting was used (data not shown).

The most cathodal candidacidal fractions coincided with the gel regions that contained lysozyme and a more cathodal protein with esterase activity (see below). By decreasing the polyacrylamide concentration of the running gels, it was possible to separate these constituents widely (Fig. 6). Eluates corresponding to the cationic esterase were more active against *C.*

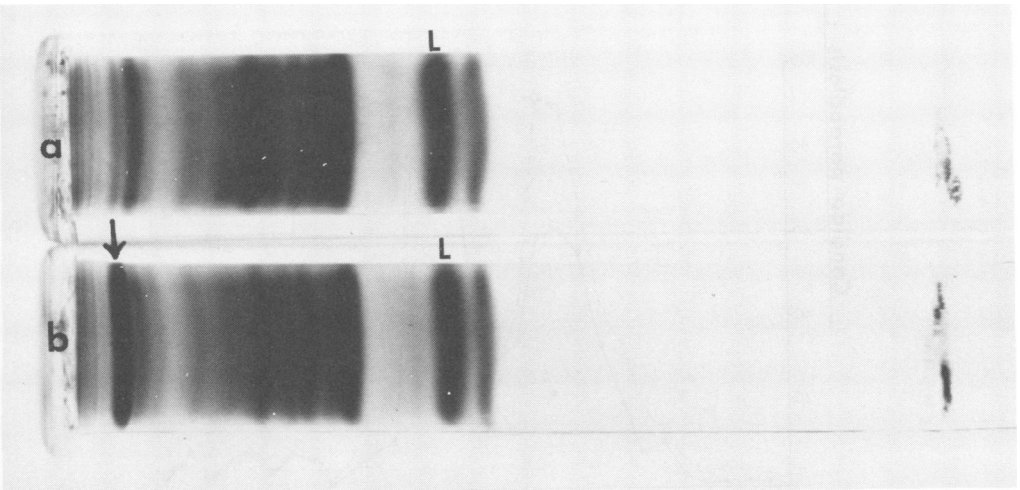


FIG. 3. *Polyacrylamide gels of normal and MPO-deficient neutrophils. Citric acid extracts from granule-rich fractions of MPO-deficient (a) and normal (b) human neutrophils were electrophoresed as described in the text, and gels were stained with amido black. The cathodal dye front (to the right) is indicated by an irregular dark line where it was marked by india ink. The positions of lysozyme (L) and MPO (↓) are shown. Note the presence of a protein band more cathodal than lysozyme.*

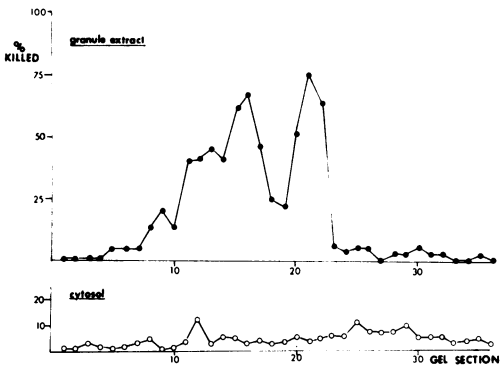


FIG. 4. *Ability of human neutrophil components to kill C. parapsilosis. Gels containing 400 μg of protein derived from a citric acid extract of the granule-rich fraction or from the 27,000 × g postgranule supernatant were electrophoresed on polyacrylamide as described in the text. The gels were sectioned and eluted, and eluates were tested against C. parapsilosis at pH 5.0. Candidacidal activity was evaluated by dye exclusion. The cathodal fractions are to the right. Lysozyme and the more cathodal protein correspond to sections 20 to 22 of the gel.*

*parapsilosis* than were the eluates with lysozyme activity (data not shown).

**Rabbit and guinea pig heterophils.** Electrophoretic patterns of GRF extracts from rabbit and guinea pig heterophils differed from those obtained with human neutrophils. Rabbit heterophils contained at least five proteins, extractable from the GRF with 0.01 M citric acid,

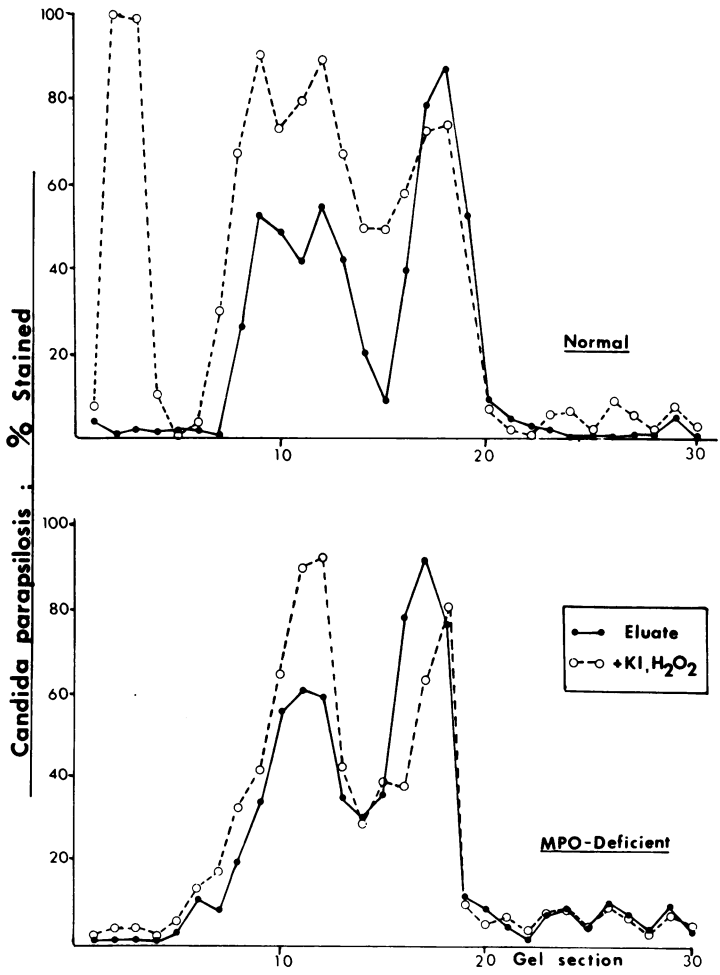


FIG. 5. *Candidacidal components of normal and MPO-deficient neutrophils. Citric acid extracts of granule-rich fractions were electrophoresed, eluted, and tested against C. parapsilosis at pH 5 with the dye exclusion assay. Solid lines show the effects of the eluates. Interrupted lines show the effects of supplementing of the eluates with $10^{-5}$ M KI and $10^{-3}$ M $H_2O_2$. These concentrations of KI and $H_2O_2$ lacked intrinsic candidacidal activity.*

whose cathodal migration greatly exceeded that of lysozyme (Fig. 7). Some, but not all, of these highly cathodal proteins killed *C. parapsilosis* in vitro (Fig. 8). Candidacidal activity was also observed in fractions containing lysozyme.

Guinea pig heterophils also contained cathodal proteins whose migration considerably exceeded that of lysozyme. These were not as abundant or heterogeneous as the cationic proteins of the rabbit cells. *C. parapsilosis* was killed by the major cathodal protein of the guinea pig extracts, and additional candidacidal activity was present in fractions containing lysozyme and other less cathodal proteins.

**Esterase stains.** GRF extracts of human, guinea pig, and rabbit granulocytes were electrophoresed and the gels were stained for naphthol-ASD acetate esterase activity (Fig. 9). Human extracts contained at least seven clearly distinguishable bands of esterase activity, including one whose migration coincided with that of the protein band whose cathodal migration exceeded that of lysozyme. Two major and two minor bands of activity were present in

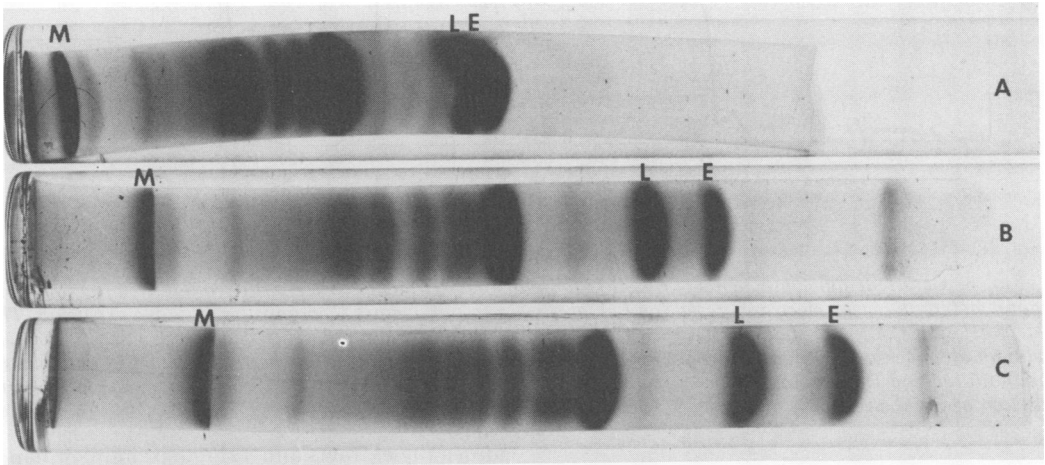


FIG. 6. *Effect of polyacrylamide concentration on resolution of GRF extracts. Electrophoresis was carried out as described in the text, except that in addition to the standard running gel containing 15.3% polyacrylamide (A), gels containing 13.0% (B) and 10.8% (C) polyacrylamide were also used. Abbreviations: M, Myeloperoxidase; L, lysozyme; E, esterase.*

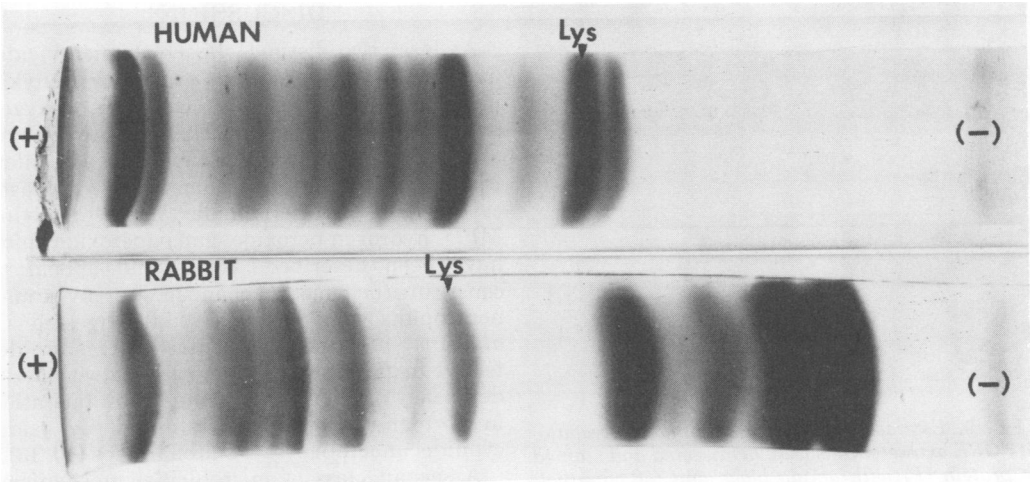


FIG. 7. *Components of human and rabbit heterophils. Citric acid extracts of granule-rich fractions from human and rabbit granulocytes were eletrophoresed and stained for protein with amido black. The cathodal dye front (−) and lysozyme (Lys) are indicated. Note the multiple highly cathodal proteins in the rabbit extracts without direct counterpart in the human material.*

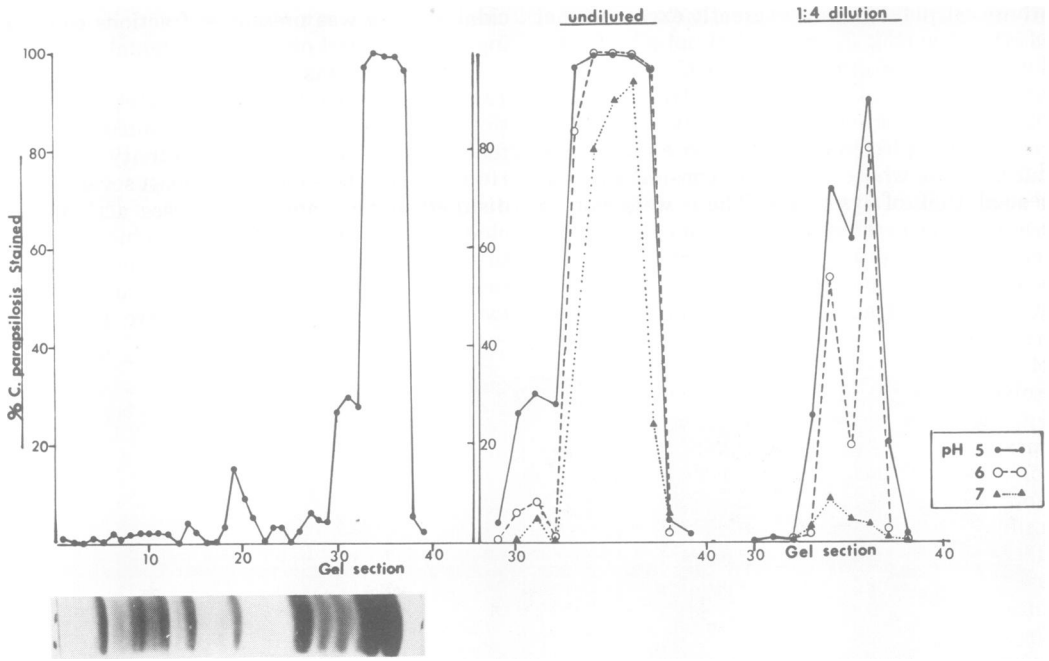


FIG. 8. *Candidacidal components of rabbit heterophils. A citric acid extract of the granule-rich fraction from rabbit heterophils was electrophoresed, and eluates were tested against C. parapsilosis by dye exclusion. The left portion of the figure shows activity by the eluates. The companion half of the gel was stained for protein with amido black and is shown to scale beneath the abscissa. The effect of pH on activity against C. parapsilosis of undiluted and 1:4 diluted eluates corresponding to the group of highly cationic proteins is shown in the right portion of the figure.*

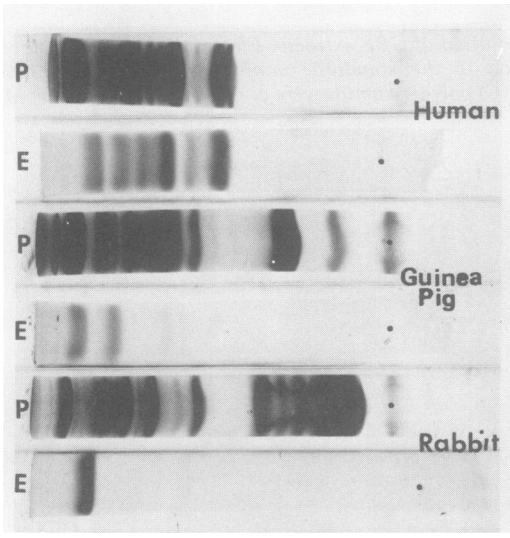


FIG. 9. *Esterase staining of mammalian granulocyte GRF extracts. Gels were hemisected and stained for protein (P) with amido black and for naphthol-ASD acetate esterase activity (E) as described in the text. The position of the dye front is marked by a dot.*

guinea pig extracts and a single prominent band was present in rabbit extracts. The highly cationic proteins of rabbit and guinea pig granules did not show esterase activity, nor did purified human or hen egg white lysozyme.

## DISCUSSION

At least two distinct classes of microbicidal mechanisms enable human granulocytes to kill ingested fungi and bacteria. One class is oxygen dependent and is integrated with the striking alterations in oxidative metabolism that follow the uptake of particulate material by phagocytic cells (2, 7). Microbicidal systems involving MPO, hydrogen peroxide, and superoxide anion have been described (8, 9), and appear to constitute the major mechanism whereby human neutrophils and monocytes kill ingested *C. albicans* (14). Such fungicidal systems are defective in granulocytes from subjects with MPO deficiency or CGD (13–15), and their operation in normal neutrophils is inhibited by azide, cyanide, anaerobiosis, or sulfonamides (10, 13).

A second class of microbicidal mechanisms functions in the absence of oxygen (18) and may account for certain of the residual microbicidal

activity displayed by MPO-deficient or CGD granulocytes (11). *C. parapsilosis* was found to be highly susceptible to such mechanisms (11) and therefore was selected for this investigation.

There is much information concerning the presence of microbicidal constituents in mammalian and other vertebrate granulocytes. Citric acid extracts of granules from rabbit heterophils kill gram-positive and gram-negative organisms in vitro (6), and this is attributable, in large part, to a family of highly cationic proteins of low molecular weight that have been characterized by the studies of Zeya and Spitznagel (24-27). The proteins are rich in arginine, can be resolved into multiple bands by electrophoresis, and appear to be localized to the peroxidase-containing primary granules of the rabbit heterophil (24-27). These components are not known to have enzymatic activity, and they exhibit some specificity in their antibacterial effects. Similar cationic proteins have been described in the granules of guinea pig heterophils and were noted to be active against *C. albicans* (24).

Our limited investigations with guinea pig and rabbit heterophils confirm the existence of these highly cationic proteins and show them also to be effective against *C. parapsilosis*. Other candidacidal components were also noted in our studies, but their effects were relatively less prominent. It is of interest that fractions containing lysozyme also killed *C. parapsilosis*. Collins and Pappagianis recently reported *Coccidioides immitis* spherules to be killed by human or hen egg white lysozyme in vitro (3), and Gadebusch and Johnson had earlier noted lysozyme to be effective against *Cryptococcus neoformans* in vitro (4).

Welsh and Spitznagel used cellulose acetate electrophoresis to demonstrate that certain granule-rich fractions prepared from peripheral blood leukocytes of normal human subjects contained proteins whose cathodal migration exceeded that of lysozyme (23). Although extracts of these granule fractions inhibited the growth of a strain of *E. coli* in vitro, fractions lacking these components were also inhibitory, and the contribution of cationic proteins to antibacterial effect was not established.

We found that citric acid extracts of granule-rich fractions prepared from normal and MPO-deficient human neutrophils killed *C. parapsilosis* and *C. albicans* in vitro. However, polyacrylamide electrophoresis indicated marked differences between the active components of human neutrophil granules and those of rabbits and guinea pigs. In agreement with Welsh and Spitznagel (23), we did not detect in human granulocytes the abundant low molecular weight, highly cationic proteins characteristic of the rodent heterophils. A single component whose cathodal migration exceeded that of lysozyme was observed under the conditions of our electrophoretic separation. This component had esterase activity toward naphthol ASD-acetate, and was among the candidacidal components detected in these experiments. This conclusion is based on the association of candidacidal and esterase activities under two conditions of electrophoresis. It is strengthened by the observation of similar electrophoretic mobility and candidacidal and esterase activity by a highly purified preparation of protein from leukemic granulocytes, as discussed below. However, experiments with highly purified cationic esterase from normal granulocytes will be required for definitive attribution of candidacidal activity to this protein.

Our experiments do not exclude the existence of other constituents, more cationic than lysozyme, in human neutrophils. Polyacrylamide electrophoresis separates proteins on the basis of molecular size as well as charge; thus, components that were both more cationic and of larger molecular dimensions than lysozyme might exhibit lesser cathodal migration.

Olsson and Venge (19) examined granulocytes from patients with chronic myelogenous leukemia and purified seven proteins whose cathodal migration on cellulose acetate or agarose exceeded that of lysozyme. The four most cationic proteins (fractions I–IV) had virtually identical amino acid compositions, showed immunological identity, and ranged in molecular weight from 25,500 to 28,500. We tested several of these components in collaboration with these investigators, and found their fractions I and II to correspond in migration to the most highly cathodal component on our gels. Like it, their preparation had naphthol ASD-acetate esterase activity and killed *C. parapsilosis* in vitro. Their fractions III and IV also possessed demonstrated esterase and candidacidal activity, but migrated less cathodally than lysozyme on the polyacrylamide system used in our studies. The fungicidal activity of these purified fractions will be described in a future communication. The presence of highly cationic esterases in human neutrophils was also demonstrated by Rindler and Braunsteiner (21) and Li et al. (16).

We believe our studies to be significant in several general areas. By demonstrating that *C. parapsilosis* is killed by multiple components present in human neutrophils, they could ac-

count for the ability of MPO-deficient and CGD granulocytes to kill this microorganism and explain how normal granulocytes may kill certain microorganisms under conditions inimical to the operation of oxygen-dependent microbicidal mechanisms. They underline the presence of marked interspecies differences involving the microbicidal components of granulocytes. They may also provide methodology suited to an examination of factors influencing phagocyte performance in man.

## ACKNOWLEDGMENTS

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