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Heterogeneous Activity of Immature and Mature Cells of the Murine Monocyte-Macrophage Lineage Derived from Different Anatomical Districts against Yeast-Phase *Candida albicans*

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Mature mononuclear phagocytes have been receiving much attention as effectors of spontaneous candidacidal activity, although with controversial results due to differences in the effector populations and the methods used in different laboratories. We here systematically compare the fungistatic activity of immature and mature cells of the murine macrophage series. The results show that nonadherent, nonphagocytic precursor cells (isolated either [90% purity] from bone marrow liquid cultures or from the organs of mice in which inflammatory conditions had been elicited in vivo) exerted a strong extracellular candidastatic activity. In contrast, mature macrophages, either obtained from different anatomical areas (spleen, liver, lung, peritoneal cavity) or matured in vitro from the precursor populations, displayed striking heterogeneity in their ability to inhibit the growth of *Candida albicans*, depending on the anatomical site they were derived from. Lymphokine activation did not alter the fungistatic pattern of the untreated cells. The different macrophage populations behaved very differently also in the production of reactive oxygen intermediates (ROI) in response to phagocytosis of *C. albicans*. The amounts of ROI generated, however, showed no correlation with candidastatic ability. Low levels of candidastatic activity exerted by resident peritoneal macrophages (good ROI producers) were inhibited by catalase, whereas high levels of growth inhibition by Kupffer cells (poor ROI producers) after 8 h of assay were hardly influenced by the enzyme. Our data suggest the existence of two different effector mechanisms in macrophage-mediated *C. albicans* growth inhibition, a rather inefficient ROI-dependent one, and a second, very efficient oxygen-independent mechanism. The implications of these findings are discussed.

Candida albicans is a ubiquitous dimorphic fungus whose pathogenicity is restricted in normal individuals to self-limited mucocutaneous infection. This microorganism has been gaining more and more clinical significance as an opportunistic pathogen due to the increase in the incidence of various induced immunodepressive conditions (10, 12, 24, 47, 52). In an immunodepressed patient, in fact, this fungus is able to cause serious systemic diseases which are particularly difficult to handle.

For the past few years our group has been studying systemic acute *C. albicans* infections in normal and immunomodulated mice (4, 8). In this particular model of systemic challenge, the role played by the natural defense mechanisms in reducing the invading pathogenic burden seems to be decisive. Among the cells mediating natural candidacidal activity, polymorphonuclear leukocytes are doubtlessly the best known and the most studied (17, 21, 25, 51). Mature macrophages have also been receiving attention, although the results obtained with these cells have always been controversial, due to either the different methods used for assessing candidacidal activity or the different degree of purity of the effector cell populations (5, 14, 22, 30, 43). In addition to these mature phagocytes, which are in any case expected to deal with particulate matter such as *C. albicans* cells, we recently described a novel natural effector mediating strong candidacidal activity, the macrophage precursor (2). Macrophage precursors are nonadherent, nonphagocytic cells in the early stages of macrophage differentiation, which are already capable of spontaneously killing many different target cells (tumor cells, microorganisms, and protozoa [2, 29; M. Baccarini, A. F. Kiderlen, and M.-L. Lohmann-Matthes, submitted for publication]). These cells have been identified in bone marrow liquid cultures growing under the influence of CSF-1. In normal, untreated animals they are present, although in limited numbers, in the peripheral organs, but these cells can also be elicited in many different anatomical districts under recruitment or inflammatory conditions (3, 15).

In this paper we analyze the interactions of immature and mature cells of the monocyte-macrophage system with the yeast phase of the fungus *C. albicans*. We show that all macrophage precursors display the same strong candidastatic potential, whereas mature phagocytes, freshly isolated from the animal or matured in vitro from their precursor cells, behave heterogeneously in their ability to inhibit the growth of the yeast. This heterogeneity was not overcome when the macrophages were stimulated with lymphokine, was not observed in the case of other targets, such as the P815 tumor cell line, and appeared not to be linked to the production of reactive oxygen intermediates (ROI) following phagocytosis of the yeast. The possibility that oxygen-independent mechanisms are crucial for candidacidal activity is discussed.

MATERIALS AND METHODS

Mice. Inbred C57BL/6 (H-2^b) mice 6 to 8 weeks old of either sex were obtained from the Zentrale Versuchstieranstalt, Hannover, Federal Republic of Germany (FRG).

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Drugs. MVE-2 (pyran) copolymer (25 mg/kg, intravenous) and polyvinosilic:polycytidilic acid [poly(I:C); Sigma Chemical Co., St. Louis, Mo.; 5 mg/kg intraperitoneal [i.p.]) were injected 3 days before harvesting the effector cells to induce inflammatory conditions.

C. albicans. The *C. albicans* strain used throughout this study was isolated from a clinical specimen and identified by the taxonomic criteria of Lodder and others (28, 31). The yeasts were grown at 28°C under slight agitation in low-glucose Winge medium composed of 0.2% (wt/vol) glucose and 0.3% (wt/vol) yeast extract (BBL Microbiology Systems, Cockeysville, Md.) until a stationary phase of growth was reached (about 24 h). Under these conditions, the cultures gave a yield of approximately 2.7×10^8 cells per ml, and the organism grew as an essentially pure yeast-phase population (32). After a 24-h subculture on Sabouraud-dextrose agar at 37°C, the cells were washed twice, diluted to the desired concentration, and used in the assays.

Tumor cell line. P815, a mastocytoma induced by methylcholanthrene in DBA/2 mice, was maintained as a tissue culture in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 0.1% gentamicin (hereafter referred to as complete medium). All components were purchased from GIBCO, Grand Island, N.Y.

Isolation of effector cells. Bone marrow (BM) cultures were established by a slight modification of the technique described by Meerpohl et al. (2, 33). Briefly, the BM of two femurs from each mouse was collected, and 5×10^6 cells in 10 ml of Dulbecco modified Eagle medium supplemented with 15% FCS and 5% horse serum (GIBCO) were plated in 9-cm untreated petri dishes (Greiner, Nurtingen, FRG). After 18 h of incubation, 20% conditioned medium, consisting of the supernatant of a monolayer of fibroblasts from an L-929 cell line cultivated for 4 days in RPMI medium plus 2.5% FCS, was added to the cultures.

Nonadherent cells were harvested after 3 days in culture and passed through a nylon wool column (45 min at 37°C in 5% CO_2) as previously described (19) to eliminate mature polymorphonuclear cells. Thereafter, since immature elements of the granulocyte lineage were still present in the effluent, cells were applied to a discontinuous Percoll density gradient by a modification (2) of the method of Timonen et al. (49). In short, a 60.6% Percoll solution (osmolarity 285 mOsM of H_2O per kg) was diluted in RPMI with 10% FCS to five different concentrations, and 2 ml of each solution was carefully layered in a plastic conical tube (Falcon plastics), with a maximum of 60% at the bottom of the tube and then grading by 2.25% concentration diminuition from 45.75 to 39%. A total of 50×10^6 nylon wool-passed cells in 2 ml of RPMI medium supplemented with 10% FCS were placed on top of the gradient, and the tube was spun at $550 \times g$ for 30 min. Cells from the first three fractions (F_{1-3}), consisting of about 95% BM-derived macrophage precursors (BMMP), were pooled and used as effector cells in the assays. Fraction 4 contained 85% young granulocytes and granulocyte precursors and 15% small, very dense early macrophage precursors. The total cell recovery after the gradient was about 90% of the input.

Preparation of spleen cells and liver NPC suspensions. Spleen cell suspensions were obtained by standard techniques and deprived of erythrocytes by hypotonic lysis. Liver nonparenchymal cells (NPC) were obtained by collagenase digestion of the liver by the method of Richman et al. (42). In brief, livers of pentobarbital-anesthetized mice were blanched by perfusing 10 ml of HEPES-buffered Hanks balanced salt solution (pH 7.4) via the portal vein, followed by perfusion of 5 ml of 0.05% collagenase (Sigma, type IV, 300 U/mg) in Hanks balanced salt solution plus 5% FCS as a competitive inhibitor of nonspecific proteases. The perfused liver was excised, passed through a 50-gauge stainless steel mesh, and then incubated for 45 min at 37°C in 40 ml of the collagenase solution used for perfusion. Liver cells in suspension were then centrifuged for 3 min at $50 \times g$, and the pellet, containing the bulk of hepatocytes, was discarded. The remaining cells were then washed twice in cold medium, and NPC were obtained by Percoll gradient centrifugation. One volume of Percoll (Pharmacia Fine Chemicals, Freiburg, FRG) was mixed with 1 volume of twofold-concentrated RPMI 1640 to obtain a 50% Percoll solution. The resulting solution was diluted 1:1 with complete medium to obtain a 25% Percoll solution. A 5-ml amount of each step was then layered in a 15-ml plastic conical tube (Falcon Plastics, Oxnard, Calif.) and 50×10^6 to 70×10^6 cells were placed on top of the gradient, and the tubes were spun at 550 $\times g$ for 30 min, NPC migrating between the two layers were collected, washed twice, and further processed.

Isolation of organ-associated macrophage precursors. Spleen cells and liver NPC obtained as described above were passed over a nylon fiber column (45 min at 37°C in 5% CO_2) as previously reported (19) to select for nonadherent cells. Cell recovery was 30 to 50%.

Fractionation of effector cells through a discontinuous Percoll gradient. Nylon wool-nonadherent cells were applied to a discontinuous density gradient of Percoll. Two volumes of Percoll and one volume of three fold-concentrated RPMI 1640 were mixed and 10% FCS was added, giving a final 60.6% Percoll solution of 285 mOsm of H_2O per kg. The gradient was prepared by diluting the starting solution with complete medium to seven different concentrations, ranging from 38.6 to 60.6%, in the case of the spleen, and to six different concentrations, ranging from 31.95 to 60.6%, in the case of the liver. After the careful layering of 2 ml of each solution in a 15-ml plastic conical tube (Falcon Plastics) 60×10^6 to 80×10^6 cells in 2 ml were placed on top of the gradient and the tube was spun at $550 \times g$ for 30 min. Cells from the seven layers were collected from the top with a syringe and washed twice in medium. Total cell recovery was about 80 to 90%.

Fractionation of effector cells by rosetting techniques. (i) Antisera. M143, a highly specific rat-anti mouse macrophage monoclonal antibody of subclass IgG2a has been produced in our lab (48) and was used as a 1:5 dilution of hybridoma culture supernatant. F4/80, a rat anti-mouse macrophage monoclonal antibody of subclass IgG2b whose specificity has been previously described (20) was a kind gift of Siamon Gordon, Sir William Dunn School of Pathology, Oxford University. This antibody, provided as a dialyzed ammonium sulfate precipitate from tissue culture supernatant, was used at a 1:20 dilution.

Purified rat IgG (Miles Laboratories, Slough, U.K.) was used at a concentration of 50 μg/ml. Rabbit-purified IgG fractions of anti-rat IgG were obtained as a lyophilized powder from Miles (lot no. S607) and were diluted to a concentration of 1 mg/ml.

(ii) Antibody coupling of SRBCs. One volume of packed sheep erythrocytes (SRBC) previously washed four times in sterile saline was incubated with 1 volume of chromium chloride solution ($CrCl_3$, 0.1% [wt/vol] in saline) and 1 volume of rabbit anti-rat IgG purified antibodies (1 mg/ml) for 5 min at room temperature. The SRBCs were then washed three times in saline and stored for up to 10 days at 4°C.

(iii) Indirect rosette assay. Cells from the three lowest Percoll fractions (10×10^6/ml), containing <1% esterase-positive mature macrophages, were incubated with an equal volume of an appropriate dilution of monoclonal antibodies or of purified rat IgG (as a specificity control) in normal medium for 60 min at 23°C, as described (18). Cells were then washed six times and mixed with an equal volume of a 4% suspension of SRBCs coupled with rabbit anti-rat IgG antibody. The mixture was centrifuged at $200 \times g$ for 4 min, the supernatant was discarded by aspiration, and the pellet was suspended by vigorous pipetting to disperse the nonspecific aggregates. No unspecific binding took place, as shown by the inability of cells reacted with purified rat IgG to form rosettes under these conditions. Cells were then applied to a Percoll gradient to separate the rosetting from the nonrosetting fraction.

(iv) Separation of rosetting from nonrosetting cells. A two-step Percoll density gradient was devised to separate the two fractions. The two steps were a 50% and then a 60% Percoll solution. A 3-ml amount of each solution was carefully layered in 15-ml tubes, and 2 ml of the cell suspension (20×10^6 cells) was layered on top. Tubes were spun at 500 $\times g$ for 20 min. Nonrosetting cells did not enter the gradient, rosetting cells formed a band between the two layers, and free SRBCs migrated in the pellet. The two cell fractions were collected from the top with a syringe. The rosetting fractions, representing the splenic macrophage precursors (SMP) and the liver macrophage precursors (LMP), were washed twice in medium and used in the assay.

The organ-associated macrophage precursor populations obtained in this way display >90% purity, as described elsewhere (3, 15). Contaminant cells are small lymphocytes (~7%) and polymorphonuclear leukocytes (~3%).

Isolation of PCMP. The peritoneal cavity of mice injected with poly(I:C) (5 mg/kg i.p., 3 days before the assay) was washed with 5 ml of cold heparinized medium (10 U/ml, Liquemin Roche). The cell suspension obtained in this way was allowed to adhere to tissue culture-treated 9-cm petri dishes (Falcon) for 90 min at 37°C; the nonadherent cells were removed and again allowed to adhere. This procedure was repeated two times. The nonadherent cells were then reacted with the macrophage-specific monoclonal antibody M143 and further processed in the same way as the organ-associated macrophage precursors. The resulting rosetting fraction represented the peritoneal cavity-derived macrophage precursors (PCMP) and contained, like the organ-derived M143- and F4/80-positive fractions, ~90% macrophage precursors, ~7 to 8% small lymphocytes, and 2 to 3% polymorphonuclear cells.

Isolation of in vitro-differentiated mature macrophages. The different precursor populations were allowed to mature to macrophages in the liquid culture system described above for the BM. After 7 to 10 days, all cells had become adherent. The in vitro-differentiated macrophages were detached by incubating the plates at 4°C for 30 min and then vigorously flushing the bottom of the plates with cold complete medium.

Separation of adherent cells. Liver macrophages (Kupffer cells) were obtained from the NPC suspension by selective adherence. Splenic macrophages were obtained by applying SRBC-deprived spleen cell suspensions to a discontinuous Percoll gradient consisting of four steps from 30 to 60% in 10% steps. Cells from the first two layers were harvested and further purified by selective plastic adherence. Peritoneal macrophages were obtained by washing the peritoneal cavity of normal mice with 5 ml of cold heparinized medium and allowing the cell suspension obtained to adhere to plastic surfaces.

Alveolar macrophages were harvested by cannulating the trachea of exsanguinated mice with a catheter (22 by 32 mm) (Deutschel Abbott, Wiesbaden, FRG) and washing the lungs with 2 ml of cold heparinized medium. This last procedure was repeated five times. Cells were then further purified by selective plastic adherence. All macrophage populations were cultivated in vitro for at least 2 days before the assays and were homogeneous (>95%) as judged by phagocytosis of 1.2-μm latex beads and nonspecific esterase staining.

PMN. Peritoneal polymorphonuclear neutrophils (PMN) were induced by i.p. injection of 1 ml of 10% thioglycolate broth (Bacto brewer thioglycolate medium; Difco Laboratories, Detroit, Mich.) 18 h before testing as previously described (5). The elicited cells were harvested by peritoneal lavage with cold heparinized medium, and approximately 95% were found to be PMN by morphological examination.

Macrophage activation. Cells in 96-well flat-bottomed microtiter plates were incubated for 18 h before the assay in the presence of recombinant gamma interferon (rIFN-γ; Genentech; kindly supplied by Boehringer, Ingelheim, FRG) at a concentration of 10^2 U/ml, plus 10 ng of *Escherichia coli* lipopolysaccharide (LPS) (Difco) per ml as previously described (39). Before the assay, the supernatant was removed and replaced with fresh complete medium.

***C. albicans* growth inhibition assay.** Different numbers of macrophage precursor cells in 0.1 ml were infected with yeast-phase *C. albicans* (5×10^4/well in 0.1 ml) (2). After a 12-h incubation period (unless otherwise stated) at 37°C in 5% CO_2, plates were vigorously shaken and serial dilutions were made in distilled water from each well. When mature macrophages were used as effector cells, macrophage monolayers (10^5 cells per well) were infected with different numbers of *C. albicans* microorganisms. Phagocytosis of yeast cells, as determined microscopically, was complete after 1 h. After a 12-h effector-target cell contact, unless otherwise stated, plates were centrifuged (10 min at 800 $\times$ *g*), the supernatant was removed, and lysis of the phagocytes in distilled water was performed and microscopically controlled. After all effector cells had been lysed, serial dilutions were made in distilled water from each well. Plates (duplicate samples) were made on Sabouraud-dextrose agar (Difco). The number of CFU was determined after 18 h of incubation at 37°C. Control cultures consisted of *C. albicans* incubated alone in complete medium (spontaneous growth) or, with identical results, *C. albicans* incubated with 2.5 $\times$ 10^5 thymocytes from untreated mice. Data were expressed as percent inhibition by effector cells by the following formula: % candidastatic activity = 100 − [(CFU expt group)/(CFU spontaneous growth)] $\times$ 100.

^{51}Cr release assay against tumor cells. The ^{51}Cr release assay has been described previously (38). Effector cells in triplicate microtiter wells were incubated with 5×10^3 radiolabeled (200 μCi for 1 h at 37°C in 5% CO_2) tumor cells for 18 h (P815) at 37°C in 5% CO_2. A portion of the supernatant was collected and its radioactivity was measured in a gamma-counter. The spontaneous release was determined by incubation of tumor cells without effector cells and never exceeded 30% of the total radioactivity incorporated by the target cells (cpm_{tot}). The percentage of specific lysis was then calculated by the formula % specific lysis = $(cpm_{expt} - SR)/(cpm_{tot}) \times 100$.

Enzymes. Catalase and superoxide dismutase (SOD) were purchased from Sigma, Deisenhofen, FRG (catalog numbers C-100 and S 4761, respectively). Where indicated, enzymes

TABLE 1. Candidastatic activity of different macrophage precursor populations[a]

Effector cells[b]	% *C. albicans* growth inhibition at E:T ratio:		
	10:1	5:1	2.5:1
BMMP	89.7	85.9	69
SMP	65.2	50.4	43.2
LMP	64.7	56.7	49
PCMP	55.9	50.2	36.7

[a] *C. albicans* growth inhibition was measured in a 12-h CFU assay. Standard errors, usually lower than 5%, have been omitted.

[b] Abbreviations: BMMP, Bone marrow liquid culture-derived precursors; SMP, precursors isolated from the spleens of normal mice; LMP, precursors isolated from the livers of Pyran-treated mice; PCMP, precursors isolated from the peritoneal cavities of poly(I:C)-treated mice (See Materials and Methods).

were added to CFU inhibition assays at the given concentrations (see below) prior to infection with *C. albicans* and were then present throughout the assay.

The percent reduction of macrophage candidastatic activity due to the presence of the enzymes was calculated by the following formula: % reduction of CFU inhibition = 100 − [(% CFU inhibition in the presence of the enzyme)/(% CFU inhibition in the absence of the enzyme)] × 100.

Measurement of luminol-dependent CL. A total of 2×10^5 macrophages cultured for 48 h in HEPES-buffered RPMI plus 5% FCS were used for measurement of luminol-dependent chemiluminescence (CL). A 15-μl amount of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Boehringer, Mannheim, FRG) at 2 mg/ml was added, and the background CL was monitored in six-channel Berthold Biolumat (7). When background activity, always below 15,000 cpm, had reached constant values, 10^6 viable yeast form *C. albicans* organisms in 20 μl of incubation medium were added and quickly spun down on the effector cells (30 s, 300 × *g*). CL resulting from subsequent ROI generation was then measured until it had dropped back to near background values (ca. 2 h). Software for computerized calculation of peak activities and integrals was supplied by Berthold, Wildbad, FRG.

Statistical analysis. Standard errors of the mean (SEMs) have been calculated for triplicate samples. In the CFU inhibition assay they did not exceed 5%. For measurement of CL they did not exceed 10% (peak values) and 8% ($integral_{60}$ values). They are omitted for reasons of clarity. Differences in CL emittance, CFU inhibition, and percent specific ^{51}Cr release have been analyzed by Student's *t* test. Each experiment was repeated three to five times.

RESULTS

Candidastatic activity of different macrophage precursor populations. We recently described the ability of immature cells of the monocyte-macrophage lineage obtained from in vitro BM liquid cultures to inhibit the growth of yeast-phase *C. albicans*. We directly compared the fungistatic activity of these cells with that of macrophage precursors freshly isolated from different anatomical sites (see Materials and Methods) (Table 1). All the cells tested, obtained either from normal animals (in the case of the SMP) or from mice in which inflammatory conditions had been induced in vivo, displayed good inhibitory activity. None of the organ-associated populations, however, reached the high levels of growth inhibition exerted by the cells derived from in vitro BM cultures. This is probably due to the fact that organ-associated cells, although homogeneously consisting of macrophage precursors, are composed of cells in different stages of maturation along the monocyte-macrophage lineage, whereas BMMP obtained from in vitro cultures represent a more synchronized population; in fact, as we previously demonstrated in the BM liquid culture system (2), candidastatic activity is not exerted by all the immature cells of the monocyte-macrophage lineage, but is rather a feature of a definite maturation stage.

Candidastatic activity of macrophages matured in vitro from distinct macrophage precursor populations. Macrophage precursors obtained either in vitro from BM cultures or in vivo from different anatomical sites were cultivated in the presence of L-cell-conditioned medium as a source of CSF-1 for 1 week. Under these conditions, the cells proliferated and finally matured to adherent phagocytes. The candidastatic activity of these mature macrophages was subsequently tested. Only macrophages derived from organ-associated precursors (SMP derived and LMP derived) were able to inhibit the growth of the yeast to the same or even greater extent as the corresponding immature cells (Fig. 1). BMMP-derived and PCMP-derived mature macrophages showed an extremely reduced fungistatic potential.

Candidastatic activity of distinct macrophage populations freshly isolated from different anatomical areas. To assess whether the heterogeneity in the behavior of macrophage populations matured in vitro from precursors isolated from different organs somehow reflected the situation in vivo, we further investigated the behavior of macrophages in response to *C. albicans* challenge, using as effectors mature cells freshly isolated from different anatomical sites. The same heterogeneity in candidastatic ability reported above for the cells matured in vitro from precursors induced in the various organs was displayed by the mature phagocytes obtained from the corresponding anatomical sites. Hepatic and splenic macrophages exerted strong fungistatic activity, comparable to that displayed by the cells matured in vitro from LMP and SMP, whereas macrophages freshly obtained from the peritoneal cavity (PCMP) were unable, like their in vitro PCMP-derived counterparts, to inhibit the growth of the yeast (Fig. 2). A fourth population investigated, the alveolar macrophages, displayed an intermediate behavior in which the effector-target cell (E:T) ratios used in our experiments, unlike in all other cases, played a crucial role. Only the highest concentration of effector cells, in fact, proved to be efficiently candidastatic.

Effect of in vitro rIFN-γ stimulation on the candidastatic activity of different macrophage populations. Different macrophage populations, freshly isolated from various anatomical sites, were exposed to rIFN-γ and trace amounts (10 ng/ml) of LPS to obtain optimal activation. Under these conditions, all macrophages became cytotoxic against P815 tumor cells (Table 2). When the candidastatic ability of these effectors was tested, the pattern summarized in Table 2 was obtained. Significant activation could be evidenced in macrophages which already displayed good activity, although only at the lowest E-T ratios, at which the candidastatic property of nonactivated macrophages was suboptimal. In contrast, with peritoneal macrophages, which displayed in the resting stage very little or no candidacidal ability, a 10% increase was observed as a consequence of activation only at the highest E:T ratio.

Respiratory burst activity of different macrophage populations. To assess whether the heterogeneity displayed in the candidastatic activity by the different macrophage popula-

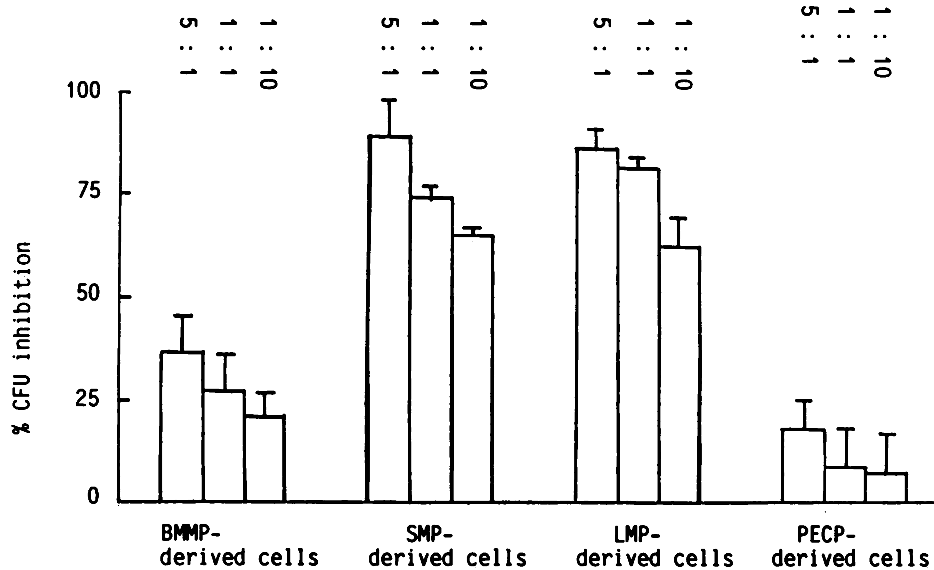

FIG. 1. Candidastatic activity of macrophages matured in vitro from macrophage precursor populations isolated from different anatomical sites. Candidastatic activity, measured in a 12-h assay, is expressed as percent CFU inhibition at the E:T ratios indicated at the top. PECP = PCMP. Vertical bars represent SEMs.

tions, activated or not, could be in some way correlated to their efficiency in responding to the phagocytosis of *C. albicans* with a respiratory burst, macrophages derived from lung, liver, and peritoneal cavity were challenged with yeast cells at an E:T ratio of 1:5, and the CL generated through ROI production was monitored. Luminol was chosen as an amplifying substrate because it detects CL by O_2^- radical as well as by H_2O_2 (1) and thus allows the determination of the oxygen-dependent cytotoxic potential in toto. Table 3 shows the CL peak and total activities over 1 h (integral 0–60, I_{60}). This period was chosen because it always contained the bulk of the emitted CL, as will be shown below (Fig. 3), and thus provided additional information concerning the burst intensity. The macrophages reacting with the highest CL in terms of both peak activity and I_{60} were the resident macrophages from the peritoneal cavity. This finding accounted for both in vitro-activated and nonactivated macrophages.

The two other resident organ-associated macrophage populations tested, alveolar macrophages and Kupffer cells, emitted CL I_{60} values of roughly one-sixth and one-fourth of the amount emitted by resident peritoneal macrophages, respectively. In contrast to ROI production, alveolar macrophages and Kupffer cells efficiently inhibited *C. albicans* growth, whereas only little candidacidal activity was exerted by resident PCMP.

Some findings held for all macrophage populations, as could be expected (35, 37), CL emittance was always higher for rIFN-γ- or LPS-activated macrophages than for untreated ones, the degree of the response to activation varying between the populations. All macrophages tested could also be activated to different extents by LPS alone. The lower limit of sensitivity of all macrophages to LPS in the absence of lymphokine was between 0.1 and 0.01 μg/ml (data not shown).

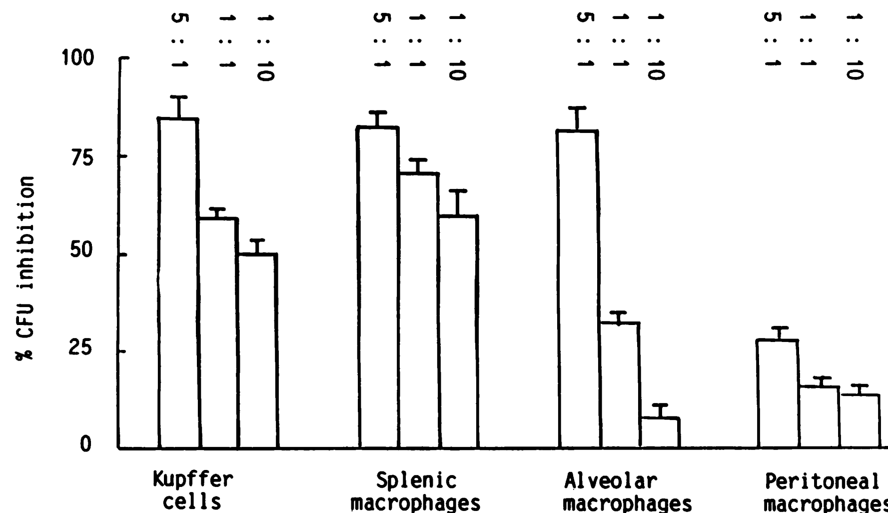

FIG. 2. Candidastatic activity of mature macrophage populations freshly isolated from different anatomical sites. Candidastatic activity, measured in a 12-h assay, is expressed as percent CFU inhibition at the E:T ratios indicated at the top. Vertical bars represent SEMs.

TABLE 2. Effect of rIFN-γ on in vitro stimulation of the candidastatic and tumoricidal activity of different macrophage populations[a]

Effector cells	Stimulation	% CFU inhibition[b] at E:T ratio:			% ^{51}Cr release[c] at E:T ratio of 10:1
		10:1	1:1	1:10	
Peritoneal macrophages	None	16.6	17.4	12	0.07
	rIFN-γ	27.3**	28.2	15	41.64*
Alveolar macrophages	None	78.7	30.7	7	1.3
	rIFN-γ	81.7	35	29.3*	29.8*
Splenic macrophages	None	82	71	55.8	2.7
	rIFN-γ	79.8	74.4	69.2**	39.2*
Liver macrophages (Kupffer cells)	None	86	66.7	50.7	1.3
	rIFN-γ	86	73.4	71.52*	38.7*

[a] Macrophages were exposed in vitro to 10^2 U of rIFN-γ plus 10 ng of LPS per ml for 18 h as previously described (21) or not exposed. Supernatants were removed, and the cells were used as effectors in the assays. Significance levels (Student's *t* test) for stimulated versus unstimulated cells are indicated as follows: *, $P < 0.01$; **, $P < 0.05$.

[b] Candidastatic activity, expressed as percent CFU inhibition, was determined in a 12-h assay.

[c] Tumoricidal activity, expressed as percent ^{51}Cr specific release from P815 mastocytoma target cells, was determined in an 18-h assay.

Effect of catalase on macrophage-mediated inhibition of *C. albicans* growth. To further and more directly investigate the contribution of oxygen-dependent mechanisms, we performed CFU inhibition assays in the presence of SOD and catalase. SOD at 620 U/ml had very little effect on the fungistatic activity of macrophages, although they were able to inhibit the CL emittance of resident PCMP challenged with *C. albicans* by >80%. This favors the assumption that the O_2^- radical plays a minor role in *C. albicans* growth inhibition by macrophages.

CFU inhibition assays performed in the presence of 6,500 U of catalase per ml (16) are shown in Table 4. Three types of effector populations were studied: resident PCMP (good ROI response to *C. albicans* but low candidacidal efficiency), Kupffer cells (low ROI response but good effectors), and MVE-2-induced LMP (ROI response between those of resident PCMP and Kupffer cells, growth inhibition ability comparable to that of Kupffer cells). The overall candidastatic efficiency of PCMP, as outlined in Table 4, was very low compared with that of the two liver-derived populations. The high levels of CFU inhibition by these resident and inflammatory liver macrophages, activated or not, remained unaffected by catalase. Our data show that significant catalase-dependent reduction of CFU inhibition was found only for the PCMP, especially with nonactivated cells. Catalase-mediated reduction of the fungistatic ability of thioglycolate-induced murine PMN was included as a positive control for enzyme activity. Control experiments with heat-inactivated catalase were performed, in which the growth inhibition caused by the effector cells remained completely unaffected by the enzyme (data not shown).

Kinetics of ROI-dependent versus ROI-independent CFU inhibition. Experiments were performed to reveal the correlation between ROI generation in the course of the respiratory burst and candidastatic events during or near this period. The effect of catalase at different time intervals on CFU inhibition was investigated. For the reasons outlined in the above section, resident peritoneal macrophages were studied in comparison with resident and inflammatory macrophages from the liver. Figure 3 shows the kinetics of the respiratory burst response following phagocytosis of *C. albicans*. All rIFN-γ- and LPS-activated macrophages reacted with an immediate CL emittance of different intensity that reached peak values after 6 min (resident Kupffer cells), 5 min (MVE-2 induced liver macrophages), and 4.5 min (resident peritoneal macrophages). CL then declined rapidly to background levels after 2 to 3 h. Within this 3-h period significant growth inhibition was performed only by good ROI producers, such as the in vitro-activated resident peritoneal and MVE-2-induced liver macrophages (24 and 35%, respectively; Fig. 4). About 70% of this activity was indeed catalase-inhibitable for resident peritoneal macrophages (7% CFU inhibition), whereas less than half was inhibitable by the enzyme in the case of the inflammatory macrophages from the liver (20% CFU inhibition). Kupffer cells, within the first 3 hours, had little candidastatic activity. After a 6-h interval the inhibition of fungistatic efficiency through peroxide removal was negligible for both liver populations. The

TABLE 3. Luminol-dependent CL by murine macrophages[a]

Macrophage population	Treatment[b]	Peak CL (10^4 cpm)	I_{60} (10^4 cpm)	% CFU inhibition[c]
Alveolar	None	11.3	387	74
	LPS	42.6*	1,680*	76.1
	IFN plus LPS	60.1*	2,340*	81.3
Resident peritoneal	None	277.7	4,910	16.2
	LPS	665.1*	9,340*	24.3
	IFN plus LPS	825.8*	15,300*	28.4
Kupffer cells	None	22.2	983	79.4
	LPS	90.3*	3,380*	81.3
	IFN plus LPS	140.9*	4,410*	87.2
MVE-2-induced liver	None	53.9	2,040	83.2
	LPS	287.6*	9,730*	82.9
	IFN plus LPS	317.2*	10,900*	87.3

[a] CL by 2×10^5 cells challenged with *C. albicans* at an E:T ratio of 1:5. *, $P < 0.01$ (Student's *t* test), activated versus nonactivated cells.

[b] rIFN-γ (IFN) was used at 10^2 U/ml. LPS was present at 10 μg/ml alone or at 0.01 μg/ml when used with rIFN-γ.

[c] Assay performed at an E:T ratio of 5:1 for 8 h.

TABLE 4. Effect of catalase on *C. albicans* growth inhibition

Effector macrophages	Treatment[a]	% CFU inhibition[b]		% Reduction in CFU inhibition by catalase[c]
		Without catalase	With catalase	
Resident peritoneal	None	21	7	66.6*
	IFN plus LPS	28	15	46.4*
Kupffer cells	None	86	82	4.6
	IFN plus LPS	91	87	4.4
MVE-2-induced liver	None	82	78	4.8
	IFN plus LPS	91	87	4.4
Peritoneal PMN	None	75	16.9	77.5

[a] rIFN-γ (IFN), 10^2 U/ml; LPS, 0.01 μg/ml.
[b] Assays performed for 8 h at an E:T ratio of 5:1. Catalase was added to a final concentration of 6,500 Sigma U/ml before the addition of *C. albicans*.
[c] *, $P < 0.01$ (Student's *t* test), catalase-treated versus untreated cells.

levels of CFU inhibition, however, had meanwhile become half-maximal for Kupffer cells and nearly maximal for MVE-2-induced macrophages. In contrast, CFU inhibition by resident peritoneal macrophages had increased only a little compared with the 3-h value and was still catalase-inhibitable by more than 50% (30 versus 14% CFU inhibition).

The final outcome after an assay time of 8 h has been described in the above section. It confirms the tendency observed after 6 h of incubation and clearly states that the mechanisms that equip macrophages with high candidastatic activity are mostly oxygen independent.

DISCUSSION

The candidacidal ability of normal, untreated macrophages and their in vitro-activated counterparts has so far been controversial. Depending on the effectors and the target chosen and on the methods used to assess fungicidal capacity very different results have been reported by various workers (5, 14, 21, 30, 43). We here systematically analyze the interactions of the yeast phase of the opportunistic pathogen *C. albicans* with the immature and mature cells of the monocyte-macrophage system.

Immature cells of the macrophage lineage were capable of mediating extracellular growth inhibition of the yeast form of *C. albicans* regardless of their site of isolation. All macrophage precursors, when further cultivated in vitro under identical conditions in the presence of CSF-1, gave rise to mature, adherent phagocytes, displaying striking heterogeneity when reacted with *C. albicans*. SMP-derived and LMP-derived macrophages showed good fungistatic activity, even better than that of their respective immature stages. On the contrary, the candidastatic potential of BMMP-derived and PCMP-derived macrophages was significantly lower (in the case of the BMMP) or minimal (in the case of the PCMP) with respect to that of their precursors. To gain some insight into this heterogeneous behavior, which became obvious after differentiation, we tested the candidastatic ability of freshly isolated mature macrophages obtained from the corresponding anatomical sites. Each cell population behaved exactly like its counterpart matured in vitro from precursors isolated from the proper anatomical district.

The finding that macrophages have such strikingly different candidacidal activity while all of them derive from nonphagocytic precursors which are able to inhibit the growth of the yeast seems surprising, since one would expect intracellular killing to be more effective than extracellular killing and the effectors to get more potent with maturation.

Our data suggest two changes of the current view on the origin of cells of the macrophage lineage. First, macrophage precursor cells, capable of forming macrophage colonies in agar in the presence of CSF-1 and of maturing in vitro to typical macrophages, are not restricted to the BM compartment but can also be isolated from liver and spleen. Second, since these cells, when matured in vitro under identical conditions and in the absence of any particular microenvironment, give rise to mature macrophages with heterogeneous properties, the differences observed in the behavior of mature macrophages appears to be predetermined at the maturation level of the macrophage precursors. The possible existence of heterogeneous macrophage precursors has already been discussed (11, 45, 46, 50; Baccarini et al., Cell Immunol., in press). Whether the organ-associated macrophage precursors are a cell type distinct from the BM- (and peritoneal cavity-)derived precursors, or whether the microenvironment conditioning is already exerted at the level of the immature cells and is preserved under our culture conditions, remains to be clarified; the *C. albicans* model could be a very helpful tool for this kind of research.

The mechanisms underlying the differences in the candidacidal activity of distinct macrophage populations are quite complex. Differences in the phagocytosis of *C. albicans* are not involved, since all macrophages, activated or not, be-

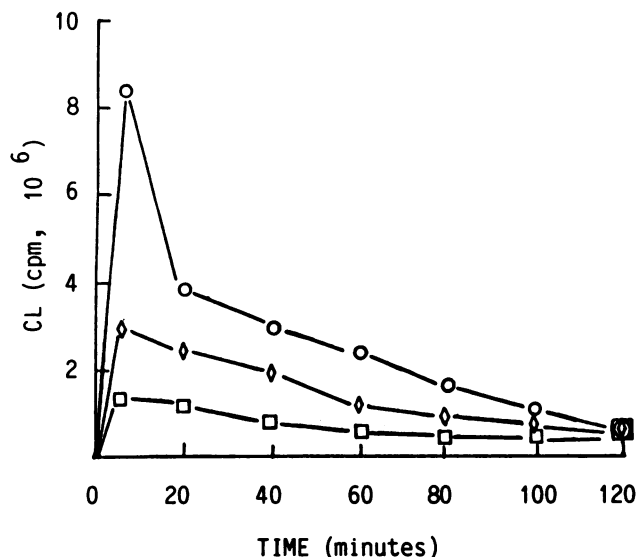

FIG. 3. Time course of luminol-dependent CL during the respiratory burst. CL in the presence of luminol was measured following the phagocytosis of *C. albicans* at an E:T ratio of 1:5. rIFN-γ- and LPS-activated resident peritoneal macrophages (○), Kupffer cells (□), and MVE-2-induced liver macrophages (◇) are shown.

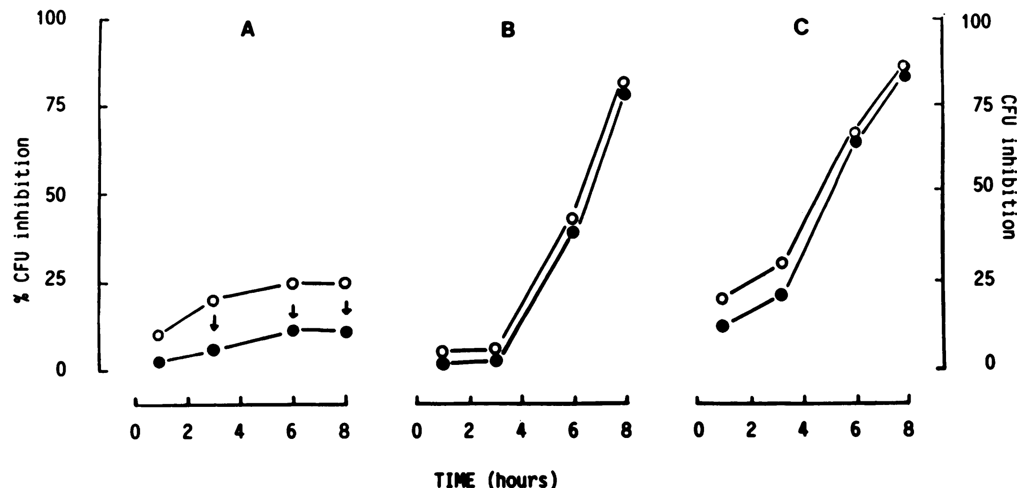

FIG. 4. Time course of *C. albicans* growth inhibition in the presence and absence of catalase. CFU inhibition assays were performed at an E:T ratio of 5:1. Where indicated, catalase was present at a final concentration of 6.5 U/ml. rIFN-γ- and LPS-activated resident peritoneal macrophages (A), Kupffer cells (B), and MVE-2-induced liver macrophages (C) are shown. Symbols: ○, no catalase; ●, catalase present; ↓, $P < 0.01$ according to Student's t test, catalase-treated versus untreated cells.

have very similarly in this respect (data not shown). Stimulation by rIFN-γ in the case of active macrophages could be evidenced only at the lowest E:T ratios and was ineffective in overcoming the impairment in the candidastatic activity of the peritoneal cells. All macrophage species, when tested for activation in a ^{51}Cr release assay against P815 mastocytoma cells as a control for the efficiency of the lymphokine, were activated, suggesting that the macrophages possess at least two distinct cytotoxic mechanisms.

Another criterion for the definition of an activated macrophage is its ability to generate ROI after phagocytosis of particulate material. ROI production, detected through luminol-dependent CL, was found to vary strikingly among the effector populations. Resident peritoneal cells played an outstanding role with regard to ROI production compared not only with Kupffer cells, whose ability to produce ROI is controversial (6, 26, 27; M.J.P. Arthur, P. Kowalski-Saunders, and R. Wright, Third Int. Kupffer Cell Symp., abstr., p. 59), but also with other organ-associated phagocytes, such as alveolar macrophages.

Activation of the various macrophage populations resulted in an increase in the levels of ROI generation, but not in a change in the observed pattern of heterogeneity. Phagocytes from sites of inflammation showed increased burst activity. Comparing the amount of ROI production with the ability of different effector macrophages to inhibit the growth of *C. albicans*, we noted a striking antiparallelism between fungistasis and the intensity of CL emittance, which was most evident for resident peritoneal macrophages on one side and for Kupffer cells on the other. CFU inhibition assays, performed in the presence of SOD or catalase, showed that the high levels of candidastasis by Kupffer cells and MVE-2-induced liver macrophages were, in an 8-h assay, unaffected by any of the enzymes, while the low CFU inhibition exerted by resident peritoneal macrophages could be further reduced through the presence of catalase. Hence, it can be deduced that peroxide-dependent mechanisms do contribute to the inhibition of yeast cell growth by high producers of ROI. However, the highly efficient fungistasis exerted by both populations of liver macrophages is likely to be performed by oxygen-independent mechanisms which are also susceptible to increase by macrophage activation.

The kinetics of ROI production of catalase-inhibitable as well as catalase-resistant macrophage-mediated *C. albicans* growth inhibition substantiate the above statements. An early, peroxide-dependent phase of irreversible microbe damage during the period of the respiratory burst existed in resident peritoneal macrophages as well as in MVE-2 induced, rIFN-γ- and LPS-activated liver macrophages which produce considerable amounts of ROI. Growth inhibition at this early time, however, was so low that it contributed only in negligible amounts to the overall activity (8 h assay). Kupffer cells, as weaker ROI producers, did not exhibit an early, catalase-inhibitable growth inhibition, which is consistent with the assumption of effective oxygen-independent mechanisms.

In summary, our results, although confirming a low level of oxygen-dependent *C. albicans* growth inhibition within the first 3 h by high ROI producers (13, 43), suggest a critical importance of oxygen-independent mechanisms for efficient destruction of the yeast. In fact, besides the ROI-mediated cell damage, a direct cytotoxicity of cationic proteins against *C. albicans* has also been shown (23, 40, 41), and correlations between the levels of lysosomal enzymes and fungicidal activity have been proposed (9). Our data fit well, too, with reports on macrophage cytocidal activity against protozoal parasites that has been demonstrated to be in part (34, 36) or even completely (44) independent of ROI.

Further studies are needed to identify all components contributing to macrophage-mediated candidastatic activity.

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