

Candida albicans Killing by RAW 264.7 Mouse Macrophage Cells: Effects of *Candida* Genotype, Infection Ratios, and Gamma Interferon Treatment†

A. Marcil,^{1*} D. Harcus,¹ D. Y. Thomas,² and M. Whiteway^{1,3}

Genetics Group, Biotechnology Research Institute, National Research Council of Canada, Montreal, Quebec H4P 2R2,¹ and Departments of Biochemistry² and Biology,³ McGill University, Montreal, Quebec H3G 1Y6, Canada

Received 1 May 2002/Returned for modification 26 June 2002/Accepted 23 July 2002

Phagocytic cells such as neutrophils and macrophages are potential components of the immune defense that protects mammals against *Candida albicans* infection. We have tested the interaction between the mouse macrophage cell line RAW 264.7 and a variety of mutant strains of *C. albicans*. We used an end point dilution assay to monitor the killing of *C. albicans* at low multiplicities of infection (MOIs). Several mutants that show reduced virulence in mouse systemic-infection models show reduced colony formation in the presence of macrophage cells. To permit analysis of the macrophage-*Candida* interaction at higher MOIs, we introduced a luciferase reporter gene into wild-type and mutant *Candida* cells and used loss of the luminescence signal to quantify proliferation. This assay gave results similar to those for the end point dilution assay. Activation of the macrophages with mouse gamma interferon did not enhance anti-*Candida* activity. Continued coculture of the *Candida* and macrophage cells eventually led to death of the macrophages, but for the RAW 264.7 cell line this was not due to apoptotic pathways involving caspase-8 or -9 activation. In general *Candida* cells defective in the formation of hyphae were both less virulent in animal models and more sensitive to macrophage engulfment and growth inhibition. However the nonvirulent, hypha-defective *cla4* mutant line was considerably more resistant to macrophage-mediated inhibition than the wild-type strain. Thus although mutants sensitive to engulfment are typically less virulent in systemic-infection models, sensitivity to phagocytic macrophage cells is not the unique determinant of *C. albicans* virulence.

Candida albicans can cause life-threatening infections in immunocompromised patients but is limited to causing primarily superficial mucosal infections in immunocompetent individuals (11, 38). This observation emphasizes that the mammalian immune system is a powerful barrier to *Candida* infections. Recent evidence has also suggested that the distinct morphological forms exhibited by *C. albicans* cells are critical for its virulence, as strains trapped in either the yeast or filamentous state in vitro are less virulent in murine infection models than cells capable of undergoing morphological changes (4, 11, 25, 26, 33, 37). It is possible that the hyphal form is required to penetrate the epithelial barrier while the yeast form allows for efficient dissemination through the bloodstream (6). However, the mutants that have been examined in these studies are pleiotropic, and characteristics unrelated to cellular morphology may determine the relative levels of virulence of the strains (7, 18, 22). Ultimately, dissection of the roles of morphology and various virulence factors in the infection process will involve the use of both in vitro and in vivo assays.

A powerful in vitro assay for the analysis of the host-pathogen interaction is the coculture of cells of the mammalian immune system with *C. albicans* cells (1, 3, 6). The usefulness of this approach is enhanced by the isolation of many mutant versions of *C. albicans* that are defective in components impli-

cated in the virulence process (3, 33, 41). Initial studies showed that cells capable of forming hyphae were able to escape from phagocytosis through the formation of germ tubes and hyphae, while cells unable to undergo the morphological switch remained engulfed (26, 33). Mutations in several components of signaling pathways have been shown to modify the regulation of this morphological switch and to influence virulence. These components include proteins involved in the production of cyclic AMP (cAMP) such as adenylyl cyclase (Cdc35p) (33) and Ras1p (24), as well as proteins required for the function of a mitogen-activated protein (MAP) kinase cascade that includes the p21-activated kinase (PAK) homolog Cst20p (23), the MEK Hst7p (23), and the MAP kinase Cek1p (12). The cAMP regulatory circuit appears to play a much more important role in the control of the hyphal switch than the MAP kinase pathway (33). However, coordinate loss of both pathways blocks the yeast-to-hypha transition under most growth conditions (26). In addition to Cst20p, a second PAK homolog, Cla4p, plays a critical role in the control of cellular morphogenesis (25).

Although the infection process is complex and involves interactions between the pathogen and many host cell types, in vitro studies involving specific cultured immune system cells can permit the analysis of interactions under controlled conditions. These studies can measure the influence of priming factors such as gamma interferon (IFN- γ) and cytokines and can provide the opportunity to monitor and compare host cell behaviors upon challenge with wild-type or mutant strains of the pathogen. A variety of methods for assessing the conse-

* Corresponding author. Mailing address: Genetics Group, Biotechnology Research Institute, 6100 Royalmount, Montreal, Quebec H4P 2R2, Canada. Phone: (514) 496-1923. Fax: (514) 496-6213. E-mail: anne.marcil@nrc.ca.

† National Research Council of Canada publication 44836.

quences of the host cell-pathogen interaction have been described, including CFU determination of harvested wells (1, 43). However, the pleiotropic nature of *C. albicans*, which can take the form of multicellular hyphae or cell aggregates, can bias such CFU measurements. Metabolic assays such as those involving radioisotope incorporation (35) and incorporation of tetrazolium salt indicators such as 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (20) and 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-([phenylamino]carbonyl)-2H-tetrazolium hydroxide (XTT) (40) involve further growth of *Candida* after the lysis of the host cells and would not be appropriate for low-multiplicity-of-infection (MOI) experiments or for strains with growth defects.

Processes such as the induction of apoptosis within cells of the host immune system can also be monitored during the interaction of the pathogen and the phagocytic cells. Apoptosis occurs via two main pathways, the death ligand receptor pathway mediated through caspase-8 activation and the stress pathway mediated through caspase-9 activation (16, 17). Some pathogens such as *Shigella* and *Salmonella* spp. (28, 32) induce apoptosis of phagocytic cells. This can trigger severe inflammation via the production and release of proinflammatory cytokines and can favor the dissemination of the pathogen deeper into host tissues. On the other hand, by undergoing apoptosis, macrophages may be able to expose the invaders to more-potent bactericidal cells such as neutrophils, as well as to the humoral arm of the immune system. Apoptosis can also prevent pathogens from using the phagocytic cells as a vehicle to evade the immune system (28); this apoptotic strategy helps control infections caused by *Mycobacterium tuberculosis* (14). It has been recently reported that apoptosis could be induced both in mouse macrophages by live *C. albicans* (36) and in human neutrophils upon challenge with killed *C. albicans* (34). However, other data suggested that *Candida* could inhibit apoptosis in human monocytic cells (19).

To be able to compare different *Candida* strains to each other in their interactions with host cells, we recently developed a rapid in vitro technique which allows for the comparison of *Candida* survival in the presence or absence of host cells at low MOIs without cell harvesting (33). We also introduced a luciferase reporter gene into five genetically defined *C. albicans* mutants and monitored their behavior in the presence of RAW 264.7 mouse macrophage cell lines at low and high MOIs. This allowed a characterization of host-pathogen interactions on a wide range of cells regardless of the pathogen phenotype observed at 37°C. We investigated the role of IFN- γ priming in enhancing the ability of macrophages to phagocytose and kill *Candida*. We also tested whether apoptosis of the RAW 264.7 macrophage cells is induced by wild-type or mutant *C. albicans* strains by using measurement of caspase-8 and -9 activity on specific substrates.

MATERIALS AND METHODS

Reagents. Dulbecco's modified Eagle's medium (DMEM), Leibovitz's L15 medium (L15), and Dulbecco's phosphate-buffered saline (PBS) were purchased from Invitrogen/Gibco (Rockville, Md.). Fetal bovine serum (FBS) was purchased from HyClone (Logan, Utah) and was heat inactivated at 56°C for 30 min.

Strains and cell lines. The *C. albicans* strains used in this study are listed in Table 1. Ura⁺ strains were grown in yeast extract-peptone-dextrose medium (YPD). Ura⁻ strains were grown in YPD supplemented with 25 μ g of uridine/ml. The RAW 264.7 mouse macrophage cell line was kindly provided by A. Desco-

TABLE 1. Strains used in this study

Strain	Genotype	Reference or source
SC5314	Wild type	13
CAI4	<i>ura3::λ imm434/ura3::λ imm434</i>	13
CDH107	CAI4 <i>ras1::hisG-URA3-hisG/ras1::hisG</i>	24
CDH108	CAI4 <i>ras1::hisG/ras1::hisG</i>	24
CaLJ1	CAI4 <i>cla4::hisG-URA3-hisG/cla4::hisG</i>	25
CaLJ5	CAI4 <i>cla4::hisG/cla4::hisG</i>	25
CDH22	CAI4 <i>cst20::hisG-URA3-hisG/cst20::hisG</i>	23
CDH25	CAI4 <i>cst20::hisG/cst20::hisG</i>	23
CR216	CAI4 <i>cdc35::hisG-URA3-hisG/cdc35::hisG</i>	33
CR276	CAI4 <i>cdc35::hisG/cdc35::hisG</i>	33
CAM 1.1	CAI4 <i>ura3 (R. reniformis luciferase gene-URA3)</i>	This study
CAM 3.1	CDH108 <i>ura3 (R. reniformis luciferase gene-URA3)</i>	This study
CAM 5.2	CaLJ5 <i>ura3 (R. reniformis luciferase gene-URA3)</i>	This study
CAM 23.3	CDH25 <i>ura3 (R. reniformis luciferase gene-URA3)</i>	This study
CAM 26.8	CR276 <i>ura3 (R. reniformis luciferase gene-URA3)</i>	This study

teaux (IAF, Laval, Canada). These cells were grown in DMEM supplemented with 10% FBS (D-10). When required, RAW 264.7 cells were treated with 100 U of mouse IFN- γ (Invitrogen Canada Inc.)/ml for 18 h prior to incubation with *Candida* cells.

Plasmid construction and transformation. An *ADHI* promoter-driven *Renilla reniformis* luciferase expression plasmid was constructed as follows. A 1.5-kb *NotI-EcoRV* fragment from pYPB1-ADHpL (23) was blunted with Klenow fragment and subcloned into *SmaI*-digested pCRW3 (39). The resulting plasmid, pAM1.3, was digested with *NcoI* and *SacI* and blunted, and the 2.8-kb fragment containing the *ADHI* promoter, luciferase gene, and *WH11* terminator sequence was subcloned into the *SmaI*-digested pVEC (27). The resulting plasmid, pAM2.5, also contained the *Candida* autonomous replicating sequence (CARS) and the *URA3* selectable marker. It was transformed in *Candida URA3*⁻ strains by the rapid lithium acetate method (8). Transformants were streaked on Ura⁻ plates and replica plated on YPD. After two rounds on Ura⁻ plates followed by replica plating to YPD, clones that stably maintained the selectable marker were identified, and plasmid integration of several of these clones was confirmed by Southern analysis (data not shown).

End point dilution survival assay. The end point dilution survival assay was performed as described previously (33). Briefly, the RAW 264.7 cells were seeded the day before at 1.5×10^5 cell per well in flat-bottom 96-well plates (Costar). When required, macrophages were treated with 100 U of mouse IFN- γ /ml (Invitrogen Canada Inc.). They were washed three times with D-10 medium before incubation with *Candida*. Overnight cultures of *Candida* cells were washed in PBS, sonicated for 1 min to disrupt any clumped cells, and resuspended at 10^7 cells/ml in cold D-10. Fifty microliters of the suspension was added to 150 μ l of D-10 in each of the first 8 wells (first column) of 96-well plates containing either medium only or medium with macrophages. Serial fourfold dilutions were done in subsequent columns, and plates were incubated first on ice for 30 min and then at 37°C and 5% CO₂ for 48 h. One whole plate per strain with or without macrophages was considered one experiment. Colonies were visualized with a Nikon TMS inverted microscope at $\times 20$ or $\times 100$ magnification. Passive lysis buffer (Promega, Madison, Wis.) at $1 \times$ final concentration was added immediately before counting the wells containing macrophages to facilitate colony visualization. Dilutions (without macrophages) where colonies could be distinctively visualized were counted toward the lower limit and compared to the same dilutions with macrophages. Colonies from a total of at least 48 wells per condition were used to provide data. Survival assays using luciferase-expressing strains were done similarly with serial twofold dilutions, starting at a 1:1 ratio for a final volume of 150 μ l per well. Wells in triplicate were harvested after 12 h of incubation.

Renilla luciferase detection. Microplates were centrifuged at $3,000 \times g$ for 5 min, 75 μ l of supernatant was removed, and 25 μ l of $4 \times$ passive lysis buffer was added to each well. After two freeze-thaw cycles at -80°C , luciferase activity was assayed with a Promega Dual-Luciferase reporter kit in accordance with the manufacturer's instructions and detected with a Turner Designs model TD20/20 luminometer with a 20-s measurement period.

Time-lapse microscopy. Biotech's petri dishes were segmented into three parts with silicone (Dow Corning Corp., Midland, Mich.), left to dry for 2 to 3 days, washed three times with PBS, and sterilized with 70% ethanol. RAW 264.7 cells were seeded the day before at 3×10^5 cells per section of 1 cm². *Candida*

TABLE 2. Virulence, phenotype, and end point dilution survival assay

Parental strain	Luciferase-expressing strain	Virulence ^a	Phenotype ^b	End point survival (%) ^c of:	
				Parental strain	Luciferase-expressing strain
SC5314	CAM 1.1	Virulent	Hyphal	32.2 ± 2.5 (5)	27.6 ± 5.0 (5)
<i>ras1/ras1</i>	CAM 3.1	Reduced	Pseudohyphal	9.9 ± 4.9 (3)*	11.2 ± 1.6 (3)*
<i>cla4/cla4</i>	CAM 5.2	Avirulent	Pseudohyphal, aberrant	48.5 ± 9.9 (5)*	56.8 ± 11.3 (3)*
<i>cst20/cst20</i>	CAM 23.3	Reduced	Hyphal	22.0 ± 3.5 (4)	25.2 ± 4.1 (6)
<i>cdc35/cdc35</i>	CAM 26.8	Avirulent	Yeast	7.0 ± 2.1 (4)*	7.0 ± 4.4 (4)*

^a Hematogenously disseminated murine model for parental strains.

^b At 37°C, in D-10 medium.

^c Data represent percentages of survival ± standard deviations of the means and are expressed as the numbers of colonies in the presence of RAW264.7 macrophages divided by the number of colonies in the absence of macrophages. Numbers in parentheses indicate numbers of experiments. *, significantly different from the wild-type strain of each group ($P < 0.01$).

cells were added at the desired ratio in the CO₂-independent L15 medium containing 10% FBS and 2.5 µg of propidium iodide (Molecular Probes, Eugene, Oreg./ml). Phase-contrast and epifluorescence pictures were taken every 30 min with a DMIRE2 inverted microscope (Leica Microsystems Canada) equipped with a Hamamatsu cooled charge-coupled device camera, a Biotechs temperature-controlled stage adapter, and a Ludl motorized stage at ×400 magnification. Openlab software (Improvision) was used for image acquisition.

Caspase activity determination. RAW 264.7 cells were cultured overnight from an initial inoculum of 2.5×10^6 cells per well in six-well plates to obtain a monolayer at 80% confluence. Medium was then removed and replaced by 2 ml of fresh medium just before the experiment. Overnight culture of *C. albicans* strain SC5314 and *cla4/cla4* and *cdc35/cdc35* strains were prepared as described earlier. One milliliter of a *Candida* suspension containing 5×10^7 or 5×10^6 cells was added to appropriate wells and incubated at 37°C and 5% CO₂. Cells were collected with a cell scraper (Sarstedt) and centrifuged at $3,000 \times g$. The supernatant was removed, and the pellet was frozen at -80°C until the caspase activity determination. Macrophages without *Candida* cells were used as negative controls. Positive controls consisted of UV treatment of 10 mJ/cm² (CL-1000 UV cross-linker; UVP). Caspase-8 and caspase-9 activities were assayed with ApoAlert caspase fluorescence assay kits (Clontech) in accordance with the manufacturer's instructions. Caspase-8 specifically cleaves the peptidic substrate IETD-7-amino-4-trifluoromethyl coumarin, and the release of 7-amino-4-trifluoromethyl coumarin was measured. Similarly, caspase-9 activity was detected through the release of 7-amino-4-methyl coumarin from LEHD-7-amino-4-methyl coumarin. Samples were incubated in black microtiter plates (Labsystems) and read with a Spectramax Gemini fluorimeter (Molecular Devices) at the appropriate excitation and emission wavelengths.

Statistical analysis. For end point dilutions, percentages of survival were compared to those for a wild-type strain (SC5314 or CAM 1.1) according to the Dunnett multiple-comparison test with the PRISM program (GraphPad Software). P values <0.01 were considered significant.

RESULTS

End point dilution analysis of macrophage-*C. albicans* interaction. Cell viability assays provide a measure of the consequences of the interaction between phagocytic cells such as macrophages and pathogens such as *C. albicans*. We used an end point dilution assay to monitor the survival of *C. albicans* cells in the presence of RAW 264.7 macrophage cells. *Candida* colonies were counted starting from the MOI where they could be distinctly visualized and at all dilutions below this point that still contained cells. Survival was expressed as the number of colonies in the presence of macrophages divided by the number of colonies in the absence of macrophages. Several mutant lines that compromise hyphal formation in vitro were tested. Their respective phenotypes and levels of virulence in mouse studies are shown in Table 2. This end point survival assay showed that the colony-forming capacity of *C. albicans* cells defective in components of the cAMP signaling pathway, such as Ras1p and Cdc35p, was reduced in the presence of the

macrophage cells (Table 2). Mutant strains with *CST20* deleted, which fail to form hyphae under some growth conditions but which form hyphae normally during serum stimulation (23), showed a survival similar to that of the wild type. Surprisingly, mutant cells with *CLA4* deleted, which are defective in filament formation and avirulent in a mouse systemic-infection model (25), survived better than even the wild-type cells when subjected to the macrophage challenge.

Luciferase assays. The end point dilution assay provides a measure of the loss of CFU due to macrophage killing of *C. albicans* cells cocultured at low MOIs. Figure 1 shows the appearance of colonies in the end point dilution assay after 48 h of incubation in D-10 at 37°C. However, as can be seen in Fig. 1, the macrophages can also greatly inhibit the proliferation of cells that are capable of forming colonies since the colony sizes are reduced, and thus the macrophage effect is not limited to reducing colony number due to initial phagocytosis and cell killing. To provide an assay that would allow investigation of the macrophage-*C. albicans* interaction at higher MOIs and that could measure the influence of the macrophages on *Candida* cell proliferation, we inserted the *Renilla* luciferase reporter gene into the *C. albicans* cells. The strains containing the luciferase reporter have phenotypes similar (cell shape and colony morphology) to those of their parental strains (data not shown) and have similar survival patterns in the end point dilution assay (Table 2).

We selected the inoculation conditions for the *C. albicans* cells by determining the initial cell densities that permitted exponential growth of the *Candida* cells in the absence of macrophages for the length of the experiment. All the cell lines were capable of exponential growth over the 12-h period of the experiments when they were inoculated at an initial cell density of 3.75×10^5 cells/well (150 µl). Shorter periods of incubation did not allow for evaluation of the slow-growing *cdc35/cdc35* strain, and longer periods of incubation result in saturation and an eventual decrease in luciferase activity for several of the strains (data not shown).

Macrophages inhibit *Candida* growth. We used the strains with the luciferase reporter gene to investigate growth inhibition in the presence of the mouse macrophage cell line RAW 264.7 at different MOIs. After 12-h incubation periods, wells in triplicate were harvested as described in Materials and Methods. The inhibition of growth of the wild-type strain in the presence of macrophages is illustrated in Fig. 2A and was determined by comparison with luciferase units measured from

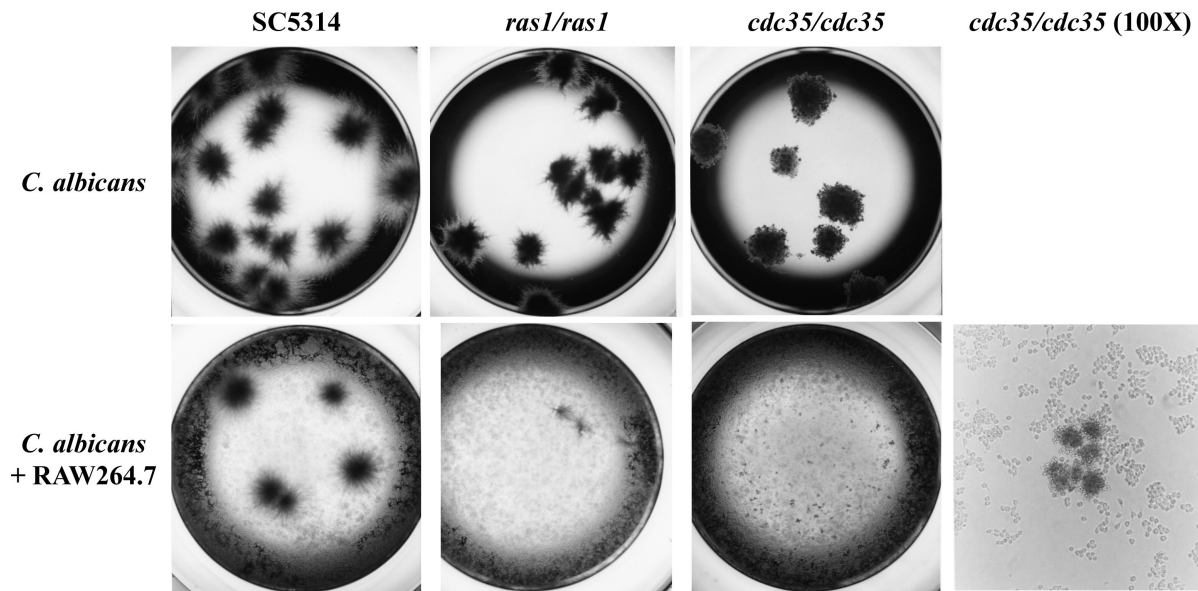


FIG. 1. Appearance of *C. albicans* colonies in the absence (top) or presence (bottom) of RAW 264.7 macrophage cells after a 48-h incubation time in 96-well plates. Photomicrographs were taken at $\times 20$ magnification except where indicated.

the same *Candida* strain grown in the absence of macrophages. The wild-type reference strain CAI4 showed a growth inhibition that is dependent on the MOI used, with an 80% survival rate for MOIs of 1 and 1/2, which is reduced to 30% for MOIs of 1/64 and 1/128. At MOIs higher than 1/16, the *cst20/cst20* strain is more sensitive than the wild-type CAI4. However, below this MOI, the behavior of that strain is similar to that of the control. Growth of the *ras1/ras1* strain and that of the *cdc35/cdc35* strain are more inhibited than that of CAI4 at MOIs lower than 1. The *cdc35/cdc35* strain is the most sensitive strain, with a growth inhibition of 55% at an MOI of 1. The *cla4/cla4* strain is up to two times more resistant than the wild-type CAI4 strain; the *cla4/cla4* strain forms aberrant pseudohyphae compared to the hyphae observed in CAI4.

IFN- γ treatment of macrophages does not enhance *Candida* growth inhibition. *Candida* phagocytosis and killing activity have been shown to increase upon IFN- γ priming of macrophages, alone or in combination with bacterial lipopolysaccharides (LPS) (5, 15, 30, 42). Figure 2B shows *Candida* survival when RAW 264.7 mouse macrophages were primed the day before with IFN- γ . The efficiency of IFN- γ priming could be observed by changes in RAW 264.7 cell morphology (data not shown). Surprisingly, this treatment seemed less efficient in causing growth inhibition of *Candida* cells than that of not priming RAW 264.7 cells, especially for the *cla4/cla4* strain at MOIs lower than 1/16, where macrophages seemed inefficient in growth inhibition (80 to 100% survival). As shown in Fig. 2C, leaving IFN- γ in the medium during *Candida* challenge did not improve growth inhibition for MOIs higher than 1/32 for all the strains tested. However, the CAI4 strain was more inhibited at lower MOIs (1/64 and 1/128). IFN- γ had no effect on *Candida* cell proliferation when added directly to the medium (data not shown). Figure 3 shows the effect of those treatments on survival of *C. albicans* strains at different MOIs, compared to survival in untreated macrophages. A value of 1

indicates identical survival rates. No substantial variation could be visualized for MOIs at or above 1/32. Below this MOI, results are more variable due to lower luciferase activities detected in wells containing macrophages (near detection limit) than in wells without macrophages.

Macrophages are killed by virulent *Candida* strains. Time-lapse microscopy experiments were performed to monitor the macrophage-*Candida* interactions as described in Materials and Methods. The ability of *Candida* to form hyphae or pseudohyphae allows its escape from the macrophage phagolysosome (21, 26). As shown in Fig. 4A, at low MOIs (1/20) *cla4/cla4* strain pseudohyphae could impale many macrophage cells without any apparent damage to either cell type for over a 9-h period or longer (not shown). The *cdc35/cdc35* mutant is unable to form pseudohyphae or hyphae and therefore could not escape from the macrophage upon phagocytosis. In some observations (Fig. 4B), both macrophage (at 15 h) and ingested *cdc35/cdc35* cells (at 8.5 h) were able to undergo cellular division. Propidium iodide staining was used to monitor macrophage cell death. As shown in Fig. 5, *Candida* at high MOIs (1 or 10) rapidly overgrew the macrophage monolayer. When RAW macrophages were challenged with SC5314 or *cla4/cla4* *Candida* strains at an MOI of 10 (Fig. 5A), propidium iodide staining indicates that they died after 6 h; they died after 9 to 12 h when challenged by strains at an MOI of 1 (Fig. 5B). When macrophages were challenged with the avirulent, non-hyphal *cdc35/cdc35* strain, macrophage death still occurred at MOIs of 10 and 1 but at later time points (10 to 12 h and 15 h, respectively) (Fig. 5A and B). At an MOI of 1/20, macrophage death occurred after 15 to 18 h of incubation (data not shown). The control RAW 264.7 macrophage cell line cultured without *Candida* died after 18 to 22 h under these culture conditions (Fig. 5C).

***C. albicans* does not induce apoptosis of macrophages.** We analyzed whether the macrophage cell death observed (Fig. 5A

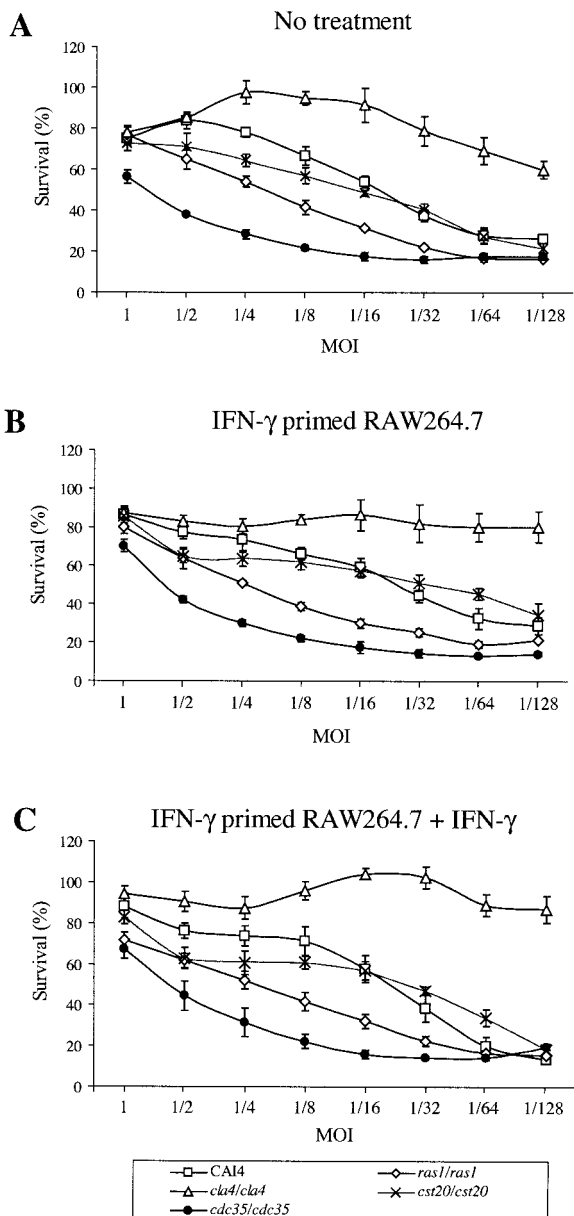


FIG. 2. Survival rate of *C. albicans* strains in the presence of RAW 264.7 mouse macrophage cells. Macrophages untreated (A), IFN- γ primed (B), or IFN- γ primed and left in media (C) were incubated with twofold dilutions of *C. albicans* strains for 12 h and harvested as indicated in Materials and Methods. The percentage of survival was determined as residual *Renilla* luciferase activity compared to that for the same *C. albicans* strains grown in the absence of macrophages. Values obtained are the averages for three wells assayed. Bars, standard deviations. Experiments were done at least three times with similar results.

and B) was mediated through an apoptotic or a necrotic process. Caspase-8 and caspase-9 identify the two main pathways through which apoptosis could be induced, and both proteases are activated early in the apoptotic process. The death receptor pathway, which is mediated by tumor necrosis factor- α or CD95, involves cleavage of procaspase-8 to caspase-8 through a death-inducing signaling complex (17). The stress pathway, induced by nutrient deprivation, oxidant stress, UV, ischemia,

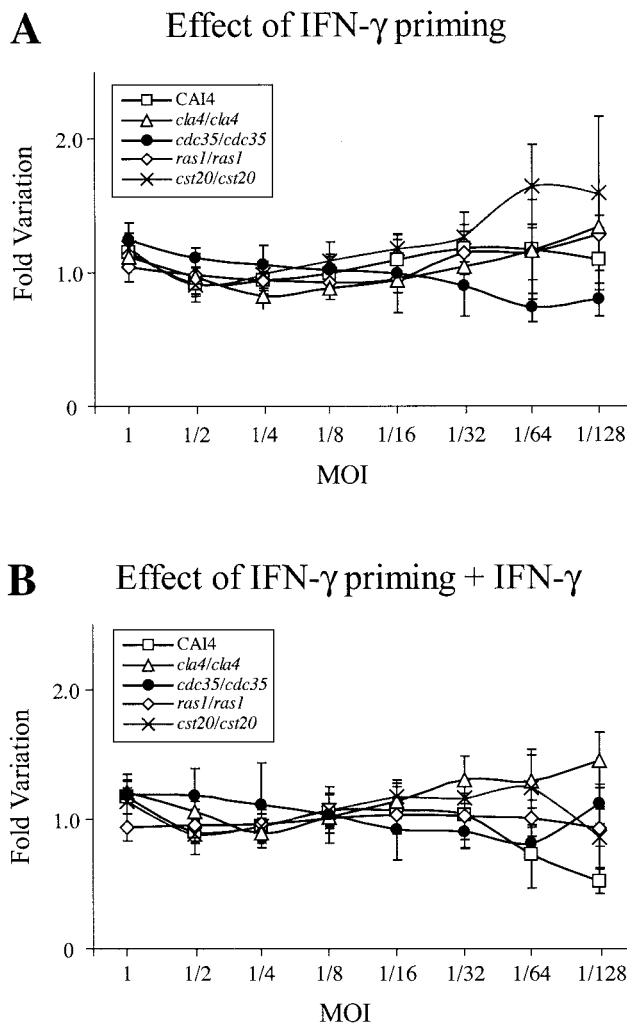


FIG. 3. Effect of IFN- γ on the survival of *C. albicans* strains when incubated with macrophages that were IFN- γ primed (A) or IFN- γ primed and left in media (B) at different MOIs, compared to their survival when incubated with unprimed RAW 264.7 macrophages. Bars, standard deviations.

toxins, or heat, involves release of cytochrome *c* from mitochondria to the apoptosome and mediates cleavage of procaspase-9 to caspase-9 (16, 17). The specific activities of these proteases could be evaluated by measurement of the fluorescent compound released through cleavage of their specific peptidic substrates, IETD-AFC for caspase-8 and LEHD-AMC for caspase-9. RAW 264.7 mouse macrophages were incubated with the *C. albicans* SC5314, *cla4/cla4*, and *cdc35/cdc35* strains at MOIs of 10 or 1 and harvested at the times indicated in Fig. 6. Caspase-8 and caspase-9 activities were measured for each sample. Negative controls consisting of macrophages only and positive controls consisting of macrophages induced with the known caspase inducer UV (44) were also included. Figure 6A and B show caspase-8 and caspase-9 activities, respectively, in RAW 264.7 cells cocultured with *Candida* strain SC5314 or the mutant *cla4/cla4* or *cdc35/cdc35* strain at MOIs of 1 or 10. As expected (44), exposure of RAW 264.7 cells to UV generated increased levels of both caspase-8 and -9 activities. For all *Candida* strains tested, caspase-8 ac-

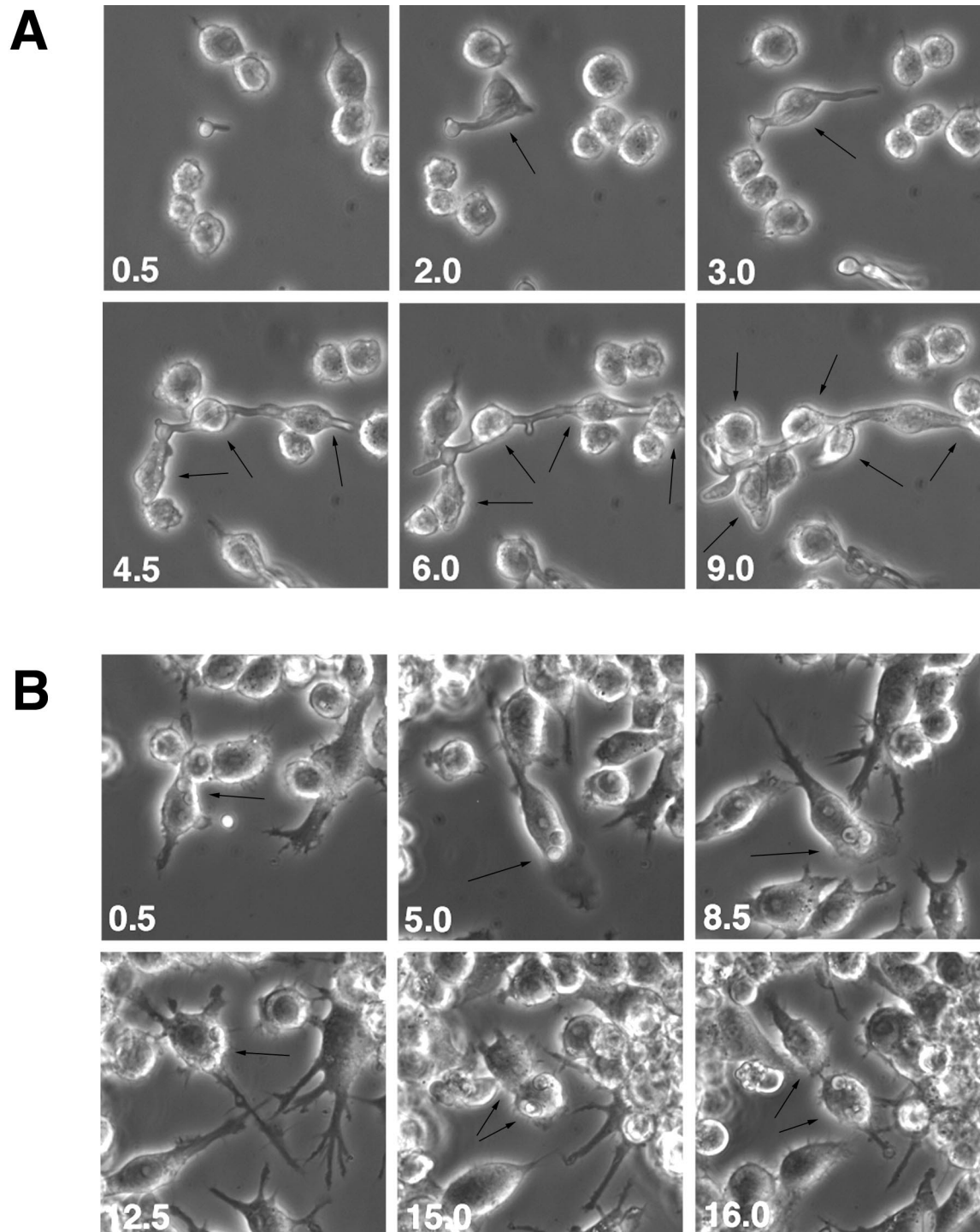


FIG. 4. Time-lapse microscopy of RAW 264.7 mouse macrophage cells in the presence of *cla4/cla4* (A) or *cdc35/cdc35* (B) *C. albicans* strains at low MOI (1/20). Times (in hours) are indicated at lower left corners. Arrows, *Candida*-macrophage interactions.

tivity was not increased in the presence of *Candida* compared to that for the control. In fact, caspase-8 activity was lower after macrophage challenge with SC5314 or the *cla4/cla4* strain at MOIs of 10 and 1. Small amounts of caspase-9 activity were detected in RAW 264.7 cells challenged with the *cdc35/cdc35* strain at an MOI of 1 in the 10- and 12-h samples. Because the RAW 264.7 macrophage cells had extensive mortality within

the assessed period (Fig. 5), it appears that macrophage cell death detected does not occur through an apoptotic process.

DISCUSSION

C. albicans is an important human fungal pathogen that can cause superficial or potentially lethal systemic infections of

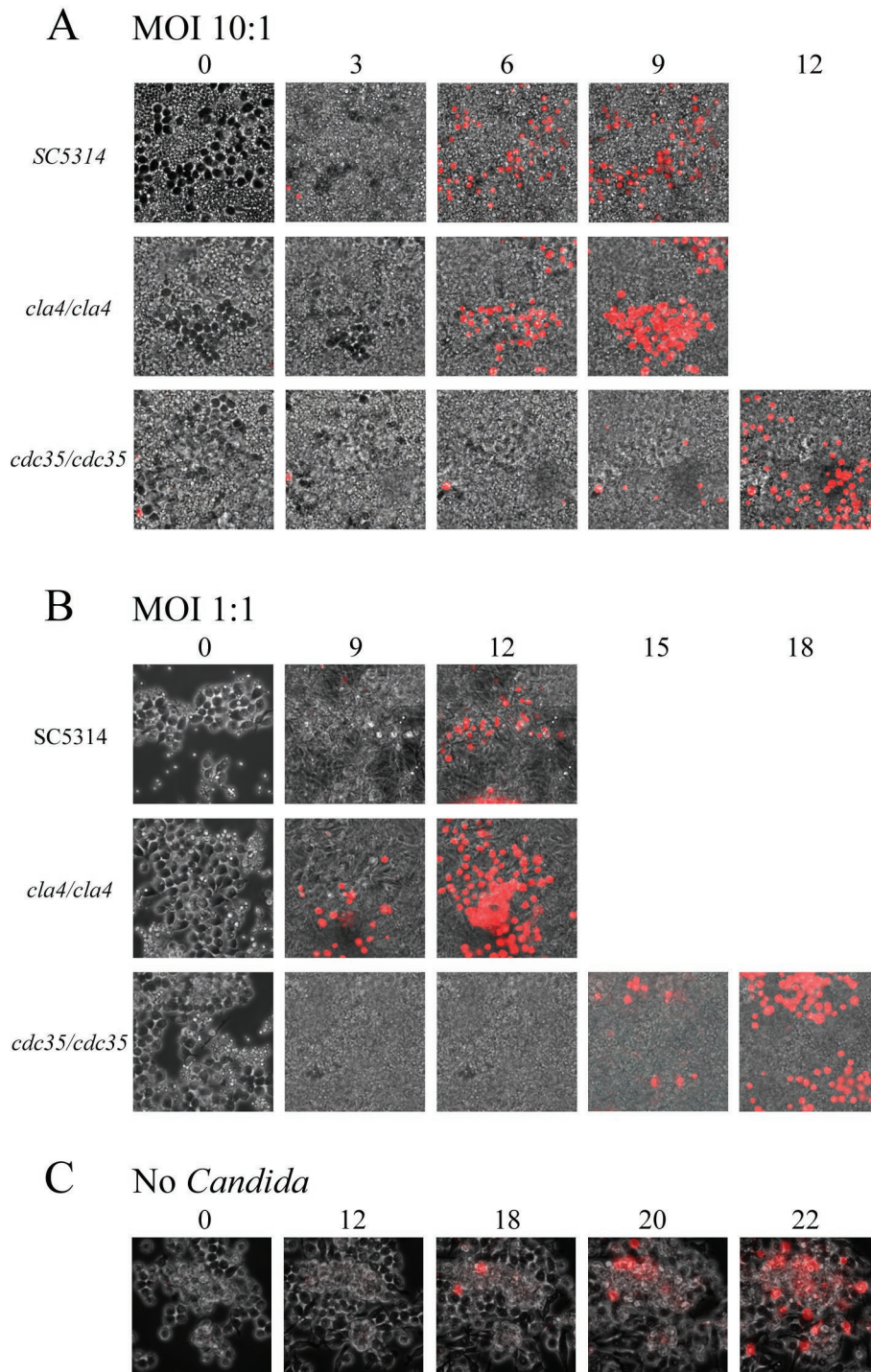


FIG. 5. Killing of RAW 264.7 mouse macrophage cells by *C. albicans* strains. Macrophages were incubated with *C. albicans* SC5314, *cla4/cla4*, or *cdc35/cdc35* strain at MOIs of 10 (A) and 1 (B) or without *Candida* (C) and monitored by time-lapse microscopy at $\times 400$ magnification for the indicated times (top, in hours) in the presence of propidium iodide. Killing of macrophages is indicated by appearance of propidium iodide-stained cells.

mammalian hosts. A powerful approach in the dissection of aspects of the infection process has been to monitor the interaction between *C. albicans* cells and cultured or purified mammalian cells (1, 5). Many of these studies have investigated the consequences of coculture of *C. albicans* cells and cells com-

prising elements of the mammalian immune system (15, 19, 36, 41). This approach permits the study of the roles of different immune system cells in killing the pathogen and also allows the analysis of the effects of signaling molecules such as cytokines on specific components of the host-pathogen interaction.

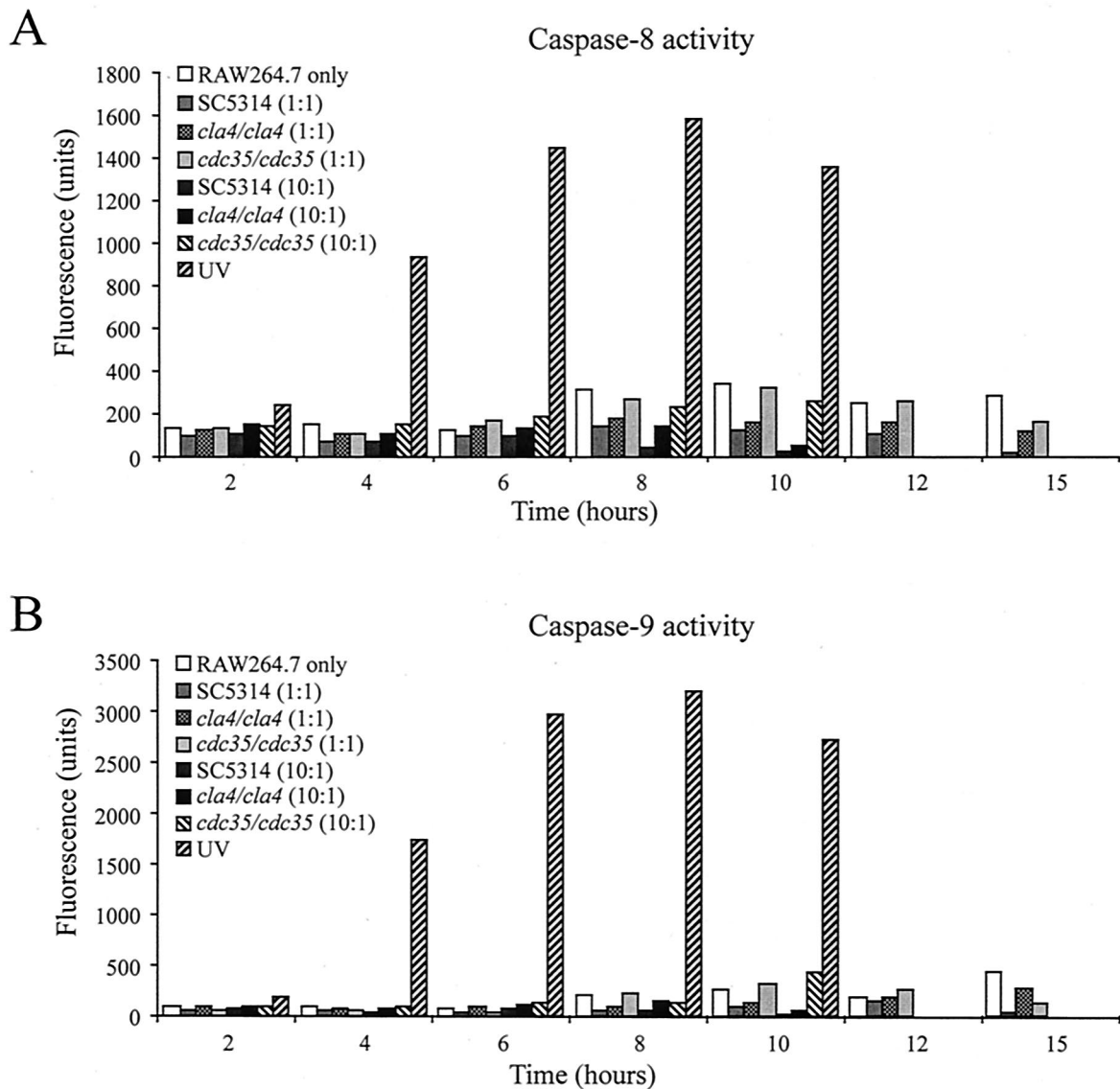


FIG. 6. Caspase-8 (A) and caspase-9 (B) activities of RAW 264.7 macrophage cells in the presence of *C. albicans* strains. Macrophage cells incubated without *Candida* and those treated with UV correspond to negative and positive controls, respectively. Cell samples were harvested at the indicated times, and caspase-8 and -9 activities were determined as described in Materials and Methods.

These studies require the measurement of the killing of *C. albicans* as well as the mammalian cells during the interaction; measurements of CFU (3, 43) and metabolic activity by dyes (20, 40) are typical strategies to determine pathogen killing.

The potential of this overall approach for the dissection of the host-pathogen interaction has been enhanced by the construction of *C. albicans* strains defective in specific molecular functions (3, 4, 23–26, 33). While these mutant strains provide important tools for the analysis of host-pathogen interactions, properties of the strains, such as changes in the ratio of yeast to hyphal forms or changes in cell wall characteristics leading to cell fragility or clumping of cells can bias assays such as CFU determinations. We have recently developed an end point dilution assay that provides a convenient measure of the effect of immune cells on *Candida* viability at low MOIs without the need for cell harvesting (33). In the present study we have

applied this assay to a series of *C. albicans* mutants (*cst20*, *cdc35*, *ras1*, and *cla4* mutants). *CDC35* and *RAS1* are primarily implicated in control of cAMP levels, while *CST20* and *CLA4* encode PAK kinase homologs that are required for proper morphogenesis. We selected these mutants because they provide a spectrum of effects on the morphological switching process. We determined that strains defective in *ras1* and adenylyl cyclase are more sensitive to killing by RAW 264.7 macrophages than are the wild-type *C. albicans* cells. This sensitivity to macrophage killing correlates well with the reduced virulence exhibited by the mutant strains in systemic-infection assays (24, 33). In contrast, strains with mutated *CST20* appear near the wild type in their resistance to macrophage killing in the end point dilution assay and exhibit virulence in the systemic-infection assays similar to that of the wild-type cells. The observation that the wild-type strains are somewhat more re-

sistant than the mutants could reflect minor influences of the Cst20p function. Alternatively, this could be due to the fact that the SC5314 (wild-type) strain contains an intact *URA3* locus, while the CAI4-derived strains such as the *cst20/cst20* mutant contain the replaced *URA3* gene but are still missing the other genes deleted during the construction of CAI4 (13). Intriguingly, strains defective in the *CLA4*-encoded PAK kinase are avirulent in the systemic-infection model (25) but exhibit a resistance to macrophage killing in the end point dilution assay that was greater than that of the wild-type strain from which the mutant was derived. Thus although sensitivity to macrophage killing is well correlated with reduced virulence in vivo, resistance to macrophages does not ensure virulence in an in vivo infection model.

To confirm the results of the end point dilution assays, as well as to provide an assay that can monitor the macrophage-*Candida* interaction at higher MOIs, we introduced a *Renilla* luciferase reporter construct into the wild-type and mutant *C. albicans* strains. Analysis of the effect of the RAW 264.7 macrophage cells on the various *C. albicans* strains shows that the inhibition of *Candida* proliferation caused by macrophages is density dependent. At high MOIs, where the number of *C. albicans* cells is similar to that of the macrophages, the wild-type *C. albicans* cells proliferate to almost the same extent as cells growing in the absence of macrophages. However, at levels where the macrophages outnumber the fungal cells by 100 to 1 there is substantial reduction in *Candida* proliferation relative to that in the cultures without macrophages. At intermediate MOIs the proliferation inhibition is also intermediate; there does not appear to be a threshold of influence but rather a constant dose dependence on the ratio of *Candida* cells to macrophages. This pattern is not dramatically influenced by treating the macrophages with IFN- γ , suggesting that this cytokine does not play a significant role in activating these mouse macrophage cells against *Candida*. Previous evidence on the role of IFN- γ in candidacidal activity by macrophages has been controversial. The priming of macrophages with IFN- γ was found to enhance their candidacidal potential (5, 15, 30, 42). However, systemic infection of IFN- γ knockout mice showed that *Candida* clearance was not impaired (31). Baltch et al. (1) recently demonstrated that, in vitro, this priming was efficient against *Candida* only when used in combination with fluconazole.

The luciferase assay confirms the relative sensitivities of the *Candida* mutants determined by the end point dilution assay. The *cdc35/cdc35* mutant strain was the most sensitive to macrophage-mediated inhibition at essentially all MOIs tested, while the *ras1/ras1* mutant was more sensitive than the wild-type cells but less sensitive than the adenylcyclase-defective strain. The strain defective in *CST20* behaved similarly to the wild-type strain, while the *cla4/cla4* strain was very resistant to the influence of the macrophages. The resistance of the *cla4/cla4* strain was most evident at the lower MOIs, where the wild-type and the other mutant strains were substantially inhibited in proliferation by the macrophages. The activation of the macrophages by IFN- γ did not enhance the ability of the macrophages to inhibit proliferation of the mutant *C. albicans* cells. In fact the *cla4/cla4* strain was essentially unaffected by the IFN- γ -stimulated cells at any MOI, while the untreated macrophages showed a moderate ability to reduce prolifera-

tion of these mutant cells at low MOIs. This resistance to macrophages exhibited by the *cla4/cla4* mutant strain may reflect the altered morphology exhibited by these cells (25).

The effect of the interaction between the pathogen and the immune system cells is not limited to the growth inhibition of the pathogen cells. Coculture of the *C. albicans* and RAW 264.7 cells also enhances the death of the RAW macrophage cells. This killing is significant: high levels of wild-type *Candida* cells (MOI of 10:1) cause macrophage death within 6 h, whereas macrophages cultured without *Candida* cells die after 18 to 22 h (Fig. 5). This killing is density dependent, in that lower MOIs increase the time before macrophage death, and is also dependent on the virulence of the *Candida* cells, as the *cdc35/cdc35* mutant strains cause macrophage death at 10 h at an MOI of 10:1 and after 15 to 18 h at an MOI of 1:1.

This *Candida*-induced macrophage death does not appear to be due to apoptosis of the mouse cells. We measured both the caspase-8 and -9 activities that define the two major apoptotic pathways and showed that neither enzyme was significantly induced before the macrophage cells were killed. This observation contrasts with previous work showing that *Candida* could trigger apoptosis of mammalian cells (34, 36). Schroppe et al. (36) examined decreased mitochondrial potential and chromatin degradation of IFN- γ - and LPS-stimulated mouse peritoneal macrophages challenged with wild-type *Candida* at a low MOI (1/32). Rotstein et al. (34) examined microscopic manifestations of apoptosis such as nuclear condensation and formation of apoptotic bodies, as well as caspase-8, -9, and -3 activities of human neutrophils upon challenge with killed *Candida*. There are several possible explanations for the differences among the studies. First, mammalian cells of different origins could respond differentially to a *Candida* challenge and the response might also be MOI dependent. Second, the presence or absence of LPS priming could have a major influence on the activation of apoptotic pathways in response to *Candida*. In this work we challenged macrophages with live *Candida* without prior IFN- γ and LPS priming. IFN- γ and LPS are strong inducers of inducible nitric oxide synthase (2), and a high level of production of NO could potentially induce apoptosis in macrophages through downregulation of antiapoptotic proteins (10). As well, macrophages challenged with *Candida* have been shown to reduce the level of inducible nitric oxide synthase (9, 36); this reduction could help prevent macrophage apoptosis. Because LPS can bind to Toll-like receptors and because these receptors can activate proapoptotic pathways through activation of caspase-8 (28), LPS priming of macrophages could stimulate apoptotic processes. A third difference among the studies is the use of heat-killed *Candida*; this treatment modifies *Candida*-macrophage interactions both biochemically and physiologically (9), and thus comparisons between live and heat-killed cells may not be valid. Finally, apoptosis could be induced by nutrient limitation. In the present study we selected conditions to minimize the effect of nutrient limitation, but this could have influenced other studies.

It is interesting that cultured monolayered RAW 264.7 macrophages are not particularly efficient in killing *Candida*: up to 30% of the wild-type pathogen cells still survived the interaction even at low MOIs (Table 2 and Fig. 2). In addition, RAW 264.7 cells can both phagocytose *Candida* and be impaled by

Candida filaments without any immediately apparent cell damage (Fig. 4A), and macrophages can continue to divide in the presence of *Candida* (Fig. 4B). Overall then, the *Candida*-macrophage interaction is not dramatically detrimental to either cell type; this may reflect the fact that *Candida* is usually a common but innocuous component of mammalian flora (38). It is also clear from the observation that *CLA4* mutants are avirulent but also insensitive to macrophage killing that the whole organism's response to *Candida* involves much more than macrophages. Other cell types such as neutrophils or dendritic cells, as well as the humoral immune response, presumably play a significant role in host defense against *Candida* systemic infections (6, 29). Because in vitro experiments allow for the dissection and measurement of individual processes involved in the host-pathogen interaction, further studies should provide insight into the importance of each individual component in the process. Ultimately, a combination of in vitro and in vivo studies will be necessary to provide the framework for understanding this complex interaction.

ACKNOWLEDGMENTS

We thank D. R. Soll, B. Magee, and A. Brown for plasmids, W. A. Fonzi, C. Rocha, and E. Leberer for providing *C. albicans* strains, and Antoine W. Caron for help with time-lapse microscopy. We are especially grateful to Albert Descoteaux for providing the RAW 264.7 macrophage cell line and for helpful discussion and André Nantel for critical reading of the manuscript. Finally, we thank members of the laboratory for fruitful discussions.

REFERENCES

- Baltch, A. L., R. P. Smith, M. A. Franke, W. J. Ritz, P. B. Michelsen, and L. H. Bopp. 2001. Effects of cytokines and fluconazole on the activity of human monocytes against *Candida albicans*. *Antimicrob. Agents Chemother.* **45**:96–104.
- Beck, K. F., W. Eberhardt, S. Frank, A. Huwiler, U. K. Messmer, H. Muhl, and J. Pfeilschifter. 1999. Inducible NO synthase: role in cellular signalling. *J. Exp. Biol.* **202**:645–653.
- Borg-von Zepelin, M., S. Beggah, K. Boggian, D. Sanglard, and M. Monod. 1998. The expression of the secreted aspartyl proteinases Sap4 to Sap6 from *Candida albicans* in murine macrophages. *Mol. Microbiol.* **28**:543–554.
- Braun, B. R., and A. D. Johnson. 1997. Control of filament formation in *Candida albicans* by the transcriptional repressor TUP1. *Science* **277**:105–109.
- Brummer, E., and D. A. Stevens. 1989. Candidacidal mechanisms of peritoneal macrophages activated with lymphokines or gamma-interferon. *J. Med. Microbiol.* **28**:173–181.
- Calderone, R., and N. A. Gow. 2002. Host recognition by *Candida* species, p. 67–86. In R. Calderone (ed.), *Candida and candidiasis*. ASM Press, Washington, D.C.
- Calderone, R. A., and W. A. Fonzi. 2001. Virulence factors of *Candida albicans*. *Trends Microbiol.* **9**:327–335.
- Chen, D. C., B. C. Yang, and T. T. Kuo. 1992. One-step transformation of yeast in stationary phase. *Curr. Genet.* **21**:83–84.
- Chinen, T., M. H. Qureshi, Y. Koguchi, and K. Kawakami. 1999. *Candida albicans* suppresses nitric oxide (NO) production by interferon-gamma (IFN-gamma) and lipopolysaccharide (LPS)-stimulated murine peritoneal macrophages. *Clin. Exp. Immunol.* **115**:491–497.
- Chung, H. T., H. O. Pae, B. M. Choi, T. R. Billiar, and Y. M. Kim. 2001. Nitric oxide as a bioregulator of apoptosis. *Biochem. Biophys. Res. Commun.* **282**:1075–1079.
- Corner, B. E., and P. T. Magee. 1997. *Candida* pathogenesis: unravelling the threads of infection. *Curr. Biol.* **7**:R691–R694.
- Csank, C., K. Schroppel, E. Leberer, D. Harcus, O. Mohamed, S. Meloche, D. Y. Thomas, and M. Whiteway. 1998. Roles of the *Candida albicans* mitogen-activated protein kinase homolog, Cek1p, in hyphal development and systemic candidiasis. *Infect. Immun.* **66**:2713–2721.
- Fonzi, W. A., and M. Y. Irwin. 1993. Isogenic strain construction and gene mapping in *Candida albicans*. *Genetics* **134**:717–728.
- Fratuzzi, C., R. D. Arbeit, C. Carini, M. K. Balcewicz-Sablinska, J. Keane, H. Kornfeld, and H. G. Remold. 1999. Macrophage apoptosis in mycobacterial infections. *J. Leukoc. Biol.* **66**:763–764.
- Gaviria, J. M., J. A. van Burik, D. C. Dale, R. K. Root, and W. C. Liles. 1999. Comparison of interferon-gamma, granulocyte colony-stimulating factor, and granulocyte-macrophage colony-stimulating factor for priming leukocyte-mediated hyphal damage of opportunistic fungal pathogens. *J. Infect. Dis.* **179**:1038–1041.
- Goyal, L. 2001. Cell death inhibition: keeping caspases in check. *Cell* **104**:805–808.
- Green, D. R., and J. C. Reed. 1998. Mitochondria and apoptosis. *Science* **281**:1309–1312.
- Haynes, K. 2001. Virulence in *Candida* species. *Trends Microbiol.* **9**:591–596.
- Heidenreich, S., B. Otte, D. Lang, and M. Schmidt. 1996. Infection by *Candida albicans* inhibits apoptosis of human monocytes and monocytic U937 cells. *J. Leukoc. Biol.* **60**:737–743.
- Jahn, B., E. Martin, A. Stueben, and S. Bhakdi. 1995. Susceptibility testing of *Candida albicans* and *Aspergillus* species by a simple microtiter menadione-augmented 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay. *J. Clin. Microbiol.* **33**:661–667.
- Kaposzta, R., L. Marodi, M. Hollinshead, S. Gordon, and R. P. da Silva. 1999. Rapid recruitment of late endosomes and lysosomes in mouse macrophages ingesting *Candida albicans*. *J. Cell Sci.* **112**:3237–3248.
- Kobayashi, S. D., and J. E. Cutler. 1998. *Candida albicans* hyphal formation and virulence: is there a clearly defined role? *Trends Microbiol.* **6**:92–94.
- Leberer, E., D. Harcus, I. D. Broadbent, K. L. Clark, D. Dignard, K. Ziegelbauer, A. Schmidt, N. A. Gow, A. J. Brown, and D. Y. Thomas. 1996. Signal transduction through homologs of the Ste20p and Ste7p protein kinases can trigger hyphal formation in the pathogenic fungus *Candida albicans*. *Proc. Natl. Acad. Sci. USA* **93**:13217–13222.
- Leberer, E., D. Harcus, D. Dignard, L. Johnson, S. Ushinsky, D. Y. Thomas, and K. Schroppel. 2001. Ras links cellular morphogenesis to virulence by regulation of the MAP kinase and cAMP signalling pathways in the pathogenic fungus *Candida albicans*. *Mol. Microbiol.* **42**:673–687.
- Leberer, E., K. Ziegelbauer, A. Schmidt, D. Harcus, D. Dignard, J. Ash, L. Johnson, and D. Y. Thomas. 1997. Virulence and hyphal formation of *Candida albicans* require the Ste20p-like protein kinase CaCla4p. *Curr. Biol.* **7**:539–546.
- Lo, H. J., J. R. Kohler, B. DiDomenico, D. Loebenberg, A. Cacciapuoti, and G. R. Fink. 1997. Nonfilamentous *C. albicans* mutants are avirulent. *Cell* **90**:939–949.
- Magee, B. B., and P. T. Magee. 1997. WO-2, a stable aneuploid derivative of *Candida albicans* strain WO-1, can switch from white to opaque and form hyphae. *Microbiology* **143**:289–295.
- Navarre, W. W., and A. Zychlinsky. 2000. Pathogen-induced apoptosis of macrophages: a common end for different pathogenic strategies. *Cell. Microbiol.* **2**:265–273.
- Newman, S. L., and A. Holly. 2001. *Candida albicans* is phagocytosed, killed, and processed for antigen presentation by human dendritic cells. *Infect. Immun.* **69**:6813–6822.
- Puliti, M., D. Radzioch, R. Mazzolla, R. Barluzzi, F. Bistoni, and E. Blasi. 1995. Influence of the Beg locus on macrophage response to the dimorphic fungus *Candida albicans*. *Infect. Immun.* **63**:4170–4173.
- Qian, Q., and J. E. Cutler. 1997. Gamma interferon is not essential in host defense against disseminated candidiasis in mice. *Infect. Immun.* **65**:1748–1753.
- Raupach, B., and S. H. Kaufmann. 2001. Immune responses to intracellular bacteria. *Curr. Opin. Immunol.* **13**:417–428.
- Rocha, C. R., K. Schroppel, D. Harcus, A. Marcil, D. Dignard, B. N. Taylor, D. Y. Thomas, M. Whiteway, and E. Leberer. 2001. Signaling through adenylyl cyclase is essential for hyphal growth and virulence in the pathogenic fungus *Candida albicans*. *Mol. Biol. Cell* **12**:3631–3643.
- Rotstein, D., J. Parodo, R. Taneja, and J. C. Marshall. 2000. Phagocytosis of *Candida albicans* induces apoptosis of human neutrophils. *Shock* **14**:278–283.
- Scaringi, L., E. Blasi, P. Cornacchione, C. Bietta, and F. Bistoni. 1991. A rapid *Candida albicans* hyphal-form growth inhibition assay: determination of myelomonocytic-mediated antifungal activity. *Mycoses* **34**:119–123.
- Schroppel, K., M. Kryk, M. Herrmann, E. Leberer, M. Rollinghoff, and C. Bogdan. 2001. Suppression of type 2 NO-synthase activity in macrophages by *Candida albicans*. *Int. J. Med. Microbiol.* **290**:659–668.
- Schweizer, A., S. Rupp, B. N. Taylor, M. Rollinghoff, and K. Schroppel. 2000. The TEA/ATTS transcription factor CaTec1p regulates hyphal development and virulence in *Candida albicans*. *Mol. Microbiol.* **38**:435–445.
- Soll, D. R. 2002. *Candida* commensalism and virulence: the evolution of phenotypic plasticity. *Acta Trop.* **81**:101–110.
- Srikantha, T., A. Klapach, W. W. Lorenz, L. K. Tsai, L. A. Laughlin, J. A. Gorman, and D. R. Soll. 1996. The sea pansy *Renilla reniformis* luciferase serves as a sensitive bioluminescent reporter for differential gene expression in *Candida albicans*. *J. Bacteriol.* **178**:121–129.
- Tellier, R., M. Krajden, G. A. Grigoriev, and I. Campbell. 1992. Innovative end point determination system for antifungal susceptibility testing of yeasts. *Antimicrob. Agents Chemother.* **36**:1619–1625.
- Torosantucci, A., P. Chiani, F. De Bernardis, A. Cassone, J. A. Calera, and R. Calderone. 2002. Deletion of the two-component histidine kinase gene

- (CHK1) of *Candida albicans* contributes to enhanced growth inhibition and killing by human neutrophils in vitro. *Infect. Immun.* **70**:985–987.
42. **Vazquez-Torres, A., and E. Balish.** 1997. Macrophages in resistance to candidiasis. *Microbiol. Mol. Biol. Rev.* **61**:170–192.
43. **Vonk, A. G., C. W. Wieland, M. G. Netea, and B. J. Kullberg.** 2002. Phagocytosis and intracellular killing of *Candida albicans* blastoconidia by neutrophils and macrophages: a comparison of different microbiological test systems. *J. Microbiol. Methods* **49**:55–62.
44. **Vu, C. C., C. D. Bortner, and J. A. Cidlowski.** 2001. Differential involvement of initiator caspases in apoptotic volume decrease and potassium efflux during Fas- and UV-induced cell death. *J. Biol. Chem.* **276**:37602–37611.

Editor: T. R. Kozel