INFECTION AND IMMUNITY, Mar. 1992, p. 832–837
0019-9567/92/030832-06$02.00/0

Vol. 60, No. 3

Early Differential Molecular Response of a Macrophage Cell Line to Yeast and Hyphal Forms of *Candida albicans*

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Received 12 August 1991/Accepted 13 December 1991

The dimorphic transition of *Candida albicans* from the yeast (Y-*Candida*) to the hyphal (H-*Candida*) form is a complex event; the relevance of this transition in fungal pathogenicity is still poorly understood. By using a cloned macrophage cell line (ANA-1), we questioned whether the interaction between macrophages and Y-*Candida* or H-*Candida* could affect specific cell functions, i.e., tumor necrosis factor and lysozyme production. We found that ANA-1 macrophages selectively responded to H-*Candida* with increased tumor necrosis factor and downregulated lysozyme, as assessed by measurement of relative mRNA levels and secreted biological activities. The H-*Candida*-mediated effects were (i) dependent upon the ratio between ANA-1 macrophages and H-*Candida*, (ii) detectable after 1 h of coincubation, and (iii) accomplished without fungal ingestion. Conversely, Y-*Candida*, which was found inside the ANA-1 macrophages, did not affect tumor necrosis factor and lysozyme production, nor did it prevent the macrophage response to other stimuli. Overall, these results indicate that a macrophage can distinguish between Y-*Candida* and H-*Candida* and that only the latter is able to modulate specific functions. H-*Candida* is recognized and probably processed as an extracellular target. The possible implication of macrophages as autocrine and paracrine regulatory cells during *Candida* infections is discussed.

The dimorphic fungus *Candida albicans* is an interesting microorganism because of the enigmas that surround its relationship with the host, particularly the pathogenicity, dimorphic transition, and mechanisms of host response to colonization and/or invasion (19; for reviews, see references 15 and 37). Although usually harbored as a harmless commensal in the gastrointestinal tract of healthy humans, *C. albicans* causes morbidity and, in some cases, mortality in immunocompromised hosts (37). In this respect, candidosis is becoming a life-threatening disease in patients undergoing aggressive chemotherapy and in immunocompromised patients, such as those with human T-cell leukemia virus-associated immunodeficiencies (16, 26, 30, 36). Morphogenetic studies document the dimorphic properties of *C. albicans*, which undergoes a transition from the yeast form (Y-*Candida*) to the hyphal form (H-*Candida*) (15, 20, 37). This transition is accompanied by remarkable changes in the expression of surface structures. Antigenic determinants exclusively or predominantly expressed on either Y-*Candida* or H-*Candida* have been generally characterized as high-molecular-weight mannoproteins (12, 14, 40, 42). However, the significance of these antigenic variations in *Candida* pathogenicity is not clear.

During the early stages of infection, the phagocytic clearance of *C. albicans* enables the host to remove and destroy most of the Y-*Candida*. However, the *Candida* cells that escape the phagocytosis process rapidly convert into H-*Candida* and invade the organism, where they produce granulomas and cause tissue damage (37). In this context, any potential ability of the host immunoeffectors to discriminate between the two forms of *Candida* cells, thus exhibiting different biological responses, is an important issue that remains to be elucidated. The initial information indicated that inflammatory cells may distinguish between yeast cells and hyphae. In particular, neutrophils selectively respond to the latter with a delayed rise in cytosolic inositol 1,4,5-trisphosphate and subsequent O_2^- release (32, 33, 35). In studies of the interaction between *Candida* cells and phagocytes we showed that murine effector cells of distinct anatomical compartments exhibit different patterns of anti-*Candida* activities (2, 3, 5, 46), which, in turn, can be modulated by appropriate in vitro and/or in vivo treatments (6, 44, 45, 47). Furthermore, using cloned macrophage cell lines obtained in vitro by recombinant retroviral infection of primary bone marrow or microglial cultures (7, 9, 19), we found that a macrophage accomplishes its anti-*Candida* activity through a cascade of events that are distinct in biochemical demands and susceptibility to certain cytokines (8, 10). Since our studies have mainly focused on Y-*Candida*, it became important to assess the interaction of macrophages with H-*Candida*.

We show here that ANA-1 macrophages respond to H-*Candida* but not to the Y-*Candida*, with increased tumor necrosis factor (TNF) and downregulated lysozyme (LZM) transcription and production. These findings provide the first evidence that macrophages can discriminate between the two forms of *C. albicans*.

MATERIALS AND METHODS

Cell lines. The ANA-1 murine macrophages, derived by immortalization of bone marrow cells from C57BL/6 mice with a recombinant retrovirus carrying the v-*raf* and v-*myc* oncogenes (9, 19), 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). L-929 fibroblast cells (CCL 1; American Type Culture Collection, Rockville,

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Md.) were maintained in complete medium. All reagents were obtained from Flow Laboratories (Rockville, Md.).

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 (34). Under these conditions, the organism grew as an essentially pure Y-*Candida* population.

To obtain H-*Candida*, a pure Y-*Candida* population was harvested from Winge medium, washed twice in saline, resuspended in complete medium, dispensed in 60-mm tissue culture plates (5×10^5 cells per ml, 5 ml 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 determined by examination with an inverted microscope and as extensively described in a recent report (41).

Experimental protocol. ANA-1 macrophages and H-*Candida* or Y-*Candida* were coincubated in 60-mm tissue culture plates at 37°C in 5% CO_2. All experiments were performed in complete medium. The incubation times and the macrophage/*Candida* cell ratios, hereafter referred to as E/T ratios, varied from experiment to experiment as detailed in the tables and figure legends. The cultures were then employed in biological assays or Northern RNA blot analysis.

LZM assay. LZM activity was determined by the modified lysoplate method of Osserman and Lawlor (38). The LZM content of test samples was extrapolated from a standard curve of purified egg white LZM, established under identical conditions, and expressed as units per milliliter per 10^6 cells.

TNF assay. The quantitation of TNF activity was performed by a bioassay with L-929 cells, as described previously (39). Briefly, L-929 cells were seeded into 96-well flat-bottom plates (4×10^4 cells per well) and incubated for 24 h at 37°C. The spent medium was then removed and replaced with a test sample or a standard TNF preparation containing actinomycin D (3 μg/ml). After 20 h of incubation, the plates were stained with 0.5% crystal violet in 20% methanol for 15 min and washed in tap water. After the plates were dried, the A_{450} was determined by using a Titertek Multiskan platereader (Flow). All determinations of TNF activity in test samples were compared with those of commercially available preparations of TNF with known titers; the results are expressed as units per milliliter.

RNA extraction and Northern blot analysis. Total cellular RNA was isolated from stimulated and unstimulated ANA-1 cells solubilized with guanidine isothiocyanate as previously described (17). Samples of 10 μg of total RNA were electrophoresed in denaturing conditions, blotted onto nylon membranes (Amersham International, Amersham, United Kingdom), cross-linked by UV irradiation, and heated for 1 h at 60°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.5% sodium dodecyl sulfate. Filters were prehybridized for 6 h at 37°C in prehybridization buffer containing formamide and denatured salmon sperm DNA (100 μg/ml); then 10 ng of the specific ^{32}P-labeled probe was added for 18 h in hybridization buffer containing dextran sulfate. Filters were washed four times at room temperature for 5 min and four times at 60°C for 30 min in 1× SSC–0.5% sodium dodecyl sulfate and then autoradiographed with Kodak X-AR5 films (Eastman Kodak Co., Rochester, N.Y.) with intensifying screens at −80°C. Probes were labeled by nick translation with a commercial kit (Amersham) as suggested by the manufacturer. The specific activity was always higher than 10^8 cpm/μg. For LZM mRNA detection, the 0.642-kb *Hin*dIII-*Eco*RI fragment (from the pGBR2.4 plasmid) was used (18). For TNF detection, the 1.2-kb *Pst*I-*Eco*RI fragment (cloned in from the pUC9 plasmid) was used (13).

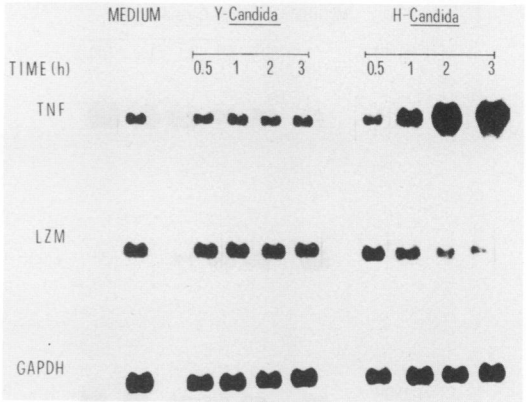

FIG. 1. Kinetics of TNF and LZM mRNA levels in ANA-1 macrophages exposed to H-*Candida* or Y-*Candida*. ANA-1 macrophages (10^6 cells per ml) were incubated alone (medium) or with H-*Candida* or Y-*Candida* (E/T ratio, 1:1) for the indicated times and processed for Northern blot analysis as described in Materials and Methods. Blotted RNA (10 μg per lane) was hybridized with ^{32}P-labeled probes for TNF, LZM, and GAPDH.

RESULTS

Experiments were performed to assess whether ANA-1 macrophages could differentially modulate TNF or LZM mRNA expression in response to Y-*Candida* or H-*Candida*. Cytokine mRNA levels were monitored in cells exposed for 0.5, 1, 2, and 3 h to Y-*Candida* or H-*Candida*. ANA-1 cells constitutively expressed detectable levels of both TNF and LZM mRNA (Fig. 1). Exposure of cells to Y-*Candida* did not significantly affect the levels of either LZM or TNF mRNA at any of the time points tested. In contrast, ANA-1 cells responded to H-*Candida* with a marked increase in TNF and downregulation of LZM mRNA levels; the effects were time dependent. No differences in GAPDH mRNA expression were observed when the blots were rehybridized with a GAPDH-specific probe (Fig. 1). These data indicate that macrophages could differentially modulate gene expression in response to Y-*Candida* or H-*Candida* and that only the latter is biologically active.

To determine the dose response of macrophage-gene modulation by H-*Candida*, ANA-1 cells were exposed to H-*Candida* for 3 h at E/T ratios of 10:1, 5:1, 1:1, 1:5, and 1:10 and then assessed for TNF and LZM mRNA levels. H-*Candida* enhanced TNF levels and reduced LZM mRNA levels in a dose-dependent manner (Fig. 2). Although the E/T ratio of 10:1 was ineffective, the E/T ratio of 5:1 produced partial effects and plateau levels were reached when the effector cell number was equal to or lower than the number of *Candida* cells. Y-*Candida* failed to produce any effect at any of the E/T ratios employed (data not shown).

To establish whether the changes in mRNA levels were associated with parallel variations of secreted biological activities, supernatants of ANA-1 cells, incubated for 3 h with H-*Candida* at various E/T ratios, were tested for TNF or LZM activity. A major increase in TNF levels and a decrease in LZM activities were detected in the supernatants (Table 1); the effects were dependent upon the E/T ratios employed. Under the same conditions, Y-*Candida* did not affect the secretion of either LZM or TNF. These results

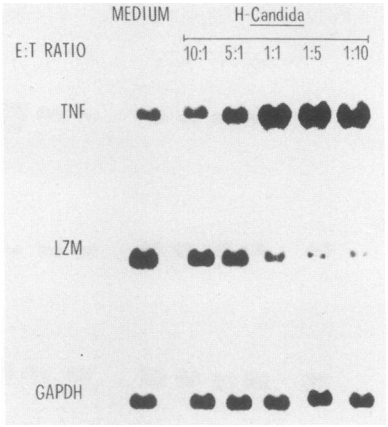

FIG. 2. TNF and LZM mRNA levels in ANA-1 macrophages exposed to H-*Candida* at various E/T ratios. ANA-1 macrophages (10^6 cells per ml) were incubated for 3 h with H-*Candida* at the indicated E/T ratios and then processed for Northern blot analysis as described in Materials and Methods. Blotted RNA (10 μg per lane) was hybridized with ^{32}P-labeled probes for the TNF, LZM and GAPDH.

demonstrated that the pattern of secreted biological activities paralleled the changes in mRNA levels. To assess whether Y-*Candida*, which did not affect gene expression, could interact with ANA-1 macrophages, its susceptibility to phagocytosis was measured. ANA-1 cells were incubated with Y-*Candida* for 1 h (E/T ratio, 1:10), stained with Giemsa, and scored for the presence of phagocytic cells. Y-*Candida* cells were ingested (Fig. 3A), whereas H-*Candida* cells were totally refractory to phagocytosis by ANA-1 macrophages (Fig. 3B). Similar results were obtained at different E/T ratios and after longer times of coincubation (data not shown). These results demonstrated that the macrophage-activating properties of *C. albicans* do not correlate with its susceptibility to phagocytosis.

The failure of Y-*Candida* to modulate gene expression in macrophages could be due to the lack of stimulatory moieties or to a concomitant expression of inhibitory signals. To address this issue, we tested whether ANA-1 macrophages exposed to Y-*Candida* were still capable of responding to a

TABLE 1. TNF and LZM activities in supernatants from ANA-1 macrophages exposed to Y-*Candida* or H-*Candida*[a]

Treatment	E/T ratio	Activity (U/ml) of:	
		TNF	LZM
None		4 ± 2	11.9 ± 2.3
Y-*Candida*	1:20	4 ± 4	10.7 ± 3.2
	1:10	8 ± 4	12.1 ± 2.2
	1:1	2 ± 4	11.5 ± 1.5
	10:1	4 ± 2	11.0 ± 2.1
H-*Candida*	1:20	110 ± 20[b]	3.5 ± 1.3[b]
	1:10	90 ± 20[b]	3.8 ± 0.8[b]
	1:1	100 ± 15[b]	6.5 ± 2.4
	10:1	2 ± 4	10.6 ± 2.8

[a] ANA-1 macrophages were incubated at 10^6 cells per ml with Y-*Candida* or H-*Candida* at the indicated E/T ratios for 3 h at 37°C in 5% CO_2. Cell supernatants were then collected and tested for TNF and LZM activities as described in Materials and Methods. The values are the means of five experiments ± standard deviations.

[b] $P < 0.01$ (*Candida*-treated versus untreated cells).

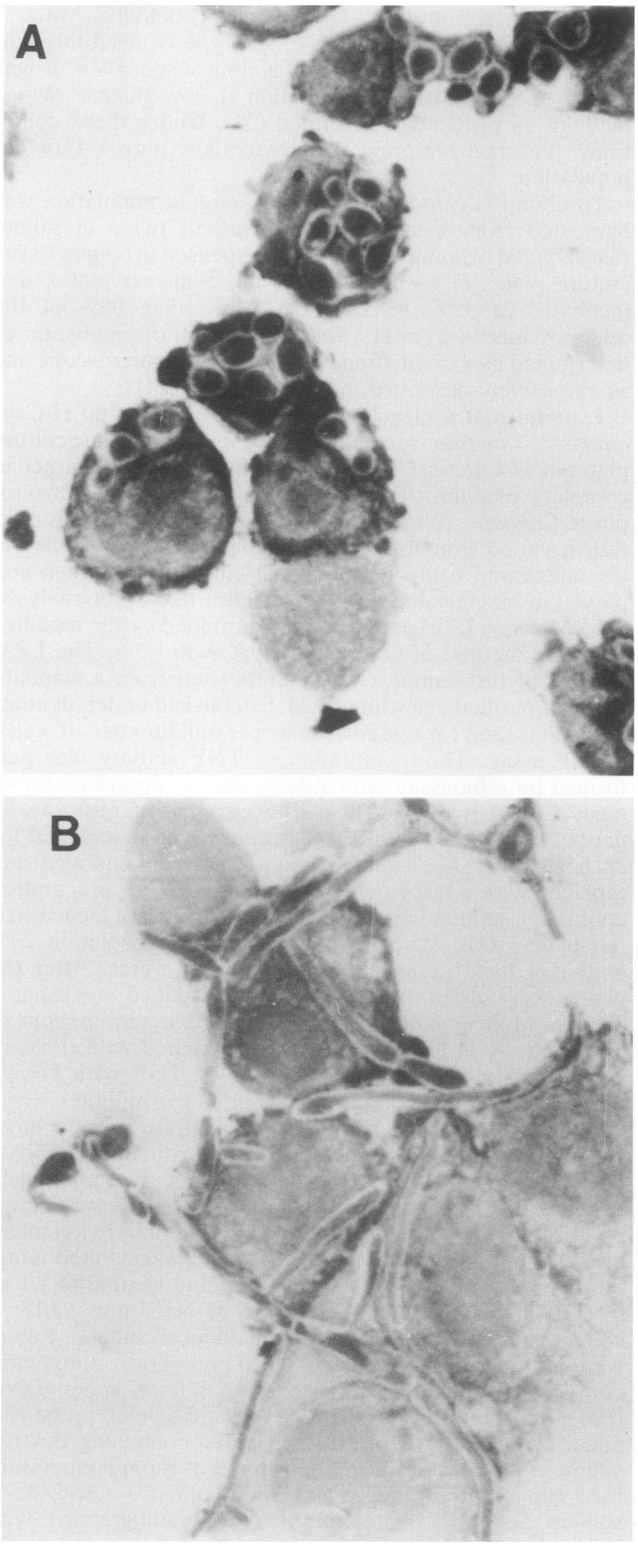

FIG. 3. Photomicrographs of ANA-1 macrophages exposed to Y-*Candida* (A) or H-*Candida* (B). ANA-1 macrophages (10^6 cells per ml) were incubated for 1 h with Y-*Candida* or H-*Candida* at an E/T ratio of 1:10. Cytospin preparations were made, stained with Giemsa, and photographed at 1,000-fold magnification by using a Zeiss microscope.

TABLE 2. TNF and LZM production by ANA-1 macrophages preexposed to Y-*Candida* and then stimulated with H-*Candida* or LPS[a]

Cells	Treatment	TNF activity (U/ml)
Control ANA-1 cells	None	2 ± 2
	H-*Candida*	60 ± 20
	LPS	130 ± 25
ANA-1 cells preexposed to Y-*Candida*	None	4 ± 4
	H-*Candida*	75 ± 30
	LPS	160 ± 25

[a] ANA-1 macrophages (10^6 cells per ml) were or were not preexposed to Y-*Candida* (E/T ratio, 1:10) for 30 min at 37°C. Then, H-*Candida* (10^7 cells per ml) or LPS (1 μg/ml) was added, and the cultures were incubated for an additional 3 h. Cell supernatants were collected and tested for TNF activity as detailed in Materials and Methods. The values are the means of three separate experiments ± standard deviations.

subsequent stimulation by H-*Candida* or lipopolysaccharide (LPS). Control cells and cells preexposed to Y-*Candida* for 30 min were incubated with H-*Candida* or LPS for 3 h and then assessed for TNF production. We found that H-*Candida* enhanced TNF levels in control macrophages as well as in macrophages preexposed to Y-*Candida* (Table 2). Similarly, LPS was active in both cell populations (Table 2).

Overall, our findings indicate that a macrophage recognizes H-*Candida* but not Y-*Candida* as a stimulating signal; however, Y-*Candida* per se is devoid of inhibitory properties, since it does not prevent the macrophage response to other stimuli.

DISCUSSION

The main purpose of this study was to investigate whether a macrophage could discriminate between the yeast and hyphal forms of the dimorphic fungus *C. albicans*. We show that ANA-1 macrophages selectively respond to H-*Candida* with enhanced TNF levels and downregulated LZM levels, as assessed by evaluation of relative mRNA levels and biological activities.

Firm evidence exists that natural defense mechanisms are crucial in reducing the pathogenic burden of *C. albicans* in both normal and immunodepressed hosts (15, 37). Among the cells with potential candidacidal activity, polymorphonuclear leukocytes are undoubtedly the best known and most studied (1, 21, 23, 26, 31). Macrophages also exert anti-*Candida* activity; however, their contribution to host defenses is less well defined. Using in vivo models, we previously showed that tissue macrophages may play an important role in protecting the host against *C. albicans* infections (5, 6, 11, 43–45). Furthermore, in vitro data demonstrate that macrophages of different districts constitutively exhibit anti-*Candida* activity (2, 3, 43, 47). Such activity can be modulated upon appropriate treatment (8, 10, 47) and is retained after in vivo transfer into syngeneic hosts (11). Although most of the studies performed have dealt with Y-*Candida*, initial evidence indicates that macrophages (41) and polymorphonuclear leukocytes and other natural effector cells (4, 22) can affect the hyphal form of the fungus.

In the present report, we demonstrate that the interaction between ANA-1 macrophages and H-*Candida* results in a consistent augmentation of TNF mRNA levels. The effect is time and dose dependent. Concomitantly, downregulation of LZM mRNA levels occurs.

The pattern of results observed at the mRNA level is superimposable on that obtained by assessment of LZM and TNF secretory products. These data imply that, after interaction with H-*Candida*, macrophages modify specialized cell functions and, consequently, their potential immunomodulatory role as secretory cells. In vivo evidence suggests an important role for macrophages as autocrine and paracrine regulatory cells. Kindler et al. (29) showed that macrophages produce TNF during *Mycobacterium bovis* BCG infection, which favors local macrophage accumulation and differentiation and leads to bacterial elimination.

When ANA-1 macrophages are exposed to Y-*Candida*, no significant modulations in TNF and LZM mRNA levels or in cytokine production have ever been detected. Two major hypotheses can be formulated to explain these results. First, Y-*Candida* possesses macrophage-stimulating properties that are masked by concomitant inhibitory signals. Second, Y-*Candida* per se lacks macrophage-stimulating properties. The experiments showing that ANA-1 macrophages preexposed to Y-*Candida* respond as well as control ANA-1 cells to stimuli such as H-*Candida* or LPS strongly argue against the first hypothesis. Therefore, our data provide the first evidence that a macrophage can discriminate between Y-*Candida* and H-*Candida*; only H-*Candida* behaves as a macrophage-stimulating signal. Although qualitative and quantitative changes in surface determinant expression have been described during *Candida* morphogenesis (12, 14, 15, 37), their immunological or pathogenic role is still a matter of controversy. Evidence indicates that cell wall mannoproteins and cell surface hydrophobicity are involved in the adhesion of the microorganism to host cells (14, 15, 37). We are currently investigating whether H-*Candida*-derived cell wall and/or secretory products may be identified as mediators of the phenomenon described in this report.

Other authors have shown that murine splenic macrophages (46) and human peripheral blood monocytes (24) produce TNF upon in vitro exposure to Y-*Candida*. It is conceivable that in these experimental models there is cooperation among different cell types that is responsible for TNF production and that this cooperation is absent in a single cell population, such as that used in our experimental system. Alternatively, unlike tissue macrophages, ANA-1 macrophages may have additional properties that are responsible for the TNF induction. Extensive studies involving macrophages from different anatomical compartments and/or at different stages of activation will definitively clarify this issue. To date, experiments performed with macrophage cell lines obtained from other anatomical regions by the v-*raf* and v-*myc* oncogenes (7, 9) have shown results similar to those described herein for ANA-1 macrophages (data not shown).

Although Y-*Candida* is unable to modulate TNF and LZM production, it interacts with ANA-1 macrophages to the extent of being ingested. Most of the microorganisms are internalized after 30 min of coculture. On the contrary, H-*Candida* is not susceptible to phagocytosis; only H-*Candida* is detectable outside the effector cell, regardless of the length of time in coculture (data not shown). These results indicate that ANA-1 macrophages are comparable to polymorphonuclear leukocytes (22) in their ability to engulf yeast cells but not hyphae. As previously suggested, the most likely explanation is that hyphae are too large to be internalized. Alternatively, structures selectively expressed in H-*Candida* have phagocytosis-inhibiting properties. In any case, hyphae are processed as extracellular targets. Gene products involved in phagocytosis and/or phagosome-related

systems, such as LZM, are likely not needed and thus become downregulated in macrophages exposed to H-*Candida*. Meanwhile, an alternative macrophage strategy occurs, since ANA-1 cells augment TNF mRNA levels in response to H-*Candida*. This strategy closely resemble that employed against tumor targets. It has been recently shown that monocytes and/or macrophages exhibit enhanced TNF production when exposed to tumor cells (27, 28).

To our knowledge, there is no evidence for toxic effects of TNF on hyphae, nor have changes been observed in hyphal growth or hyphal susceptibility to macrophage inhibitory effects (data not shown). However, TNF is known to play an important role as an activating factor during infections (29, 44). Macrophages and other immune system elements respond to TNF with potentiation of a variety of functions, including anti-*Candida* activity (8, 23, 25). We have previously shown that, by infecting a mouse with a low-virulence *Candida* strain, it is possible to establish protection against a subsequent challenge with a lethal variant (44). This phenomenon is associated with and probably mediated by high levels of cytokines, including TNF, in serum. Our in vitro data demonstrate that ANA-1 macrophages are early TNF producers in response to *Candida* infection, provided that the fungus has undergone dimorphic transition to the hyphal form. The demonstration that tissue macrophages may share this peculiarity with ANA-1 macrophages will provide straightforward proof that in vivo the encounter of a macrophage with *C. albicans* triggers an early differential response, depending upon the morphogenetic status of the fungus.

ACKNOWLEDGMENTS

We are grateful to L. Varesio and A. Cassone for helpful suggestions and critical reading of the manuscript. Thanks are also due to L. P. Chung and S. Gordon for a generous supply of the LZM probe and to L. Varesio for providing the TNF probe. We express our appreciation to P. Fabbri and R. Siniscalchi for typing the manuscript and to E. Mahoney for editorial assistance.

This work was partially supported by contract 91.00088.PF41 within the Progetto Finalizzato FATMA from the Consiglio Nazionale delle Ricerche, Italy.

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