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Differential Susceptibility of Yeast and Hyphal Forms of *Candida albicans* to Proteolytic Activity of Macrophages

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The dimorphic transition of *Candida albicans* from the yeast (Y-*Candida*) to the hyphal (H-*Candida*) form is a complex event whose relevance in fungal pathogenicity is still poorly understood. Using a cloned macrophage (Mϕ) cell line (ANA-1), we have previously shown that a Mϕ can discriminate between the two fungal forms, eliciting different secretory responses. In the present study, we investigated the susceptibility of Y-*Candida* and H-*Candida* to Mϕ proteolytic activity. In particular, sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot (immunoblot) techniques were employed to analyze the patterns of lyticase proteinaceous extracts from cell walls of Y-*Candida* and H-*Candida* which had been unexposed or exposed to ANA-1 Mϕs for 3 h. Silver staining allowed detection of a complex protein pattern in both forms of *C. albicans*, qualitatively and quantitatively differing from each other, mainly at molecular masses below 106 kDa. Western blot staining with anti-*C. albicans* mannan antibodies and convalescent-phase sera of mice previously infected systemically or intracerebrally with *C. albicans* showed that, after contact with Mϕs, Y-*Candida* but not H-*Candida* proteinaceous cell wall components are profoundly modified, with substantial reduction and/or disappearance of many bands. Our experimental approach provides initial insights into the differential susceptibility of Y-*Candida* and H-*Candida* to the proteolytic activity of Mϕs.

The opportunistic pathogen *Candida albicans* is a serious agent of infection in immunocompromised hosts (28). *C. albicans* exhibits dimorphic growth, being capable of reproducing by budding, which leads to the formation of yeast cells (Y-*Candida*), or by germination, which gives rise to hyphae (H-*Candida*) (29). Although both morphologies are found in tissues affected with candidiasis, the ability to form hyphae is generally considered an important virulence factor (29).

With murine models of systemic or intracerebral candidiasis, we identified some of the immunological events crucial in the outcome of infection in healing (self-limited) or lethal disease (2, 5, 27, 39). Phagocytes as well as $CD4^+$ T helper type 1 cells certainly play key roles, through a cascade of effector and secretory functions also involving antibody production (5, 27, 33, 34, 39). Moreover, initial evidence that *C. albicans* affects host immune responses differently depending on its morphologic status has been provided. In particular, although both forms are susceptible to macrophage (Mϕ) antifungal activity (3, 35), only the nonphagocytable H-*Candida*, but not the rapidly ingested Y-*Candida*, stimulates Mϕ tumor necrosis factor and interleukin 1 transcription and production (6, 7), thus providing initial insight into the dichotomy of the host response against the dimorphic fungus *C. albicans.*

Besides conferring the peculiar shape to each morphologic form, the *C. albicans* cell wall is the site of the initial interactions between the microorganism and the environment or host (36). Information on the identity, composition, and distribution of the cell wall components is important for understanding of both the structural role of the cell wall and its function in host-fungus interaction. Several studies have previously shown that proteinaceous cell wall constituents, mainly glycoproteins containing N- and O-linked mannose polymers, i.e., mannoproteins, play an essential role in the regulation of cell wall morphogenesis (10, 14, 19, 25, 36) as well as in the control of cell surface properties, such as antigenicity, adherence to inert surfaces or to animal cells, extracellular proteolytic activities, and cell surface hydrophobicity (10, 15, 16, 18, 20, 23, 30). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) alone or in association with Western blotting (immunoblotting) techniques has been used to analyze proteinaceous constituents solubilized from whole cells as well as from isolated cell walls, by various chemical and/or enzymatic treatments (11–13, 23, 24). These studies underlined the complexity of the protein-containing components and identified quantitative and qualitative differences between Y-*Candida* and H-*Candida* cell walls in protein composition.

The encounter between *C. albicans* and phagocytes is generally considered the initial step through which cell-mediated host immune defenses begin. While there exists evidence concerning the interaction between phagocytes and H-*Candida* or Y-*Candida*, the precise sequence of events allowing fungal elimination, likely through destruction of cell wall components, is uncertain. Taschdjian et al. (37), using fluorescence microscopy, found that the cell wall is removed first, while Belcher et al. (1) noted cytoplasmic changes preceding cell wall changes, as seen by electron microscopy. Richardson and Smith (32) found that within 2 h, the cell wall layers as well as the cytoplasmic organelles of Y-*Candida* are destroyed within phagocytes, while the overall elliptical outline of the microorganism remains intact.

In this report, we show that, after contact with Mϕs, Y-*Candida* and H-*Candida* exhibit differential modification or reduction of their proteinaceous cell wall components, further strengthening the hypothesis of a dichotomy in the host immune response against dimorphic fungi. The possible implications of this phenomenon are discussed.

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MATERIALS AND METHODS

Cell lines. ANA-1 Mϕs, derived by immortalization of bone marrow cells from C57BL/6 mice with a recombinant retrovirus carrying v-*raf* and v-*myc* oncogenes (4, 17), were cultured in RPMI 1640 medium supplemented with glutamine (4 mM), gentamicin (50 μg/ml), and 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, Utah) (complete medium).

C. albicans. *C. albicans* CA-6 serotype A, used throughout this study, was isolated from a clinical specimen. It was grown at 28°C with mild agitation in low-glucose Winge medium as previously described (7). Under these conditions, the organism grew as an essentially pure Y-*Candida* population. To obtain H-*Candida*, Y-*Candida* cells were harvested from Winge medium, washed twice in saline, suspended in complete medium, dispensed in 60-mm-diameter tissue culture plates (10^7 cells per plate), and incubated at 37°C in 5% CO_2. More than 98% of the microorganisms were H-*Candida* after 3 h of incubation, as detailed elsewhere (7). The agerminative *C. albicans* strain PCA-2, a kind gift of D. Kerridge (Department of Biochemistry, University of Cambridge, Cambridge, England), is an echinocandin-resistant mutant of the parental strain 3153 (26). It was maintained by daily passages on Sabouraud dextrose agar plates.

Experimental protocol. The experimental cultures were (i) Y-*Candida*, (ii) H-*Candida*, (iii) Y-*Candida* plus Mϕs, (iv) H-*Candida* plus Mϕs, and (v) Mϕs. Each experimental group consisted of 10^7 microorganisms and/or 10^7 Mϕs in a 5-ml final volume of complete medium. The cultures were incubated in 60-mm-diameter tissue culture plates at 37°C in 5% CO_2. After 3 h of incubation, the cultures were subjected to the enzymatic treatment described below and then employed in SDS-PAGE and Western immunoblotting techniques.

Enzymatic treatment of cells. Enzymatic lysis of cells was carried out by the method of Goud et al. (21) with minor modifications. The cells were centrifuged twice in cold Hanks' balanced salt solution (without phenol red and without sodium bicarbonate) (pH 7.4), 10 mM NaN_3, and 0.3 mM phenylmethylsulfonyl fluoride at 6,000 × *g* for 10 min. The cell pellets were then converted to spheroplasts by addition of 1 ml of 50 mM phosphate-buffered saline (PBS) (pH 7.5) containing 1.4 M sorbitol, 100 U of (1-3)-β-glucanase complex (lyticase from *Arthrobacter luteus*; Sigma Chemical Co., St. Louis, Mo.), 0.6% 2-mercaptoethanol, and 0.3 mM phenylmethylsulfonyl fluoride. After 30 min at 37°C, spheroplasts were sedimented at 12,000 rpm for 15 min, and the supernatants (lyticase extracts) were carefully harvested. Equal volumes (150 μl) of lyticase extracts from each experimental group were mixed with 30 μl of 6× SDS-sample buffer (6× SB) (0.3 M Tris-HCl [pH 6.8], 10% SDS, 36% glycerol, 0.012% bromophenol blue, 6% 2-mercaptoethanol). The mixture was heated at 100°C for 5 min and subjected to SDS-PAGE and Western blotting techniques as described below.

SDS-PAGE and Western blotting techniques. SDS-PAGE was performed as described by Laemli (22), using slab gradient gels (5 to 15%; acrylamide-bisacrylamide, 30:0.8) with 4 to 5% polyacrylamide stacking gels. Electrophoresis was performed at a constant current of 25 mA for 18 h. The following prestained molecular weight standards (Bio-Rad, Richmond, Calif.) were used: myosin (205,000), β-galactosidase (116,000), phosphorylase *b* (106,000), bovine serum albumin (80,000), ovalbumin (49,500), carbonic anhydrase (32,500), soybean trypsin inhibitor (27,500), and lysozyme (18,500). Subsequently, the gels were either silver stained, as described by Bloom et al. (8), or electrophoretically transferred to polyvinylidene difluoride (PVDF) transfer membranes (Immobilon-P; Millipore, Bedford, Mass.) for 3 h at 4 mA/cm^2 by using an electroblotter (Bio-Rad Trans-blot cell) according to the method of Towbin et al. (38). The transfer was complete as judged by restaining of the gel after transfer. The PVDF membrane was then soaked in blocking solution (5% [wt/vol] nonfat dried milk, 0.02% Tween 20, 0.02% NaN_3 in PBS) for 1 h at room temperature, washed in PBS, incubated overnight at room temperature in a 1:100 dilution of mouse convalescent-phase serum (see below), washed three times with PBS, and then incubated in a 1:500 dilution of peroxidase-conjugated goat anti-mouse immunoglobulins (Southern Biotechnology Associates Inc., Birmingham, Ala.) for 2 h at room temperature. For mannan immunostaining, PVDF membranes were incubated overnight at room temperature in a 1:500 dilution of peroxidase-conjugated rabbit anti-*C. albicans* mannan antibody (DAKO A/S, Glostud, Denmark). Then the PVDF membranes were rinsed three times with PBS and developed with H_2O_2 and diaminobenzidine tetrahydrochloride in Tris-HCl (pH 7.6).

Preparation of convalescent-phase sera. CD1 female mice, systemically (intravenously [i.v.]) or intracerebrally (i.c.) infected with the agerminative mutant *C. albicans* strain PCA-2 (10^6 cells per mouse), were challenged 14 days later i.v. or i.c. with the parental strain CA-6 (10^6 yeast cells per mouse), as detailed elsewhere (27, 39). After an additional 14 days, the animals were sacrificed and sera were collected from individual mice, pooled, and stored at −20°C until use in Western blots.

RESULTS

We compared the electrophoretic patterns of lyticase extracts from Y-*Candida* and H-*Candida* which had been unexposed to Mϕs or exposed to ANA-1 Mϕs for 3 h at an effector-to-target cell ratio of 1:1. Such extracts were separated by SDS-PAGE, under reducing conditions, on 5 to 15% acrylamide slab gradient gels and silver stained. As depicted in Fig. 1, fungal as well as Mϕ extracts showed at least 20 major bands with molecular masses ranging from >205 to <18.5 kDa. The comparison of Y-*Candida* (lane 1) and H-*Candida* (lane 2) silver-stained profiles revealed the existence of many bands common to both morphological forms. Although equal volumes of lyticase extracts were loaded, some bands, ranging between 120 and 27.5 kDa, were quantitatively more abundant in H-*Candida* than in Y-*Candida* extracts. Moreover, four bands (bands 1, 2, 4, and 5; >120 kDa) were present in only the Y-*Candida* form, and six bands, one of which was >120 kDa (band 3) and five of which were <27.5 kDa (bands 21 and 23 to 26), were detectable in only the H-*Candida* form (Fig. 1). By comparing the protein patterns of Y-*Candida* or H-*Candida* which had been exposed or unexposed to Mϕs, we observed substantial enhancement of the diffused background and increased thickness of some of the bands in extracts of H-*Candida* plus Mϕs in comparison with those of Mϕs alone (Fig. 1, lane 5 versus lane 3). In contrast, only minor differences were evident between the extracts of Y-*Candida* plus Mϕs versus Mϕs alone (lane 4 versus lane 3).

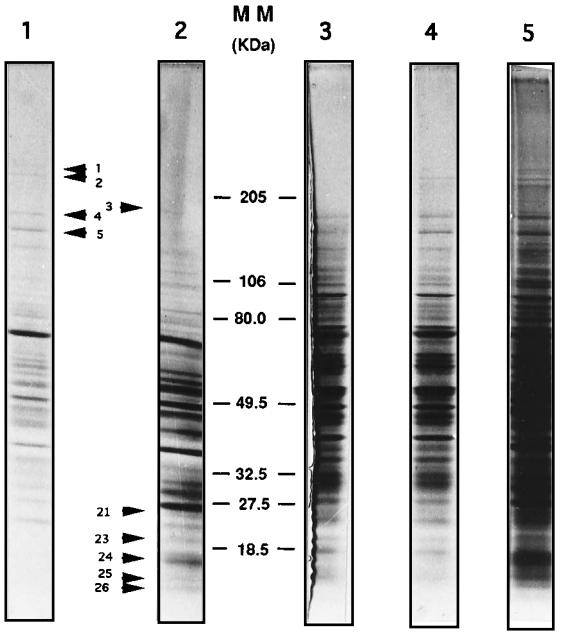

FIG. 1. Electrophoretic patterns of lyticase extracts from Y-*Candida* and H-*Candida* unexposed to Mϕs or exposed to ANA-1 Mϕs. Lyticase extracts were obtained from Y-*Candida* (lane 1), H-*Candida* (lane 2), ANA-1 Mϕs (lane 3), Y-*Candida* plus ANA-1 Mϕs (lane 4), and H-*Candida* plus ANA-1 Mϕs (lane 5), as detailed in Materials and Methods. For each experimental group, 150 μl of lyticase extract was run in 5 to 15% acrylamide gradient SDS-PAGE. After electrophoresis, silver staining was performed. Molecular masses (MM) are indicated in kilodaltons. Relevant bands (numbered arrowheads) are indicated. The results of one representative experiment of three performed are shown.

In parallel experiments, immunostaining of blotted lyticase extracts was performed by using peroxidase-conjugated rabbit anti-*C. albicans* mannan antibody. As depicted in Fig. 2, both Y-*Candida* (lane 5) and H-*Candida* (lane 3) extracts displayed mannoproteins of >49.5 kDa. In particular, three major polydisperse bands (I through III), located in the high-molecular-mass range (i.e., >106 kDa), were evident in H-*Candida* as well as in Y-*Candida* extracts (Fig. 2, lanes 3 and 5). As expected (4), Mϕ extracts did not react with antimannan antibodies (lane 1). By comparing the mannoprotein patterns of

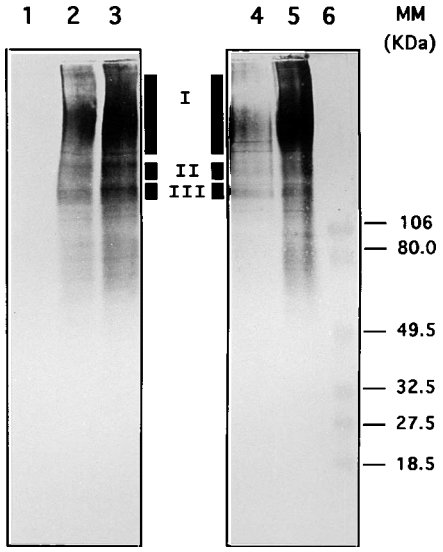

FIG. 2. Characterization of mannoproteins in lyticase extracts from Y-*Candida* and H-*Candida* unexposed to Mϕs or exposed to ANA-1 Mϕs by antimannan antibodies. Extracts were obtained from cultures of ANA-1 Mϕs (lane 1), ANA-1 Mϕs plus H-*Candida* (lane 2), H-*Candida* (lane 3), ANA-1 Mϕs plus Y-*Candida* (lane 4), and Y-*Candida* (lane 5) as described in Materials and Methods. For each experimental group, 150 μl of lyticase extract was run in 5 to 15% acrylamide gradient SDS-PAGE. After electrophoresis, blotting and immunostaining with antimannan antibodies were performed as detailed in Materials and Methods. Lane 6 shows a mixture of prestained molecular weight standards run in parallel. Molecular masses (MM) are indicated in kilodaltons. Relevant bands (I through III) are indicated. The results of one representative experiment of three performed are shown.

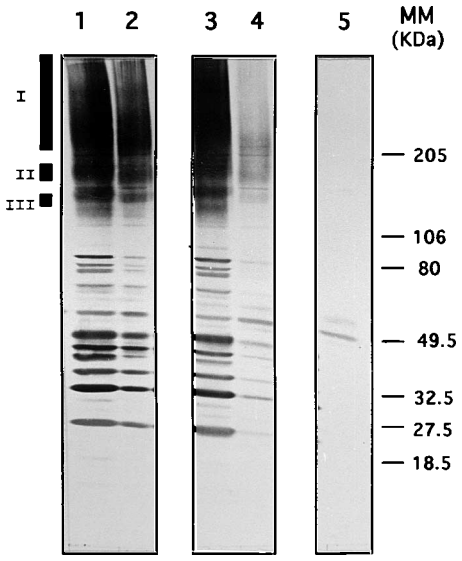

FIG. 3. Western blot analysis of extracts from Y-*Candida* and H-*Candida*, unexposed to Mϕs or exposed to ANA-1 Mϕs, with convalescent-phase sera from i.v.-infected mice. Lyticase extracts were obtained from H-*Candida* (lane 1), Y-*Candida* (lane 2), H-*Candida* plus ANA-1 Mϕs (lane 3), Y-*Candida* plus ANA-1 Mϕs (lane 4), and ANA-1 Mϕs (lane 5), as detailed in Materials and Methods. For each experimental group, 150 μl of lyticase extract was run in 5 to 15% acrylamide gradient SDS-PAGE. After electrophoresis, blotting and immunostaining with convalescent-phase sera of i.v.-infected mice were performed as described in Materials and Methods. Molecular masses (MM) are indicated in kilodaltons. Relevant bands (I to III) are indicated. The results of one representative experiment of three performed are shown.

H-*Candida* in the absence (Fig. 2, lane 3) and in the presence (lane 2) of Mϕs, a slight decrease in the intensity of bands I through III was observed. In contrast, when Y-*Candida* was analyzed, major differences between the mannoprotein patterns of Y-*Candida* incubated alone and in the presence of Mϕs (Fig. 2, lane 5 versus lane 4) were evident. In particular, mannoproteins of <106 kDa disappeared and those in the high-molecular-mass range showed a marked reduction of intensity.

In the next series of experiments, convalescent-phase sera of mice previously i.v. or i.c. infected were used as probes to establish whether antigenically relevant moieties were modulated during in vitro Mϕ-*C. albicans* interaction. Figure 3 shows that immunostaining of blotted extracts by convalescent-phase sera of mice previously infected i.v. allowed the detection of polydisperse high-molecular-mass proteins in both forms of *Candida*, while resolution of individual bands occurred within the 106- to 27-kDa range (lanes 1 and 2). As already shown by silver staining, the investigation with convalescent-phase sera also demonstrated that in the molecular mass range between 106 and 32.5 kDa, Y-*Candida* (Fig. 3, lane 2) and H-*Candida* (Fig. 3, lane 1) lyticase extracts had identical qualitative compositions. Furthermore, Mϕ extracts were unstained (Fig. 3, lane 5) with the exception of two bands that were detectable in all of the experimental groups. When the Y-*Candida* patterns in the absence (Fig. 3, lane 2) or presence (lane 4) of Mϕs were evaluated, major differences were detected, in that a substantial reduction in the number and intensity of bands occurred. In particular, we observed a marked reduction of the high-molecular-mass proteins. Furthermore, when the H-*Candida* patterns in the absence or presence of Mϕs were investigated, we observed only partial changes in the intensity of proteins in the medium- to low-molecular-mass range, while no appreciable changes were found in the high-molecular-mass proteins.

Figure 4 shows the immunostaining of blotted lyticase extracts with convalescent-phase sera of mice previously infected i.c. Such serum identified only three bands in Y-*Candida* extracts (Fig. 4, lane 2), while it reacted with polydisperse high-molecular-mass proteins of >205 kDa (I and II) and with four bands of <49.5 kDa when H-*Candida* extracts were used (lane 1). This is in agreement with the silver-staining cell bands detected in both forms of *Candida*, which were less represented in Y-*Candida* than in H-*Candida*. Lyticase extracts from Mϕs (Fig. 4, lane 5) were unstained. The presence of Mϕs caused the complete disappearance of all bands in Y-*Candida* (Fig. 4, lane 4), while in H-*Candida* (lane 3) we observed a marked decrease in the intensity of high-molecular-mass protein bands.

DISCUSSION

In the present report, we provide evidence that Y-*Candida* and H-*Candida* differ in their susceptibility to Mϕ proteolytic activity, with the former being profoundly affected. Comparative studies aimed at characterizing Y-*Candida* and H-*Candida* cell wall components show that their protein contents differ qualitatively and quantitatively and may have distinct locations within the cell wall (10–12, 23, 24, 36). The expression, chemical characteristics, and biological properties of such proteins appear to be dependent upon multiple environmental factors (i.e., growth conditions) as well as organism-related factors (i.e., growth state, cell morphology, strain, serotype, and phenotypic switching) (9, 10, 13, 20, 25). The enzymatic extraction procedure used in this study allowed the detection of complex protein patterns in both morphologic forms of *C. albicans*, whose profiles appear as diffused background in the high-

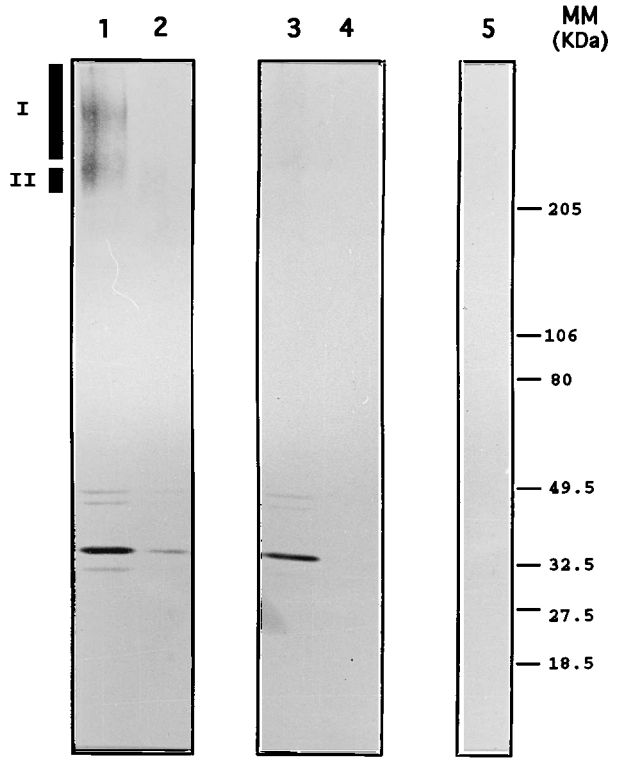

FIG. 4. Western blot analysis of extracts from Y-*Candida* and H-*Candida*, unexposed to Mϕs or exposed to ANA-1 Mϕs, with convalescent-phase sera from i.c.-infected mice. Lyticase extracts were obtained from H-*Candida* (lane 1), Y-*Candida* (lane 2), H-*Candida* plus ANA-1 Mϕs (lane 3), Y-*Candida* plus ANA-1 Mϕs (lane 4), and ANA-1 Mϕs (lane 5), as detailed in Materials and Methods. For each experimental group, 150 μl of lyticase extract was run in 5 to 15% acrylamide gradient SDS-PAGE. After electrophoresis, blotting and immunostaining with convalescent-phase sera of i.c.-infected mice were performed as described in Materials and Methods. Molecular masses (MM) are indicated in kilodaltons. Relevant bands (I and II) are indicated. The results of one representative experiment of three performed are shown.

molecular-mass range while resolving as individual bands at molecular masses below 106 kDa. Unlike silver staining, anti-mannan antibody staining of blots reveals the presence of high-molecular-weight polydisperse material, within which distinct bands are visible both in H-*Candida* and in Y-*Candida*. According to the specificity of the antibody employed (31), such bands must be mannoproteins. Immunoblotting analysis with convalescent-phase sera of *C. albicans*-infected mice further underlines differences between H-*Candida* and Y-*Candida* in such protein components. In particular, convalescent-phase sera of mice previously i.v. infected with *C. albicans* identify high-molecular-weight proteins that, although comparable in number of bands, are more marked in H-*Candida* than in Y-*Candida*, while convalescent-phase sera of mice previously i.c. infected with *C. albicans* react with only two of three high-molecular-weight polydisperse bands in H-*Candida* and with no bands in Y-*Candida*. In the medium- to low-molecular-weight range, analysis of silver-stained profiles shows that Y-*Candida* and H-*Candida* differ in that some proteins are present in only one form of *Candida* and several proteins with identical electrophoretic mobilities have greater intensity in H-*Candida* than in Y-*Candida*. While the overall qualitative protein patterns here described are in agreement with the results obtained by others (10–12, 23, 24, 36), quantitative aspects cannot be analyzed. The differences observed may be ascribed to different total amounts of proteins in H-*Candida* and Y-*Candida* extracts, with sample normalization based on cell number.

Using cloned macrophage cell lines obtained in vitro by retroviral infection of primary cell cultures, we found that a Mϕ accomplishes its anti-*Candida* activity through a cascade of events that are distinct in biochemical demands and susceptibility to cytokines (3, 6, 7). In particular, although Mϕs exert antifungal activity against both fungal forms (3, 35), only H-*Candida* is recognized and possibly processed as an extracellular target (7). In fact, interaction between Mϕs and H-*Candida*, but not Mϕs and Y-*Candida*, results in increased tumor necrosis factor and interleukin 1 transcription and production (6). Here, we show that, upon contact with Mϕs, the protein cell wall components of both forms of *C. albicans* become modified. In particular, the protein profile of Y-*Candida* exposed to Mϕs shows the disappearance of at least three bands and substantial reduction in intensity of all bands, including mannoproteins. In contrast, following exposure to Mϕs, H-*Candida* exhibits only a slight reduction of the high-molecular-mass mannoproteins and the appearance of a faint band of about 106 kDa. In parallel groups, the analysis of lyticase extracts from Y-*Candida* or H-*Candida* exposed to YAC-1, a lymphoma cell line devoid of immunological functions, including antimicrobial properties, shows no changes in the proteinaceous cell wall components of either fungal form (data not shown), implying that the phenomenon described above is not aspecific but rather the consequence of *C. albicans* interaction with an effector cell, such as a Mϕ. The finding that such activity is more pronounced against Y-*Candida* than H-*Candida* is in agreement with the demonstrations (references 3, 5, 7, and 35 and data not shown) that, under the experimental conditions employed (i.e., 3-h coincubation and an effector-to-target cell ratio of 1:1), Mϕs are proficient effector cells against Y-*Candida* (19% phagocytic cells and 35% anti-Y-*Candida* activity, as assessed by CFU assay), while their anti-H-*Candida* activity is barely detectable (no phagocytosis and 7% anti-H-*Candida* activity, as assessed by [^{3}H]glucose uptake assay). Western blot analysis documents that the interaction between Mϕs and Y-*Candida* results in the degradation of a large part of the cell wall proteins. However, a few proteins are preserved. Since they were identified by the convalescent-phase sera, such proteins may have antigenic relevance to the host, since the convalescent-phase mice have produced antibodies against them and/or some of their epitopes. Thus, it is fascinating to hypothesize that the proteins refractory to Mϕ proteolytic action have a role in vivo as immunogens in *Candida* infection and immunity.

In conclusion, our experimental approach provides initial insights into the differential susceptibility of H-*Candida* and Y-*Candida* cell wall proteins to the proteolytic activity of Mϕs. The identification of the major proteins susceptible to Mϕ proteolytic action versus those preserved will offer a novel view of the pathogenetic implications of the dimorphic transition by *C. albicans* and of the immunological role of Mϕs.

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