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Virulence for Mice of a Proteinase-secreting Strain of *Candida albicans* and a Proteinase-deficient Mutant

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A proteinase-deficient mutant of *Candida albicans*, M12, was produced by nitrosoguanidine mutagenesis of a proteinase-producing strain, ATCC 28366. The mutant was phenotypically identical to its parent in nearly all biochemical and morphological characteristics except proteinase production. The mutant was considerably less lethal than the parent when inoculated intravenously into mice and lower counts of *C. albicans* were recovered from the organs of mice infected with the mutant. Both strains were phagocytosed and killed to a similar extent by human and murine polymorphonuclear leukocytes when the yeasts were grown in a medium that did not induce proteinase production. However, in a proteinase-inducing medium, ATCC 28366 was phagocytosed and killed less well than M12. These results indicate that proteinase secretion by *C. albicans* is one factor determining the virulence of the species, but that other virulence factors are also involved in the pathogenesis of systemic candidosis.

INTRODUCTION

Candida albicans is an opportunistic pathogen, initiating infection only in persons whose host defences are in some way impaired (Odds, 1979). The mechanisms by which this fungus then causes disease in such debilitated patients are poorly understood. Although several factors, such as the ability of *C. albicans* to form hyphae and the presence of endotoxin-like substances, have been suggested as contributing to the pathogenicity of the species (Odds, 1979), neither of these features have been firmly proven to be virulence factors.

Many pathogenic bacteria secrete exotoxins and other hydrolytic enzymes that are known to be factors determining their virulence. Several similar enzymes have been described in *C. albicans* although their role as virulence factors remains equivocal. Two cell-surface enzymes have been described – acid phosphomonoesterase (Odds & Hierholzer, 1973) and phospholipase (Pugh & Cawson, 1975; Price & Cawson, 1977). An inducible, secreted acid proteinase was first described in *C. albicans* by Staib (1965), and this has been examined as a possible serodiagnostic antigen for deep-seated candidosis (Macdonald & Odds, 1980) in the belief that this enzyme might be secreted *in vivo* by the fungus in the course of tissue invasion.

Several pieces of evidence support this hypothesis. Patients with systemic candidosis frequently have serum antibodies to *C. albicans* proteinase (Macdonald & Odds, 1980). Proteolysis is seen most frequently as a property of the most pathogenic *Candida* species (Budtz-Jorgensen, 1971), and elaboration of proteinase in tissues of mice infected with *C. albicans* has been demonstrated by indirect fluorescent antibody staining (Macdonald & Odds, 1980). The role of *C. albicans* proteinase as a virulence factor has been examined further in the present study, in which the virulence of a proteinase-producing strain of *C. albicans* has been compared with that of a proteinase-deficient mutant prepared from the first strain.

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Abbreviations: EMEM, Eagle's minimal essential medium; PMN, polymorphonuclear leukocyte; SDA, Sabouraud dextrose agar; SDB, Sabouraud dextrose broth.

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METHODS

Fungus and preparation of strains. Candida albicans ATCC 28366 was used in all experiments. This strain was tested for proteinase production as previously described (Macdonald & Odds, 1980) and found to be a good producer of the enzyme. In some experiments *C. albicans* ATCC 44505, another proteinase secretor, was also used. A proteinase-deficient mutant, M12, was produced from ATCC 28366 by nitrosoguanidine mutagenesis as follows. *Candida albicans* ATCC 28366 was added to Sabouraud dextrose broth (SDB) at a concentration of 10^6 yeast cells ml^{-1} and grown for 4 h at 37 °C. The culture, in the exponential phase of growth, was centrifuged at 2500 *g* for 10 min then resuspended in 0·2 M-potassium acetate buffer, pH 5·5, containing *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Sigma) at a concentration of 500 μg ml^{-1}. After 120 min at 37 °C, the cells were washed with potassium acetate buffer containing ascorbic acid (5 mg ml^{-1}). The treated yeast cells were plated on to Sabouraud dextrose agar (SDA; Oxoid) and incubated at 37 °C for 24 h. Single colonies were inoculated into 4 ml synthetic medium containing BSA (fraction V; Sigma) as a nitrogen source to induce proteinase production (Macdonald & Odds, 1980). The cultures, in Repli Dishes (Sterilin, Teddington, Middlesex, U.K.) were incubated at 26 °C for 5 d; the supernatants were then tested for proteinase activity (Macdonald & Odds, 1980). Any isolates that did not produce detectable enzyme under these conditions were reinoculated into 100 ml batches of the same medium and assayed for growth (measured as turbidity at 550 nm) and for proteinase activity over a period of 144 h.

Characterization of C. albicans strains. In addition to quantitative tests for proteolytic activity, the parent and mutant strains were characterized by means of a number of biochemical and morphological tests. Growth at pH 1·40, production of proteinase on agar media, resistance to 5-fluorocytosine, 1·55 M-NaCl, boric acid and safranine, and utilization of urea, sorbose and citrate were all assayed in plate tests by the system described and modified by Odds & Abbott (1980, 1982). Standard assimilation and fermentation tests were set up in liquid media (Lodder, 1970). 'APIZYM' enzyme test strips (API Laboratory Products, Rayleigh, Essex, U.K.) were inoculated with washed, resuspended cells of *C. albicans* grown at 37 °C for 24 h.

The ability of strains to produce chlamydospores was tested by 'Dalmau technique' inoculation on corn meal agar (Lodder, 1970), and germ tube formation in horse serum was tested as described by Taschdjian *et al.*, 1960. Growth curves of strains in SDB were determined by bioluminescence spectrophotometry (Odds, 1980); initial cell concentrations were 10^6 ml^{-1}. The ability of strains to produce hyphae was compared in modified SDB (Evans *et al.*, 1975) at 40 °C and in Eagle's minimal essential medium (EMEM; Gibco) under 5% CO_2 in air (v/v) at 37 °C. Samples were removed from the media and the proportion of hyphae to total cell numbers estimated by haemocytometer counting as described by Chattaway *et al.* (1973). Cytoplasmic extracts, prepared by mechanical disruption of *C. albicans* (Odds *et al.*, 1975), were compared on SDS-polyacrylamide gels (Laemmli, 1970) stained with silver nitrate (Morrissey, 1981).

Mouse infections. Six groups of 10 Swiss white mice weighing 20–25 g were inoculated via the lateral tail vein with 10^7, 10^6 or 10^5 cells of *C. albicans* ATCC 28366 or M12 grown in SDB for 18 h at 37 °C. The suspensions for inoculation were prepared by an independent laboratory worker and were coded so that it was not known which *C. albicans* strain was given to which groups of mice until completion of the experiment. Death rates of mice in each of the groups were monitored daily. Two further groups of mice were inoculated as above with 10^6 *C. albicans* ATCC 28366 or strain M12. At intervals after inoculation, three mice in each group were sacrificed by cervical dislocation. A group of 15 mice was inoculated with 10^6 *C. albicans* ATCC 44505 and mice were similarly sacrificed at intervals.

Processing of tissues from mice infected with C. albicans. The heart, liver, spleen and both kidneys were removed from sacrificed animals. These organs were weighed then homogenized in 2 ml 0·15 M-saline. Samples of each homogenate (0·1 ml) were plated out on SDA containing chloramphenicol (50 mg l^{-1}). Colonies were counted after 48 h incubation at 37 °C and the specific count of yeasts (mg tissue)$^{-1}$ was calculated. The plates from viable count tests were stored at 4 °C until day 21 post infection, when samples of the colonies isolated were characterized by biochemical tests on agar plates (Odds & Abbott, 1980, 1982). Blood samples (approximately 0·5 ml) were removed from freshly killed mice by cardiac puncture and inoculated into 4 ml SDB. Blood cultures were incubated for 3 d at 37 °C then plated on to SDA plus chloramphenicol. After 2 d at 37 °C, plates were examined for the presence or absence of yeast colonies.

Serological tests. Small samples of serum were obtained from the blood of freshly killed mice and tested for precipitating antibodies to *C. albicans* cytoplasmic extract and purified proteinase antigens by counter-immunoelectrophoresis (Odds *et al.*, 1975).

Phagocytosis and intracellular killing of C. albicans by polymorphonuclear leukocytes (PMNs). Phagocytosis and killing of *C. albicans* ATCC 28366 and strain M12 were measured essentially by the radiometric assay of Bridges *et al.* (1980). EMEM buffered with 20 mM-HEPES (Sigma), pH 7·2, was used in all experiments. The two strains were grown in SDB at 30 °C overnight, and in the synthetic proteinase-inducing medium described by Macdonald & Odds (1980) for 48 h at 26 °C. Yeast cells were washed three times in EMEM before addition to leukocytes.

PMNs were prepared from human blood as described by Bridges *et al.* (1980). Mouse PMNs were obtained by intraperitoneal inoculation of Swiss white mice with 2 ml glycogen solution (50 g l^{-1} in water). Mice were

sacrificed after 4 h and PMNs were washed from the peritoneal cavity with 5 ml EMEM. The PMN suspensions were washed twice with EMEM and centrifuged at 1200 *g* before use. Assays were performed in duplicate as follows: 250 µl PMN suspension (8×10^5 ml^{-1}) was mixed with 250 µl *C. albicans* [4×10^6 ml^{-1} in EMEM, containing 10% (v/v) normal human serum] in 2 ml Eppendorf vials and incubated at 37 °C for 50 min in an end-over-end rotator. Controls containing the same concentrations of *C. albicans* cells mixed with EMEM in place of PMN suspensions were set up at the same time. Controls were processed identically to, and concurrently with, test suspensions. To determine the extent of phagocytosis of *C. albicans*, 200 µl of the assay mixture was mixed with 10 µl EMEM containing 0·2 µCi (7·4 kBq) [^{3}H]uridine (Amersham) in the wells of a Microtiter plate (Sterilin). To determine intracellular killing, 200 µl of the mixture was mixed with 50 µl EMEM containing 0·2 µCi [^{3}H]uridine plus 25 µl sodium deoxycholate (12·5 mg ml^{-1}; Sigma) and 25 µl DNAase (0·2 mg ml^{-1}; Sigma), again in the wells of a Microtiter plate. After 1 h incubation at 37 °C, cell-associated radioactivity was determined by transfer of 150 µl from phagocytosis wells or 200 µl from intracellular killing wells on to glass fibre filters (Whatman; GF/B) on a vacuum filtration apparatus (Millipore). Filters were washed with water then dried overnight at 80 °C. Radioactivity was measured in a Packard scintillation counter with Fisofluor 3 (Fisons) scintillant. The decrease in c.p.m. of *C. albicans*-associated radioactivity in the presence of PMNs was expressed as a percentage of control counts to assess the extent of phagocytosis and killing. In some experiments, purified *C. albicans* proteinase was added to the suspensions of ATCC 28366 and M12 before they were mixed with the PMNs.

RESULTS

Properties of C. albicans ATCC 28366 and its mutant strain, M12

In several attempts to produce a proteinase-negative mutant of *C. albicans* ATCC 28366, no mutants totally incapable of producing and secreting some enzyme were obtained. Strain M12 was selected as the mutant least able to secrete proteinase when it was grown in a medium with albumin as sole nitrogen source. Growth curves of ATCC 28366 and M12 in this medium are shown in Fig. 1. Both strains were able to grow in this medium, although the mutant grew far more slowly than the parent strain. Substantial amounts of proteinase were detectable in the culture filtrates from ATCC 28366 by 48 h, and enzyme levels in the culture filtrates reached a maximum by 96 h. In contrast, in culture filtrates from M12, only very low levels of proteinase activity were detected at any time, and the maximum proteinase activity, at 72 h, was less than one-tenth that of the maximal activity of the parent strain. M12 was therefore described as a proteinase-deficient rather than a proteinase-negative mutant.

To assess other phenotypic characteristics of ATCC 28366 and M12 that might reflect genetic mutations in loci other than those involved in proteinase production, the two strains were

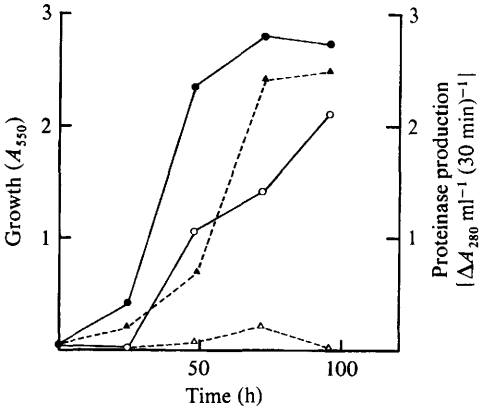

Fig. 1. Growth curves and proteinase production by *C. albicans* ATCC 28366 and M12. ●——●, Growth of 28366; ▲---▲, growth of M12; ○——○, proteinase production by 28366; and △---△, proteinase production by M12. Growth was determined by measurement of A_{550}. Proteinase was assayed as described in the text.

characterized in a wide variety of biochemical and morphological tests. Both strains behaved similarly in the majority of tests. They both gave assimilation and fermentation reactions characteristic of the species *C. albicans*; both produced germ tubes in serum and chlamydospores on corn meal agar. In modified SDB and EMEM both strains produced hyphae at essentially the same rate and to the same extent. Both strains grew at very nearly the same rate in SDB. On 'APIZYM' test strips the same enzyme patterns were produced by both strains, and on SDS–polyacrylamide gels cytoplasmic extracts from both strains gave identical patterns of protein bands with the exception of a single band that was seen at a higher intensity in extracts of M12 than in extracts of ATCC 28366.

In plate biochemical tests, M12 was proteinase-negative and ATCC 28366 was positive, which was to be expected. Three other differences were revealed in the plate tests; ATCC 28366 and M12 differed in their ability to assimilate citrate and in their resistance to safranine, and ATCC 28366 colonies varied in their salt tolerance, whereas all colonies of M12 were not salt tolerant.

Virulence of proteinase-positive and proteinase-deficient strains of C. albicans for mice

The mortality rate in groups of mice inoculated with different doses of *C. albicans* ATCC 28366 and M12 are shown in Fig. 2. The lethality of the parent strain was markedly higher than that of the proteinase-deficient mutant. With inocula of 10^7 yeast cells, all mice

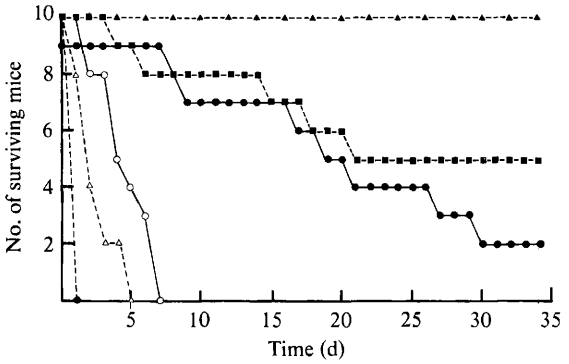

Fig. 2. Mortality rates of mice inoculated with differing doses of *C. albicans* ATCC 28366 or M12. ●---●, 28366 at 10^7 yeast cells per mouse; △---△, M12 at 10^7 yeast cells per mouse; ○——○, 28366 at 10^6 yeast cells per mouse; ■---■, M12 at 10^6 yeast cells per mouse; ●——●, 28366 at 10^5 yeast cells per mouse; and ▲---▲, M12 at 10^5 yeast cells per mouse.

succumbed to challenge with ATCC 28366 within 24 h, whereas several survived up to 5 d with this dose of M12. At 10^6 yeast cells per mouse, ATCC 28366 caused 100% mortality by 7 d, whereas the same dose of M12 had killed only half the affected mice by day 34 post infection. At 10^5 yeast cells per mouse there were no fatalities in the group inoculated with M12, whereas seven of nine mice given the same challenge of ATCC 28366 had died by day 30 post infection. From these data the LD_{50} values for ATCC 28366 and M12 were estimated as 10^5 and approximately 10^6, respectively – a virulence difference of at least 10-fold.

Pathological effects of C. albicans ATCC 28366 and M12 in mice

In a group of 20 mice inoculated with 10^6 cells of ATCC 28366, three mice were sacrificed on days 1, 3, 7 and 14 post inoculation. Of the eight mice that were not purposely killed for investigation all but one succumbed to the infection by day 7 post inoculation, and three of the four mice that survived to day 14 were moribund at this time. With the exception of the three mice killed on day 1 post infection, all mice investigated had white lesions on their kidneys, characteristic of *C. albicans* infection (Table 1). Blood cultures were positive for all three mice investigated on day 1 post infection, but no positive blood cultures were obtained from mice afterwards. At no time were antibodies to *C. albicans* cytoplasmic extract or purified proteinase

Table 1. *Pathological effects of 10^6 cells of C. albicans ATCC 28366 and M12 in mice*

		Day 1		Day 3		Day 7		Day 14		Day 21
		ATCC 28366	M12	ATCC 28366	M12	ATCC 28366	M12	ATCC 28366	M12	M12
No. of mice sacrificed		3	3	3	3	3	3	3	3	2
No. with lesions on kidney		0	0	3	0	3	0	3	1	2
No. giving positive blood culture		3	0	0	1	0	0	0	0	0
Specific count of *Candida* in tissues (average c.f.u. mg^{-1})	Left kidney	366	28	232	1	136	36	310	127	145
	Right kidney	404	22	163	1	155	44	256	188	137
	Heart	2	1	0	7	0	0	100	16	42
	Liver	19	11	4	0	1	0	16	5	2
	Spleen	9	0	9	0	1	0	8	5	2

Table 2. *Phagocytosis and killing of ATCC 28366 and M12 by PMNs*

Growth medium	No. of replicates	Source of PMNs	Percentage phagocytosis* ATCC 28366	Percentage phagocytosis* M12	Percentage killing* ATCC 28366	Percentage killing* M12
SDB	6	Human	78 ± 6	76 ± 14	49 ± 6	56 ± 16
SDB	2	Mouse	61 ± 10	60 ± 12	53 ± 7	43 ± 3
Proteinase-inducing SDB + purified proteinase (10 μg)†	6	Human	55 ± 10	74 ± 5	35 ± 7	50 ± 5
	4	Human	73 ± 20	66 ± 13	63 ± 5	62 ± 7
SDB + purified proteinase (100 μg)†	4	Human	70 ± 17	66 ± 12	53 ± 4	53 ± 9

* Data are mean values ± S.D. for replicate experiments. Control c.p.m. were between 5000 and 10000 in all experiments.

† Yeast cells were grown in SDB and purified proteinase was added to washed yeast cells before use in phagocytosis and killing experiments.

detected in serum samples from the mice. Colony counts of *C. albicans* were highest in the kidneys, but some viable *C. albicans* cells were found in the other organs investigated; in the heart, tissue counts were highest by day 14.

The biochemical characteristics of yeasts recovered from the mice infected with ATCC 28366 were those of that strain; all isolates recovered produced proteinase in plate tests.

In the group of 17 mice inoculated with 10^6 cells of M12 the pattern of infection was quite different (Table 1). Mice survived up to 21 d post inoculation, and only three mice died naturally. There were no visible white lesions on the kidneys of any mice examined up to 7 d post infection; only one of three mice examined on day 14 had visible lesions, and both mice examined on day 21 had lesions on the kidneys. Only one positive blood culture was obtained at any time, from a mouse killed on day 3 post infection. No anti-*C. albicans* precipitins were found in serum samples at any time. Colony counts of *C. albicans* in all organs were lower up to day 7 post infection than in mice infected with ATCC 28366. On days 14 and 21, higher numbers of *C. albicans* were found in the kidneys and the heart than in animals examined earlier.

Most of the *C. albicans* isolates recovered from tissues of mice infected with M12 proved to have the same biochemical phenotype as the inoculum strain. However, on day 3 post infection some of the colonies recovered from organs of infected mice showed weak proteolytic activity in plate tests.

Among 15 mice inoculated intravenously with 10^6 cells of *C. albicans* ATCC 44505, another proteinase-producing strain, the mortality rates appeared to be comparable to those in mice infected with ATCC 28366. Ten of the animals were sacrificed at days 1, 3 and 7 post infection, and nearly all of these were moribund at the time they were killed; two further animals died

naturally within this same time period, and three survived until sacrifice at day 14 post infection. Lesions were visible on the kidneys of the mice from day 3 post infection onwards, and high numbers of *C. albicans* were recovered from the kidneys of the animals at all times. Blood cultures were positive only in the three mice examined 1 d after inoculation.

Phagocytosis and killing of C. albicans ATCC 28366 and M12 by PMNs

To examine one aspect of the interaction between host defence mechanisms and *C. albicans*, the abilities of human and murine PMNs to phagocytose and kill the proteinase-positive and proteinase-deficient strains of *C. albicans* were tested. The results are shown in Table 2. When ATCC 28366 and M12 were grown in SDB they were phagocytosed and killed by both types of PMNs to the same extent, although the human PMNs appeared to be slightly more active than the murine PMNs overall. When the *C. albicans* strains were grown in proteinase-inducing medium, the yeast cells of M12 were phagocytosed and killed equally well by human PMNs as cells grown in SDB; however, the rates of phagocytosis and killing of ATCC 28366 were markedly reduced. The reductions in phagocytosis and killing were highly significant ($P < 0{\cdot}01$) when the data were analysed by the Mann–Whitney U test.

Addition of purified *C. albicans* proteinase at two concentrations to ATCC 28366 and M12 cells grown in SDB caused no statistically significant change in their susceptibility to phagocytosis and intracellular killing by human PMNs (Table 2).

DISCUSSION

This study has shown more directly than any previous publication that the secreted proteinase of *C. albicans* is a factor in the virulence of the species. Although no mutants totally devoid of proteinase activity could be produced from the parent proteinase-positive strain, we were able to obtain a mutant that synthesized only small amounts of proteinase *in vitro*. The difficulties involved in preparation of stable mutants of *C. albicans* of any type have been discussed previously, and it has been suggested that they stem from the fact that the species may be at least diploid (Olaiya & Sogin, 1979; Whelan *et al.*, 1980).

The parent *C. albicans* strain, ATCC 28366, was considerably more lethal for mice than the mutant, M12. An intravenous inoculum of 10^6 *C. albicans* per mouse should normally lead to 100% mortality within 7 d (Sandula *et al.*, 1963). Such a mortality rate was seen for the two proteinase-positive strains of *C. albicans* tested in the present study. By contrast, 50% of mice inoculated with 10^6 cells of proteinase-deficient M12 survived for at least 34 d. Counts of *C. albicans* in the kidneys of mice infected with proteinase-positive strains were high from the outset of the systemic infection, and the highest counts correlated with the appearance of visible lesions on the kidneys. In mice inoculated with the proteinase-deficient mutant, high kidney counts of *C. albicans* again correlated with the appearance of visible kidney lesions, but these only appeared by 14 d post infection. Blood cultures positive for *C. albicans* were obtained only during the early days post infection, but their prevalence was higher in animals infected with proteinase-positive strains than with M12.

It was notable that occasional colonies of M12 reisolated from tissues of infected mice appeared to have reverted to proteinase production. On one occasion a laboratory subculture of M12 reverted to proteinase production *in vitro* (unpublished results), which suggests either that the mutant genome is able to revert to a proteinase-positive genotype, or that subcultures of M12 contain subpopulations of proteinase-positive cells. In either case, it may be postulated that the development of pathological signs of candidosis late after their intravenous inoculation with M12 was partly related to reversion of some cells to proteolysis.

Thorough biochemical and morphological characterization of ATCC 28366 and M12 showed that there were some phenotypic differences between the strains apart from their ability to produce proteinase. It is therefore possible that the differences in strain virulence noted in these experiments might be related to factors other than proteinase, e.g. inability of M12 to assimilate citrate or to resist safranine. However, strain ATCC 44505 was citrate-negative and proteinase-positive, and its pattern of infection in mice was essentially the same as that of ATCC 28366, so

that it is unlikely that the property of citrate assimilation correlates with virulence in *C. albicans*.

The nonappearance of antibodies to any of the *C. albicans* antigens tested was surprising, although it is possible that, in the mice inoculated with the proteinase-positive strain, the infection was too rapidly fatal for detectable production of antibodies, or so overwhelming that there was an antigen excess in the bloodstreams of the mice.

The evidence of this study strongly suggests that secretion of proteinase is a factor in the pathogenesis of systemic candidosis, at least in mice, *in vivo*. The way in which the proteinase exerts its pathological effects *in vivo* remains unknown. It may be speculated that the enzyme could cause tissue damage and thus assist the fungus in tissue invasion. Alternatively, the enzyme might be involved in defending the fungus against host immune responses. This latter function is suggested by the experiments in which the extent to which ATCC 28366 and M12 were ingested and killed intracellularly by PMNs was measured. Both strains, when grown in SDB, in which proteinase is not induced (Macdonald & Odds, 1980), were phagocytosed and killed to a similar extent. However, when the cells were grown for 48 h in proteinase-inducing medium, at which time ATCC 28366 was producing more than 10 times as much proteinase as M12 (Fig. 1), there was a marked reduction in the extent of phagocytosis and killing of ATCC 28366, but M12 was ingested and killed to the same extent as for SDB-grown yeasts (Table 2). Addition of purified proteinase to suspensions of SDB-grown ATCC 28366 and M12 made no difference to the extent to which the two strains were ingested and killed by PMNs which indicates that the proteinase is an effective defence against phagocytosis and intracellular killing only when it is being actively produced by *C. albicans*.

One important factor that has been widely reported as significant for the virulence of *C. albicans* is the ability of this species to produce hyphae. Yeast-form *C. albicans* can grow out of macrophages and destroy them *in vitro* by producing hyphae (Louria & Brayton, 1964; Stanley & Hurley, 1969). Richardson & Smith (1981) found that strains of *C. albicans* attenuated in their virulence for mice produced hyphal germ tubes less readily than virulent strains. In the present study ATCC 28366 and M12 were equally able to produce germ tubes and hyphae *in vitro*, so it is unlikely that any morphological difference accounted for their different lethalities for mice. It is notable that in the report of Richardson & Smith (1981) the strains with lower virulence and lower germ tube-producing capability nevertheless retained some capacity for infecting mice and some capacity for producing germ tubes. In a similar way, in the present study, the proteinase-deficient strain retained some ability to kill mice and some ability to produce proteinase. It therefore seems most likely that the native virulence of *C. albicans* must be attributed to several factors, of which production of hyphae and production of proteinase are two. It seems equally likely that, since *C. albicans* appears to be rarely, if ever, capable of causing systemic infection in patients with intact immune defences, no single virulence factor can be expected to be as potent or as easily demonstrable *in vivo* as, for example, bacterial toxins.

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