INFECTION AND IMMUNITY, Feb. 1991, p. 521–528
0019-9567/91/020521-08$02.00/0
Copyright © 1991, American Society for Microbiology
Vol. 59, No. 2

# Mechanism for Candidacidal Activity in Macrophages Activated by Recombinant Gamma Interferon

KOJI WATANABE, KEIKO KAGAYA, TOSHIHIKO YAMADA, AND YOSHIMURA FUKAZAWA*

*Department of Microbiology, Yamanashi Medical College, Tamaho-cho, Yamanashi 409-38, Japan*

Received 30 July 1990/Accepted 30 October 1990

**Candidacidal activity in macrophages activated by recombinant gamma interferon was examined kinetically in relation to acidification of phagolysosomes. In resident peritoneal macrophages (PMPs) of BALB/c mice, enhanced killing activity against *Candida albicans* was demonstrated after incubation with 100 U of gamma interferon per ml for 24 h but not after incubation for 48 to 72 h. Conversely, increased generation of $H_2O_2$ was exhibited in PMPs incubated from 48 to 72 h but not in PMPs incubated for 24 h. In normal PMPs, fusion of lysosomes to candida-containing phagosomes was readily accomplished and phagosome-lysosome fusion was not enhanced further by activation. The candidacidal substance was extracted from granule-rich fractions of either normal or activated PMPs by using citric acid (pH 2.7) in equal amounts; the substance showed a noncationic, heat-stable protein nature. In addition, when phagolysosomal pH was determined by flow cytometry of intraphagolysosomal fluorescein isothiocyanate-labeled *C. albicans*, phagolysosomes with low pH (<4.0) were detected in about 40% of PMPs activated for 24 h but not in those activated for 72 h or in normal PMPs. Moreover, increasing the intralysosomal pH with $NH_4Cl$ resulted in a significant reduction of candidacidal activity in activated PMPs. These results indicate that the candidacidal activity of gamma interferon-activated PMPs correlates well with enhanced acidification of their phagolysosomes and suggest that the candidacidal activity of activated PMPs is independent from reactive oxygen molecules and is mediated by proteinaceous substance(s) generated only in a strong acidic milieu of phagolysosomes by activation.**

The endogenous pathogen *Candida albicans* is the most virulent species of the genus *Candida* and is a leading cause of human candidiasis. Most cases of human systemic candidiasis arise in compromised hosts (15). Although a major role for neutrophils in normal (nonspecific) host defense against *C. albicans* has been firmly established (1, 23), it is conceivable that cell-mediated immunity also plays an important role in the specific defense mechanism(s) against *Candida* infection (15, 16, 20).

Recently, gamma interferon (IFN-γ) was implicated as a potent macrophage-activating factor (29, 31) in cell-mediated immunity. Upon exposure to recombinant IFN-γ, macrophages develop the capacity to kill intracellular bacteria (13, 21) and fungi (5, 6, 8, 14), to secrete reactive oxygen intermediates (21), monokines (10), and cytotoxic molecules (10), and to nonspecifically kill a variety of neoplastic cells (31, 33).

Only a few researchers have reported on the killing activity of macrophages against *C. albicans*. In general, normal human (23, 36), rabbit (1), and murine (4) macrophages per se reportedly have only a limited capacity to kill *C. albicans*. Murine peritoneal macrophages (PMPs) were reported to acquire candidacidal activity when activated with IFN-γ (8, 33). The cytokine macrophage colony-stimulating factor also stimulated murine macrophages to enhance the killing activity against *C. albicans* by augmented expression of mannose receptors (22). However, the intracellular mechanisms by which activated macrophages kill *C. albicans* still remain unclear, although both oxygen-dependent (9) and oxygen-independent (26) mechanisms have been proposed.

In the present study, we provide experimental evidence to suggest that candidacidal activity of IFN-γ-activated PMPs is independent from reactive oxygen intermediates and is mediated by proteinaceous substance(s) generated only in a strong acidic milieu of phagolysosomes by activation.

* Corresponding author.

## MATERIALS AND METHODS

**Mice.** Male BALB/c mice (ages 8 to 12 weeks), obtained from Charles River Japan, Inc. (Atsugi, Kanagawa), were used for the present study.

**Culture medium.** RPMI 1640 medium (Flow Laboratories, Inc., McLean, Va.) supplemented with L-glutamine and 10% fetal bovine serum (FBS; Flow Laboratories) (RPMI-FBS) was used throughout the experiments. A 1-ng amount of lipopolysaccharide (*Escherichia coli* O111:B4; Difco Laboratories, Detroit, Mich.), 50 U of penicillin per ml, and 50 μg of streptomycin per ml were added to RPMI-FBS.

**IFN-γ.** IFN-γ (Genentech Inc., South San Francisco, Calif.) (17) was a gift from Toray Industries Inc. (Tokyo, Japan). IFN-γ was stored at 4°C in phosphate-buffered saline (PBS) at a concentration of $10^6$ U/ml. Its antiviral activity was assessed by a virus plaque reduction assay with L929 cells and the vesicular stomatitis virus system.

***Candida* strain and growth condition.** The *C. albicans* serotype A strain M1012 has been maintained in our laboratory by monthly transfers on modified Sabouraud glucose agar (2% glucose, 1% polypeptone, 0.5% yeast extract, 1.5% agar) slants. Yeast cells were cultured for 48 h at 27°C on the medium and then stored at 4°C. For experiments, stock yeasts were grown in Sabouraud glucose broth for 24 h at 27°C with constant shaking. Yeasts were harvested by centrifugation, washed thrice in physiological saline solution, counted on a hemacytometer, and suspended in physiological saline solution at a concentration of $10^8$ cells per ml.

**Activation of PMPs.** Resident PMPs were harvested from normal mice by peritoneal lavage with Eagle minimum essential medium (MEM; Nissui Pharmaceutical Co., Ltd.,

Tokyo, Japan) containing 5 U of heparin per ml. The cells were washed thrice with MEM and counted on a hemacytometer. Cell suspensions ($10^6$/ml) in RPMI-FBS were plated on 24-well tissue culture plates (Falcon 3047; Becton Dickinson Labware, Oxnard, Calif.) and incubated at 37°C in 5% $CO_2$–95% air. After a 2-h incubation, plated cells were washed thrice with MEM to remove nonadherent cells, and the monolayers were cultured in RPMI-FBS with or without 100 U of IFN-γ per ml for 4 to 72 h at 37°C in 5% $CO_2$–95% air.

**Phagocytic candidacidal assay.** The phagocytic candidacidal assay was performed by the method of Sasada et al. (36). Briefly, cultured macrophage monolayers were washed thrice with MEM, and the medium was replaced with a 1-ml suspension of $10^5$ *Candida* cells in RPMI 1640 containing 10% fresh mouse serum. After incubation at 37°C for 3 h in a $CO_2$ incubator, the culture plates were placed on ice and sonicated for 30 s at a power setting of 1 (Ultrasonic disruptor, UR-200P; Tomy Seiko Co., Tokyo, Japan). Then the numbers of viable yeast cells were determined by colony counting. During 3 h of incubation the number of yeast cells did not increase in wells without PMPs, although germ tubes were produced. Killing activity was determined by the following formula: percent killing = [1 − (number of viable yeasts in well with PMPs/number of viable yeasts in well without PMPs)] × 100.

**Assay of $H_2O_2$ generation.** The $H_2O_2$-generating activity of PMPs was determined by the method of Pick and Keisari (34) with some modifications as described previously (21). Briefly, monolayers of cultured PMPs were washed with phenol red solution (100 μg/ml) containing 17 U of horseradish peroxidase (170 U/mg of protein; Sigma Chemical Co., St. Louis, Mo.) per ml, 0.01 M potassium phosphate buffer (pH 7.0), 0.14 M NaCl, and 1 mg of glucose per ml, covered with 1 ml of the phenol red solution with 1 μg of phorbol myristate acetate (Sigma) per ml, and incubated at 37°C in 5% $CO_2$–95% air for 1 h. A 10-μl portion of 1 N NaOH was added to the cell supernatants, and the $A_{610}$ was determined. The results were expressed as nanomoles of $H_2O_2$ per milligram of macrophage protein per hour. In some experiments, viable cells of *C. albicans* ($3.0 \times 10^6$/ml) were used as a triggering agent.

**P-L fusion.** Transfer of free acridine orange label from lysosomes to phagosomes was assayed by fluoromicroscopy (6). Macrophage monolayers ($1 \times 10^6$ cells) on coverslips (15 mm in diameter) were washed with MEM to remove serum components, acridine orange (5 μg/ml) was added, and the cells were incubated for 15 min. The coverslips were washed free of acridine orange, infected with *C. albicans* ($1 \times 10^5$/ml) for 1 h, washed, and further incubated for 2 h in RPMI 1640–20% FBS. After 2 h of incubation, the coverslips were washed, air dried, and tallied for the number of yeast cells per 1,000 PMPs under a fluorescent microscope (Olympus Optical Co., Ltd., Tokyo, Japan). Phagosome-lysosome (P-L) fusion was expressed as the percentage of colored intracellular yeast cells among the total intracellular yeast cells. In some experiments, macrophage monolayers were incubated with colchicine ($10^{-5}$ M; Sigma) for 5 h (7), followed by examination of candidacidal activity and P-L fusion.

**Preparation of granule extracts.** Granule extracts were isolated from PMPs by the method of Patterson-Delafield et al. (32). Briefly, PMPs were activated with IFN-γ (100 U/ml) for 24 h and collected in PBS (pH 7.4) by scraping with a rubber policeman. After being washed with PBS, PMPs were suspended at $5 \times 10^7$ cells per 1.0 ml of 0.34 M sucrose and disrupted with a Teflon pestle homogenizer. The homogenate was centrifuged at 900 × *g* for 10 min at 4°C, and the supernatant, which was rich in cytoplasmic granules, was centrifuged at 27,000 × *g* for 20 min at 4°C. The granule pellet was stored at −20°C. For extraction, granules were suspended in 0.3 ml of 0.01 M citric acid (pH 2.7) and stirred for 2 h in an ice bath. After centrifugation at 27,000 × *g* for 20 min, the supernatant was isolated and stored at −20°C for later use. The protein contents of the extracts were determined by the method of Lowry et al. (27) with bovine serum albumin (Sigma) as the standard.

**Candidacidal activity of granule extracts.** *C. albicans* cells were washed twice with 0.01 M phosphate buffer (pH 7.4) and suspended in phosphate buffer. Assay mixtures consisted of $5 \times 10^4$ yeast cells in phosphate buffer and granule extracts at a concentration of 5 μg of protein per ml (total volume, 0.5 ml) (26). For characterizing the candidacidal molecule(s), trypsin (500 μg/ml; Sigma), polyanion (poly-L-aspartic acid, $5 \times 10^{-4}$ M; Sigma), or EDTA (0.01%; Sigma) was added to the assay mixtures, or the granule extracts were heated at 100°C for 3 min. The 0.01 M citric acid solution was added to each control. After incubation for 1 h at 37°C, the samples were serially diluted and inoculated onto Sabouraud agar plates. The plates were incubated at 37°C for 24 h, and the resulting colonies were counted. To determine the candidacidal activity of granule extracts at pH 3, 0.01 M citrate buffer (pH 3.0) was used for the assay as well. The activity was expressed as % of killing determined by the following formula: percent killing = [1 − (number of viable yeasts in assay mixture/number of viable yeasts in control mixture)] × 100.

**Intraphagolysosomal pH.** Flow cytometry (FCM) was used for the measurement of intraphagolysosomal pH of PMPs containing *C. albicans* cells by using fluorescein isothiocyanate (FITC)-labeled yeast cells. Viable *C. albicans* cells were washed with physiological saline solution and suspended in carbonate-bicarbonate buffer (pH 9.3) at a concentration of $2.0 \times 10^8$ per 2 ml. To this solution, 200 μl of fluorescein amine isomer 1 (3 mg/ml; Sigma) in dimethyl sulfoxide was added in drops with continuous stirring, and the reaction mixture was left for 2 h at room temperature protected from light. After FITC-labeled *C. albicans* cells were washed with physiological saline solution, they were stored at −80°C for later use. After conjugation, 75% of yeast cells remained viable. For calibration curves, the fluorescence intensities (FI) of FITC-labeled yeast cells at various ambient pHs were analyzed by FCM with an EPICS PROFILE (Coulter Co., Hialeah, Fla.). The relative FI of FITC-labeled yeast cells changed with their ambient pH, and a calibration curve for converting relative FI into ambient pH was obtained.

The FITC-labeled yeast cells were adjusted to a concentration of $4 \times 10^5$/ml in RPMI 1640 containing 10% fresh mouse serum, and 0.5 ml of this suspension was added to macrophage monolayers ($1 \times 10^6$; effector/target cell ratio, 5) in 24-well plates (Falcon 3047). After 30 min of incubation, monolayers were washed thrice with PBS, and PMPs were collected by pipetting in 0.01% EDTA–PBS. In this procedure, 93.0% ± 5.2% and 94.2% ± 4.4% of normal and activated PMPs, respectively, were shown to be removed from the wells by determining their protein contents. Furthermore, when the viability of PMPs in the suspension was determined by trypan blue dye exclusion, 82.3% ± 5.2% and 85.6% ± 2.8% of the normal and activated PMPs, respectively, were viable. Thus, the FI of FITC-labeled *C. albicans*

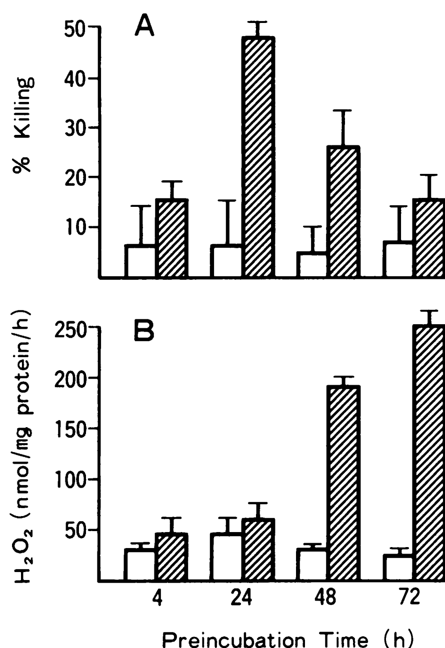


FIG. 1. Effect of IFN-γ on candidacidal activity (A) and $H_2O_2$-generating activity (B) of PMPs. Monolayers of PMPs were preincubated with (▨) or without (□) 100 U of IFN-γ per ml for 4 to 72 h. Candidacidal activity was assayed by determining the viable number of *C. albicans* 3 h after infection as described in the text. $H_2O_2$-generating activity was determined by triggering PMPs with 1 μg of phorbol myristate acetate per ml.

cells in PMPs was analyzed by FCM, and intraphagolysosomal pH was determined from the calibration curve.

**Effect of alkalization of phagolysosomes on candidacidal activity.** *C. albicans* was added to macrophage monolayers with or without 10 mM $NH_4Cl$ (35), and the candidacidal activity of PMPs was determined after a 3-h incubation as described above. $NH_4Cl$ at 10 mM was not toxic for *C. albicans*.

**Statistical analysis.** The statistical significance of the data was analyzed by Student's *t* test.

## RESULTS

**Time course of macrophage activation for candidacidal activity and $H_2O_2$ generation.** Macrophage monolayers were preincubated with IFN-γ for 4, 24, 48, or 72 h, and their candidacidal activity was determined to investigate the kinetics of macrophage activation for candidacidal activity. Control PMPs did not kill *C. albicans* at any stage during the incubation period. When PMPs were preincubated with 100 U of IFN-γ per ml for 4 h, candidacidal activity was not exhibited; however, markedly enhanced candidacidal activity ($P < 0.01$) was observed when PMPs were preincubated for 24 h with IFN-γ. The capacity of PMPs activated by IFN-γ to kill *C. albicans* decreased with stimulation time and disappeared when PMPs were preincubated with IFN-γ for 72 h (Fig. 1A).

On the other hand, $H_2O_2$ release of PMPs after triggering with phorbol myristate acetate was markedly increased ($P < 0.01$) when PMPs were preincubated for more than 48 h with IFN-γ, whereas PMPs preincubated for 4 or 24 h did not exhibit any significant increase in $H_2O_2$ generation after phorbol myristate acetate triggering, as compared with that of control PMPs (Fig. 1B), indicating that enhanced $H_2O_2$ generation requires preincubation with IFN-γ for more than 48 h. Furthermore, when $H_2O_2$ generation of PMPs was triggered by phagocytosis of viable cells of *C. albicans*, PMPs treated with IFN-γ for 24 h did not exhibit enhanced $H_2O_2$ generation, whereas PMPs treated with IFN-γ for more than 48 h exhibited enhanced $H_2O_2$ generation (data not shown). These results suggest that IFN-γ-activated PMPs are able to kill *C. albicans* when enhanced generation of reactive oxygen intermediates is not detectable.

TABLE 1. Correlation between P-L fusion and candidacidal activity

| Macrophages[a] | Colchicine treatment[b] | P-L fusion (%)[c] | % Killing |
|---|---|---|---|
| Normal | − | 77.9 ± 5.1 | 9.5 ± 6.7 |
| | + | 35.6 ± 1.6[d] | −7.9 ± 5.4 |
| Activated | − | 74.9 ± 1.0 | 42.0 ± 0.9 |
| | + | 37.6 ± 9.0[d] | 26.6 ± 0.4[d] |

[a] Macrophages were preincubated with or without 100 U of IFN-γ per ml for 24 h.
[b] Colchicine ($10^{-5}$ M) was added to macrophage monolayers for 5 h before infection and for 3 h thereafter.
[c] Determined by transfer of free acridine orange label from lysosomes to phagosomes.
[d] $P < 0.01$ compared with results from untreated PMPs.

**Effect of P-L fusion on candidacidal activity in IFN-γ-activated PMPs.** P-L fusion in PMPs after phagocytosis of *C. albicans* cells was assessed to clarify whether enhancement of P-L fusion caused candidacidal activity of activated PMPs. In spite of culturing PMPs for 24 h without IFN-γ, a high rate of P-L fusion similar to that in activated PMPs was noted, although these PMPs exhibited only a low level of candidacidal activity (Table 1). On the other hand, when activated PMPs were treated with colchicine (an inhibitor of microtubule polymerization) and infected with *C. albicans*, candidacidal activity was significantly decreased ($P < 0.01$) in relation to a decreased rate of P-L fusion (Table 1), whereas ingestion of yeast cells was not inhibited by colchicine treatment. These results indicate that P-L fusion is intrinsic in normal PMPs after phagocytizing *C. albicans*.

**Candidacidal activity of granule extracts.** The killing activities of granule extracts from normal and activated PMPs against *C. albicans* were compared. Interestingly, candidacidal activities were detected not only in extracts from lysosomes of activated PMPs but also in those of normal PMPs, and the total amounts of protein in the extracts from activated PMPs were not significantly higher than those from normal PMPs; yields of proteins were 31.6 ± 4.6 and 44.0 ± 4.8 μg per $10^7$ cells of normal and activated PMPs, respectively, in three experiments. Since the dose-response curves of candidacidal activities of granule extracts from activated and normal PMPs were similar to each other when equal amounts of protein were tested (data not shown), the total activity of the candidacidal substance in the granule extract from activated PMPs seems to be similar to that of the extract from normal PMPs.

The candidacidal activities of granule extracts from normal and activated PMPs under various conditions are shown in Table 2. The fact that EDTA did not affect killing activity made it clear that the reduction of colonies observed in this assay system was not due to aggregation of yeast cells (26). The killing activity of granule extracts was not affected by heat treatment. Moreover, the addition of a polyanion,

TABLE 2. Killing activity of granule extracts under various conditions[a]

| Treatment | % Killing at 60 min of incubation in granule extracts from: | |
|---|---|---|
| | Normal PMPs | Activated PMPs |
| None | 81.0 ± 1.0 | 80.3 ± 0.9 |
| 100°C, 3 min | 89.3 ± 1.8 | 89.9 ± 2.2 |
| Trypsin (500 μg/ml) | 23.0 ± 1.8[b] | 16.8 ± 7.0[b] |
| Polyaspartic acid (5 × $10^{-4}$ M) | 70.7 ± 10.6 | 77.4 ± 0.3 |
| EDTA (0.01%) | 77.7 ± 5.0 | 78.3 ± 3.1 |

[a] Killing activity was assayed at a concentration of 5 μg of protein per ml in 0.01 M phosphate buffer (pH 7.4) followed by heat treatment or by the addition of some chemical reagents. Results are expressed as means ± standard deviations of three experiments.

[b] $P < 0.01$ compared with results from nontreated extracts.

polyaspartic acid, at a concentration 50-fold higher than that used for inhibiting the killing activity of cationic peptide from neutrophils (11) did not affect the killing activity of the extracts tested. In contrast, the activity was abolished by trypsin (500 μg/ml). Furthermore, the sensitivities of the candidacidal substance(s) in extracts from both normal and activated PMPs to the physicochemical agents used were identical. These results indicate that the effector molecules in the granule extracts of both PMPs are noncationic heat-stable proteinaceous substances. These results suggest that these molecules are not newly synthesized by stimulation with IFN-γ but rather function effectively only in the activated PMPs, and that extraction of proteins from lysosomes with the aid of citrate at pH 2.7 induces modification of the constitutively produced lysosomal proteinaceous substance(s) to be endowed with candidacidal activity.

**Determination of acidification of phagolysosomes after ingestion of live *C. albicans*.** The pHs of phagolysosomes containing live *C. albicans* cells in normal and activated PMPs were compared by FCM analysis with FITC-labeled *C. albicans*. FITC-labeled *C. albicans* cells suspended in buffers with various pHs demonstrated specific changes in FI with their ambient pH (Fig. 2). The calibration curve of ambient pH against FI of FITC-labeled *C. albicans* is shown in Fig. 3.

When macrophage monolayers were incubated for 30 min with FITC-labeled live *C. albicans* cells (effector/target cell ratio, 5) in the presence of fresh mouse serum, removed from the wells with EDTA, and examined microscopically, all yeast cells were determined to be ingested; they were found in phagosomes surrounded by clear zone, and there was no PMP with more than two yeast cells. The percentage of candida-containing PMPs was the same in normal and activated PMPs (27.4% ± 0.8% and 26.5% ± 2.9%, respectively, in three experiments). These PMPs revealed three populations (regions I, II, and III) by FCM analysis (Fig. 4B and D). From the calibration curve in Fig. 3, we note that the high FI (>201) peak (region III) ranged from pH 6 to 8, the middle FI (11 to 200) peak (region II) ranged from pH 4 to 6, and the low FI (<10) peak (region I) ranged from pH 4 down, indicating that PMPs were divided into three populations that contain acidic (pH <4), weakly acidic (pH 4 to 6), and neutral (pH >6) phagolysosomes, respectively. Since nonspecific fluorescence was observed in PMPs without ingesting FITC-labeled yeast cells at FI below 10 (region I) (Figs. 4A and C), the FITC-labeled yeast cells at pHs of <3 were thought to fall on the above region I. Furthermore, since the FI of intracellular yeast cells is quenched by macrophages, the detected FI of intracellular yeasts was deemed to be lower than their exact value. Therefore, although the exact pH of phagosomes in region I cannot be shown, the number of phagolysosomes with low pH was calculated by subtracting nonphagocytizing macrophages from the total macrophages in region I. The precise numbers of cells in each region of Fig. 4 detected by the FCM analyzer and the frequencies of phagocytizing PMPs in each region are shown in Table 3.

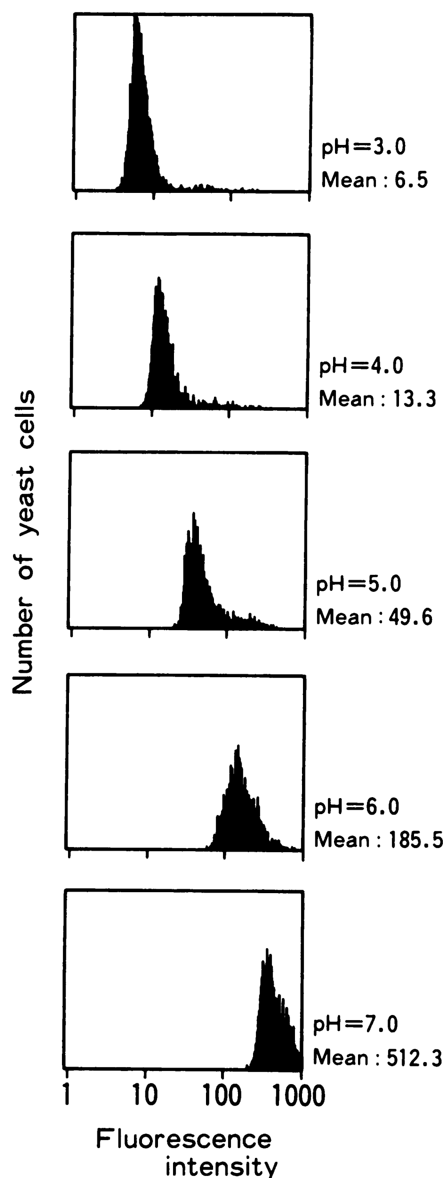


FIG. 2. FI of FITC-labeled *C. albicans* at various pHs. The FI of FITC-labeled *C. albicans* in buffers of pH 3.0 to 7.0 were analyzed by FCM, and the relative FI values are shown.

**Correlation between acidification of phagolysosomes and killing activity in PMPs.** The frequencies of incidence of phagolysosomes with each pH range were determined by calculating results of three experiments, one of which is shown in Table 3. In PMPs that were preincubated without

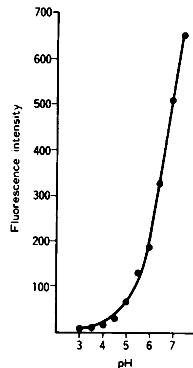


FIG. 3. Calibration curve of ambient pH as a function of the FI of FITC-labeled *C. albicans* cells. The data shown in Fig. 2 were used.

IFN-γ for 24 or 72 h, less than 2% of phagolysosomes showed a low pH (Fig. 4, region I). In contrast, in activated PMPs preincubated with IFN-γ for 24 h, which showed enhanced candidacidal activity, phagolysosomes with low pH were markedly increased (more than 40%) (Fig. 5A). The frequency of incidence of phagolysosomes with low pH was similar to that of intracellular killing; the killing rate of *C. albicans* in 24-h-activated PMPs ranged from 35 to 50% in repeated experiments (Fig. 1). On the other hand, 72-h-activated PMPs, which lacked candidacidal activity (Fig. 1), exhibited a low frequency of incidence of phagolysosomes with low pH, similar to that the control PMPs (less than 2%) (Fig. 5B). Moreover, although incubation in buffer at pH 3.0 per se did not affect viability of *C. albicans*, the extracts killed yeast cells at pH 3.0 (data not shown). These results indicate that candidacidal activity of PMPs correlated well with induction of highly acidified phagolysosomes and suggest that about 40% of the PMPs were activated by IFN-γ to induce acidic phagolysosomes, leading to candidacidal activity, and that generation of a candidacidal substance(s) may occur in phagolysosomes with low pHs in activated PMPs.

**Effect of $NH_4Cl$ on candidacidal activity of PMPs.** To further verify the correlation between candidacidal activity and acidification of phagolysosomes, we examined the candidacidal activity of the activated PMPs in the presence of $NH_4Cl$, which has been shown to raise lysosomal pH (35). The candidacidal activity of activated PMPs that were preincubated with IFN-γ for 24 h was significantly decreased ($P < 0.01$) in the presence of $NH_4Cl$; the killing of *C. albicans* by activated PMPs without $NH_4Cl$ and that with $NH_4Cl$ were 38.0% ± 3.4% and 20.2% ± 3.1%, respectively. This result further suggests that enhanced acidification of phagolysosomes is essential for killing of *C. albicans* in IFN-γ-activated PMPs.

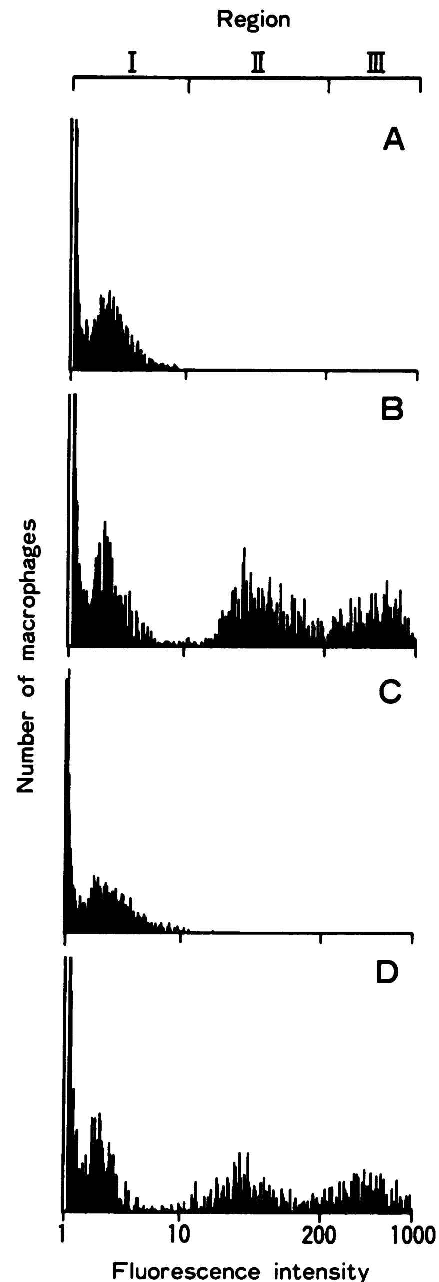


FIG. 4. FCM profiles of PMPs after ingestion of FITC-labeled *C. albicans*. Macrophage monolayers (1 × $10^6$) were preincubated with (D) or without (B) 100 U of IFN-γ per ml for 24 h, and FITC-labeled *C. albicans* cells (1 × $10^5$) in 10% fresh serum were added. After incubation for 30 min, the FI of PMPs was assayed. To determine nonspecific fluorescence, PMPs preincubated with (C) or without (A) IFN-γ were analyzed in the absence of phagocytosis. The precise numbers of particles in each region of histograms are shown in Table 3.

## DISCUSSION

The present experiments demonstrated a close correlation between killing of *C. albicans* and enhanced acidification of phagolysosomes in PMPs activated by IFN-γ, leading to generation of candidacidal proteinaceous substance(s). Oxygen-dependent killing mechanisms are deemed less important in candidacidal activity of murine PMPs, because the ability of activated murine PMPs to generate reactive oxygen intermediates is intrinsically much lower than that of neutrophils (2), although oxygen-dependent candidacidal mechanisms were reported in IFN-γ-treated human monocyte-derived macrophages (36).

P-L fusion is essential for microbial killing in phagocytes. Although some obligate or facultative intracellular microbes are known to inhibit P-L fusion (3, 18) and enhanced P-L fusion has been shown only in activated macrophages in association with enhanced killing activity (6, 21), neither

TABLE 3. FCM profiles of PMPs that had ingested FITC-labeled *C. albicans*[a]

| PMPs[b] | Yeast cells[c] | Region | pH | No. of PMPs | | | Frequency of phagocytizing PMPs (%) |
|---|---|---|---|---|---|---|---|
| | | | | Total | Nonphagocytizing[d] | Phagocytizing[e] | |
| Normal | − | I | <4 | 5,000 | 5,000 | 0 | 0 |
| | | II | 4–6 | 0 | 0 | 0 | 0 |
| | | III | >6 | 0 | 0 | 0 | 0 |
| | + | I | <4 | 3,715 | 3,678 | 37 | 3 |
| | | II | 4–6 | 750 | 0 | 750 | 57 |
| | | III | >6 | 525 | 0 | 525 | 40 |
| Activated | − | I | <4 | 5,000 | 5,000 | 0 | 0 |
| | | II | 4–6 | 0 | 0 | 0 | 0 |
| | | III | >6 | 0 | 0 | 0 | 0 |
| | + | I | <4 | 4,263 | 3,708 | 555 | 42 |
| | | II | 4–6 | 360 | 0 | 360 | 27 |
| | | III | >6 | 408 | 0 | 408 | 31 |

[a] Representative data of three experiments are shown. Histograms of this experiment are shown in Fig. 4.
[b] PMPs were preincubated with or without 100 U of IFN-γ per ml for 24 h.
[c] FITC-labeled *C. albicans* were added to macrophage monolayers at a concentration of $2.0 \times 10^5$ per 0.5 ml and incubated for 30 min.
[d] Calculated from phagocytic indices (26.3% both in normal and in activated PMPs).
[e] Calculated by subtracting the number of nonphagocytizing PMPs from the number of PMPs detected in the region.

inhibition by *C. albicans* nor enhancement by IFN-γ of P-L fusion in PMPs was observed in our case. Mor and Goren (28) reported that when PMPs were infected with live *C. albicans*, P-L fusion was not observed with acridine orange staining, whereas fusion was evident with electron microscopic observation. In our assay system, a high effector/target cell ratio of phagocytosis (ratio, 10) made possible the detection of P-L fusion by using acridine orange staining. In fact, the rate of P-L fusion detected with acridine orange staining was significantly reduced by using a low effector/target cell ratio of phagocytosis (0.2 or less; data not shown).

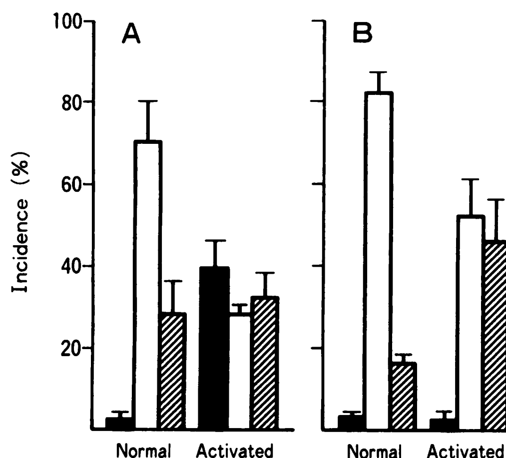


FIG. 5. Effect of macrophage activation with IFN-γ on the intraphagolysosomal pH of PMPs after ingestion of *C. albicans*. Macrophage monolayers were incubated with or without 100 U of IFN-γ per ml for 24 h (A) or 72 h (B) and infected with FITC-labeled *C. albicans* in 10% fresh serum. Macrophages were collected 30 min after infection and analyzed by FCM. The pH of each phagolysosome was obtained from the calibration curve, and phagosomes were divided into groups with pHs of <4 (■), 4 to 6 (□), and >6 (▨). The frequencies of incidence of phagolysosomes with each pH range were calculated as indicated in Table 3, and the results are expressed as the means ± standard deviations of three experiments.

In any case, our results revealed that *C. albicans* cells do not inhibit P-L fusion in PMPs.

Numerous reports have shown that neutrophils (24, 25) and alveolar macrophages (26, 32) possess several microbicidal cationic proteins or peptides in their lysosomes. Since microbicidal cationic proteins were reportedly present in very low amounts in normal rabbit PMPs (33), we expected that these proteins might be induced in PMPs by activation with IFN-γ. However, granule proteins endowed with killing ability could be obtained not only from activated PMPs but also from normal PMPs. Moreover, granule proteins from normal and activated PMPs did not vary in quality or quantity. Although the nature of the candidacidal substance(s) has not yet been defined, our experiments suggested that the effective molecule in PMPs is a noncationic proteinaceous substance(s) that differs from previously reported microbicidal cationic proteins or peptides in neutrophils and alveolar macrophages (24–26, 32); the activity was not inhibited by a high concentration of polyanion (Table 2) and was not detected in the cathodal region of polyacrylamide gel eluates, where killing activity of the neutrophil extract was detected (data not shown).

A candidacidal substance with the same physicochemical characteristics was extracted from both normal and activated PMPs by citric acid at pH 2.7, irrespective of the candidacidal activity of the PMPs. Since the phagolysosomes in normal macrophages generally exhibit a pH of approximately 5.0 (19), we speculated that the intraphagolysosomal pH is significantly low in activated PMPs, thereby modifying the constitutive lysosomal proteins that exist in both activated and normal PMPs. Therefore, we devised a method for measurement of intraphagolysosomal pH by using FCM to determine the pH of each phagolysosome separately and utilizing the pH sensitivity of fluorescence of FITC (30). Using FCM analysis, we found three populations of PMPs after FITC-labeled *C. albicans* cells were phagocytized. This method, although highly advantageous, still precludes the determination of the absolute value of phagolysosomal pHs less than 4 by interference with nonspecific fluorescence of PMPs and by quenching the fluorescence of yeast cells inside PMPs. However, the frequency of inci-

dence of phagolysosomes with pHs less than 4 among all phagolysosomes could be calculated by the data from FCM profiles (Table 3). The possibility that the low FI of yeast cells in phagolysosomes of activated PMPs may be induced by breaking of FITC molecules with some hydrolytic enzymes in lysosomes cannot be ruled out at the moment. However, the fact that the candidacidal substance in lysosomes can be extracted even from normal PMPs with low pH suggests that generation of the substance may occur in phagolysosomes with low pHs in activated PMPs. Furthermore, these extracts were found to kill *C. albicans* at pH 3.

When IFN-γ-stimulated PMPs ingested the FITC-labeled *C. albicans*, phagocytizing PMPs were divided into three populations; 40% were found to contain acidic phagolysosomes (Fig. 5). The remaining two populations contained weakly acidic and neutral phagolysosomes, respectively. The reason for the occurrence of such populations containing phagolysosomes with different pHs (not acidic) is not clear at present. It is, however, suggested that PMPs are heterogeneous with respect to the activation by IFN-γ for candidacidal activity.

Significantly, the rate of phagolysosomes with low pHs closely correlated with the candidacidal activity of PMPs (Fig. 1 and 5). The evidence that 24-h-activated PMPs exhibited 35 to 50% killing and 40 to 45% incidence of phagolysosomes with low pH supported the above-mentioned hypothesis. Additional evidence showed that the candidacidal activity of activated PMPs decreased with alkalization of phagolysosomes by $NH_4Cl$ and that prolonged stimulation of PMPs with IFN-γ induced the decay of candidacidal activity as well as the disappearance of phagolysosomes with low pH (Fig. 1 and 5). Furthermore, in J774.1 cells, macrophagelike cells that are unable to kill *C. albicans* irrespective of stimulation with IFN-γ, phagolysosomes with low pH were not detected (data not shown).

The mechanisms for acidification of phagolysosomes by IFN-γ activation and for the generation of candidacidal substance(s) in highly acidic phagolysosomes are still unclear. With regard to the latter, however, the following is hypothesized: (i) a lysosomal protein is split by a lysosomal proteinase that acts only in an acidic pH, followed by generation of an active substance; and (ii) strong acidification of phagolysosomes per se induces a conformation change to the active form of lysosomal protein(s) (12). Although numerous reports have attempted to elucidate the mechanism of enhanced microbicidal activity in IFN-γ-activated macrophages (6, 14, 21), to our knowledge this report is the first to document that enhancement of candidacidal activity is mediated by proteinaceous substance(s) generated only in a strong acidic milieu of phagolysosomes induced by activation with IFN-γ. We propose that further investigation is required to characterize such an effector substance(s) and its role in killing of intracellular pathogens in activated macrophages.

## ACKNOWLEDGMENTS

We thank Yuri Shirakura-Shibata for her technical assistance in FCM analysis and Reiko Tanaka for her assistance in the preparation of the manuscript.

## REFERENCES

1. **Arai, T., Y. Mikami, and K. Yokoyama.** 1985. Phagocytosis of *Candida albicans* by rabbit alveolar macrophages and guinea pig neutrophils. Sabouraudia **15:**171–177.
2. **Araki, S., K. Kagaya, K. Kitoh, M. Kimura, and Y. Fukazawa.** 1987. Enhancement of resistance to *Escherichia coli* infection in mice by dihydroheptaprenol, a synthetic polyprenol derivative. Infect. Immun. **55:**2164–2170.
3. **Armstrong, J. A., and P. D. Hart.** 1971. Response of cultured macrophages to *Mycobacterium tuberculosis* with observations on fusion of lysosomes with phagosome. J. Exp. Med. **134:**713–740.
4. **Baccarini, M., E. Blasi, P. Puccetti, and F. Bistoni.** 1983. Phagocytic killing of *Candida albicans* by different murine effector cells. Sabouraudia **21:**271–286.
5. **Baghian, A., and K. W. Lee.** 1988. Role of activated macrophages in resistance to systemic candidosis. J. Leuk. Biol. **44:**166–171.
6. **Beaman, L., E. Benjamini, and D. Pappagianis.** 1983. Activation of macrophages by lymphokines: enhancement of phagosome-lysosome fusion and killing of *Coccidioides immitis*. Infect. Immun. **39:**1201–1207.
7. **Bhisey, A. N., and J. J. Freed.** 1971. Altered movement of endosomes in colchicine-treated cultured macrophages. Exp. Cell Res. **64:**430–438.
8. **Brummer, E., C. J. Morrison, and D. A. Stevens.** 1985. Recombinant and natural gamma-interferon activation of macrophages in vitro: different dose requirements for induction of killing activity against phagocytizable and nonphagocytizable fungi. Infect. Immun. **49:**724–730.
9. **Brummer, E., and D. A. Stevens.** 1989. Candidacidal mechanisms of peritoneal macrophages activated with lymphokines or γ-interferon. J. Med. Microbiol. **28:**173–181.
10. **Collart, M. A., D. Belin, J.-D. Vassalli, S. de Kosscdo, and P. Vassalli.** 1986. Interferon enhances macrophage transcription of the tumor necrosis factor/cachectin, interleukin 1, and urokinase genes, which are controlled by short-lived repressors. J. Exp. Med. **164:**2113–2118.
11. **Diamond, R. D., E. Huber, and C. C. Haudenschild.** 1983. Mechanisms of destruction of *Aspergillus fumigatus* hyphae mediated by human monocytes. J. Infect. Dis. **147:**474–483.
12. **Farahbakhsh, Z. T., R. L. Baldwin, and B. J. Wisnieski.** 1987. Effect of low pH on the conformation of *Pseudomonas* exotoxin A. J. Biol. Chem. **262:**2256–2261.
13. **Flesch, I., and S. H. E. Kaufmann.** 1987. Mycobacterial growth inhibition by gamma-interferon-activated bone marrow macrophages. Infect. Immun. **56:**1464–1469.
14. **Flesch, I. E., G. Schwamberger, and S. H. E. Kaufmann.** 1989. Fungicidal activity of IFN-γ-activated macrophages. Extracellular killing of *Cryptococcus neoformans*. J. Immunol. **142:** 3219–3224.
15. **Fukazawa, Y., and K. Kagaya.** 1988. Host defence mechanisms against fungal infection. Microbiol. Sci. **5:**124–127.
16. **Giger, D., J. Domer, S. Moser, and J. McQuitty.** 1978. Experimental murine candidiasis: pathological and immune responses in T-lymphocyte-depleted mice. Infect. Immun. **21:**729–737.
17. **Gray, P. W., and D. V. Goeddel.** 1983. Cloning and expression of murine immune interferon cDNA. Proc. Natl. Acad. Sci. USA **80:**5842–5846.
18. **Horwitz, M. A.** 1983. The legionnaires' disease bacterium (*Legionella pneumophila*) inhibits phagosome-lysosome fusion in human monocytes. J. Exp. Med. **158:**2108–2126.
19. **Jacques, Y. V., and D. F. Bainton.** 1978. Changes in pH within the phagocytic vacuoles of human neutrophils and monocytes. Lab. Invest. **39:**179–185.
20. **Kagaya, K., T. Shinoda, and Y. Fukazawa.** 1981. Murine defense mechanism against *Candida albicans* infection. I. Collaboration of cell-mediated and humoral immunities in protection against systemic *C. albicans* infection. Microbiol. Immunol. **25:**647–654.
21. **Kagaya, K., K. Watanabe, and Y. Fukazawa.** 1989. Capacity of recombinant gamma interferon to activate macrophages for *Salmonella*-killing activity. Infect. Immun. **57:**609–615.
22. **Karbassi, A., J. M. Becker, J. S. Foster, and R. N. Moore.** 1987. Enhanced killing of *Candida albicans* by murine macrophages treated with macrophage colony-stimulating factor: evidence for augmented expression of mannose receptors. J. Immunol. **139:** 417–421.
23. **Lehrer, R. I., and M. J. Cline.** 1969. Leukocyte myeloperox-

idase deficiency and disseminated candidiasis: the role of myeloperoxidase in resistance to *Candida* infection. J. Clin. Invest. **48**:1478–1488.

24. **Lehrer, R. I., T. Ganz, D. Szklarek, and M. E. Selsted.** 1988. Modulation of the in vitro candidacidal activity of human neutrophil defensins by target cell metabolism and divalent cations. J. Clin. Invest. **81**:1829–1835.
25. **Lehrer, R. I., K. M. Kadra, and R. B. Hake.** 1975. Nonoxidative fungicidal mechanisms of mammalian granulocytes: demonstration of components with candidacidal activity in human, rabbit, and guinea pig leukocytes. Infect. Immun. **11**:1226–1234.
26. **Lehrer, R. I., D. Szklarek, M. E. Selsted, and J. Fleischmann.** 1981. Increased content of microbicidal cationic peptides in rabbit alveolar macrophages elicited by complete Freund adjuvant. Infect. Immun. **33**:775–778.
27. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
28. **Mor, N., and M. B. Goren.** 1987. Discrepancy in assessment of phagosome-lysosome fusion with two lysosomal markers in murine macrophages infected with *Candida albicans*. Infect. Immun. **55**:1663–1667.
29. **Nathan, C. F., H. W. Murray, M. E. Wieble, and B. Y. Rubin.** 1983. Identification of gamma-interferon as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. J. Exp. Med. **158**:679–689.
30. **Ohkuma, S., and B. Poole.** 1978. Fluorescence probe measurement of intralysosomal pH in living cells and the perturbation of pH by various agents. Proc. Natl. Acad. Sci. USA **75**:3327–3331.
31. **Pace, J. L., S. W. Russel, B. A. Toress, H. M. Johnson, and P. W. Gray.** 1983. Recombinant mouse γ-interferon induces the priming step in macrophage activation for tumor cell killing. J. Immunol. **130**:2011–2013.
32. **Patterson-Delafield, J., R. J. Martinez, and R. I. Lehrer.** 1980. Microbicidal cationic proteins in rabbit alveolar macrophages: a potential host defense mechanism. Infect. Immun. **30**:180–192.
33. **Perfect, J. R., D. L. Granger, and D. T. Durack.** 1987. Effects of antifungal agents and γ-interferon on macrophage cytotoxicity for fungi and tumor cells. J. Infect. Dis. **156**:316–323.
34. **Pick, E., and Y. Keisari.** 1980. A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. J. Immunol. Methods **38**:161–170.
35. **Poole, B., and S. Ohkuma.** 1981. Effect of weak bases on the intralysosomal pH in mouse peritoneal macrophages. J. Cell Biol. **90**:665–669.
36. **Sasada, M., A. Kubo, T. Nishimura, T. Kakita, T. Moriguchi, K. Yamamoto, and H. Uchino.** 1987. Candidacidal activity of monocyte-derived human macrophages: relationship between *Candida* killing and oxygen radical generation by human macrophages. J. Leuk. Biol. **41**:289–294.