Journal of Cell Science 112, 3237-3248 (1999)
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JCS4593

Rapid recruitment of late endosomes and lysosomes in mouse macrophages ingesting *Candida albicans*

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Accepted 7 June; published on WWW 22 September 1999

SUMMARY

***Candida albicans* is an important opportunistic pathogen, whose interaction with cells of the immune system, in particular macrophages (MØ), is poorly understood. In order to learn more about the nature of the infectious mechanism, internalisation of *Candida albicans* was studied in mouse MØ by confocal immunofluorescence and electron microscopy in comparison with latex beads of similar size, which were coated with mannosyl-lipoarabinomannan (ManLAM) to target the MØ mannose receptor (MR). Uptake of *Candida* yeasts had characteristics of phagocytosis, required intact actin filaments, and depended on the activity of protein kinase C (PKC). *Candida* phagosomes rapidly attracted lysosome-associated membrane protein (Lamp)-rich vacuoles, indicative of fusion with late endosomes and lysosomes. Rapid recruitment of late endosomes and lysosomes could be observed regardless of heat-inactivation or serum-opsonisation of *Candida*, but did not follow binding of the mannosylated-beads to MØ, which suggest that this phenotype is not MR-specific. The yeasts developed germ tubes within phagolysosomes, distended their membranes and escaped, destroying the non-activated MØ. The filamentous form of *Candida* could penetrate intact MØ even when phagocytosis was blocked, and also attracted Lamp-rich organelles. Inhibition of lysosomal acidification and associated lysosomal fusion reduced germ tube formation of *Candida* within the phagolysosomes. These data suggest that rapid recruitment of late endocytic/lysosomal compartments by internalising *C. albicans* favours survival and virulence of this pathogen.**

Key words: Endosome, *Candida*, Macrophage, Phagocytosis, Phagolysosome

INTRODUCTION

MØ play an important role in the host response to *Candida*, but little is known about cell biological and biochemical characteristics of internalisation of this opportunistic pathogen (Váquez-Torres and Balish, 1997). In tissues, MØ are recruited and yeast and filamentous forms of *Candida* can be detected both within cells and extracellularly (Káposzta et al., 1998). MØ are able to phagocytose both serum-opsonised and unopsonised *Candida* yeasts (Maródi et al., 1991a). The major opsonins for *Candida* in the serum are specific antibodies, and components of classical and alternative complement pathways (Maródi et al., 1991a; Casadevall, 1995; Ray and Wuepper, 1976). Uptake of unopsonised *Candida* is mediated by the macrophage MR, which recognises the mannan-rich outer layer of the yeast wall (Maródi et al., 1991a; Li and Cutler, 1993). The candidacidal activity of MØ largely depends on the cell source, and the mode of internalisation; i.e. murine peritoneal MØ show lower efficiency in *Candida* killing than human monocyte-derived MØ; and uptake of opsonised yeast results in higher killing activity with increased activation of respiratory burst in MØ, compared with unopsonised yeast (Sasada and Johnston, 1980; Maródi et al., 1991b, 1993).

Pathogens can evade or utilise the phagocytic process of MØ by a variety of strategies that allow them to survive and replicate. *Legionella pneumophila* and *Toxoplasma gondii*, for example, reside within vacuoles that avoid fusion with organelles of the endocytic or secretory pathway, while *Mycobacteria* spp. and *Chlamydia* can avoid acidic compartments by residing in selectively fusogenic vacuoles (Sibley, 1995; Russell, 1995; Joiner, 1997). *Salmonella* and *Trypanosoma cruzi* require an acidic environment for survival and virulence and their phagosomes fuse selectively with either Lamp-rich vacuoles or lysosomes (Rathman et al., 1996; Tardieux et al., 1992). *Coxiella burnetti* and *Leishmania* spp. are also dependent upon vacuolar acidification; they thrive in nonselectively fusogenic, acidic phagosomes, which have enhanced vesicular traffic, responsible for nutrient acquisition (Russell, 1995; Joiner, 1997). Disruption of the phagosomal membrane to escape into the cytosol has been demonstrated for *Trypanosoma cruzi*, *Shigella* spp. and *Listeria monocytogenes* (Tardieux et al., 1992; Portnoy et al., 1988).

The survival strategies of *C. albicans* in mononuclear

phagocytes are largely unexplored. *C. albicans* is more resistant to myeloperoxidase-mediated killing by monocytes than several less-pathogenic *Candida* species, and the lack of myeloperoxidase in macrophages may explain at least in part the lower killing capacity of these cells compared with monocytes, which are rich in this enzyme (Maródi et al., 1991b). The thick glycoprotein yeast cell wall, especially its glucan and covalently linked chitin-glucan components, which are highly resistant to lysosomal enzymes, may also have a role in protecting ingested *Candida* (Marquis et al., 1991). Various *C. albicans* strains secrete different proteases, hemolytic factors and phospholipases which could contribute to survival of ingested fungi (Lerner and Goldman, 1993; Ibrahim et al., 1995; Manns et al., 1994). *Candida* is a dimorphic fungus but in spite of several studies, the role of germ tube formation in pathogenicity of *Candida* has not yet been determined (Maródi, 1997).

In this study, entry mechanisms and maturation of phagolysosomes were characterised during interaction of mouse peritoneal MØ with *C. albicans*. Rapid recruitment of late endosomal/lysosomal compartments by both yeasts and germ tubes was found. Germ tubes were able to invade MØ even when phagocytosis was blocked with various inhibitors. The different entry mechanisms of the yeast and mycelial forms of *Candida* and their common ability to attract the late endosomal/lysosomal compartments suggest lysosomal fusion to their phagosome is an important mechanism in the pathogen survival strategy.

MATERIALS AND METHODS

Fungi

Stock cultures of *C. albicans* (ATCC 18804) were maintained on Sabouraud's dextrose agar (Difco Laboratories, Detroit, MI) at 4°C, inoculated into 50 ml Sabouraud's dextrose broth (Difco) and cultured at 37°C for 2-3 days to prepare stationary growth phase yeast. Heat-killed *C. albicans* was prepared by autoclaving (15 minutes, 121°C) in PBS, then washed and tested for viability by colony counting on Sabouraud's dextrose agar after 24-48 hours incubation at 37°C.

Macrophage cultures and phagocytic challenge

Peritoneal exudate cells were obtained from C57BL/6 mice of both sexes, 6-8 weeks old (Sir William Dunn School of Pathology), 4-5 days after intraperitoneal injection of 0.3 ml Biogel P-100 polyacrylamide beads, 100-200 mesh (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK). Cells were plated on acid-washed 13 mm diameter glass coverslips (BDH-Merck Ltd, Dorset, UK) at a density of 2×10^5 cells per coverslip in Optimem 1 supplemented with 2 mM L-glutamine, 100 U/ml penicillin G and 100 mg/ml streptomycin (all from Gibco Laboratories, Paisley, UK). Nonadherent cells were removed by washing after 2 hours incubation at 37°C and 5% CO_2, and cells were cultured overnight in fresh medium. For electron microscopy, 5×10^6 cells were cultured in 35 mm diameter tissue culture dishes (Falcon, Becton Dickinson UK Ltd, Oxford, UK) as described above.

MØ cultures were challenged with 10-20 live or heat-killed *C. albicans* per MØ, and incubated for 15-30 minutes at 4°C to synchronise ingestion. Unbound particles were removed by washing and uptake was initiated by incubation at 37°C and 5% CO_2. After various times, cells were fixed and processed for microscopy as described below. As internal control, we used 6 μm latex beads (Polysciences, Inc. Warrington, PA) that were coated with ManLAM (mannosyl-lipoarabinomannan), a component of *Mycobacterium tuberculosis* and ligand for the MR, or with C3bi or IgG (Schlesinger et al., 1994). Briefly, to coat latex beads with C3bi they were preincubated with phenolic glycolipid-I (PGL-1) isolated from armadillo tissues, an activator of the alternative pathway, then with mouse complement (Sigma), and to coat them with IgG they were pre-incubated with BSA (Sigma), and then mouse anti-BSA polyclonal IgG (Sigma). ManLAM and PGL-1 were obtained from Dr Patrick Brennan, University of Colorado, through the Leprosy Research Support.

Confocal immunofluorescence

Cells were fixed in 4% Hepes-buffered paraformaldehyde (Gibco Laboratories, Paisley, UK; BDH-Merck) for 10 minutes, followed by 8% paraformaldehyde for 1 hour at 4°C, then blocked and permeabilised in PBS containing 1% BSA and 0.5% saponin (Sigma Chemical Co., St Louis, MO) for 30 minutes at room temperature. Cells were incubated with monoclonal primary antibody or PBS as control for 1 hour at room temperature, washed in BSA-saponin solution, blocked in 1% normal goat serum for 15 minutes, then incubated with fluorochrome-conjugated (FITC, Texas Red, Cy5) goat anti-mouse or anti-rat $(Fab')_2$ IgG (Jackson ImmunoResearch Laboratories, INC. West Grove, Pennsylvania). Talin and vinculin were detected using mouse mAbs (Sigma), Lamp-1 and Lamp-2 with rat mAbs (1D4B, ABL93) (DSHB, University of Iowa) and the transferrin receptor with rat anti-mouse mAb (RI7217) (Sigma); F-actin was visualised using TRITC- or FITC-phalloidin (Sigma). The different endosomal compartments were labelled by using 0.2 mg/ml FITC-horseradish peroxidase (FITC-HRP) (Sigma) or 0.1 mg/ml Texas Red-70 kDa Dextran (Molecular Probes). MØ were incubated with FITC-HRP for 60 minutes on ice, then for 5 minutes at 37°C to initiate internalisation, washed and chased for 5 minutes to access the early endosomes, for 25 minutes to label late endosomes and for 2 hours for lysosomes (Alvarez-Dominguez et al., 1997). Alternatively, MØ were incubated with Texas Red-70 kDa Dextran for 2 hours at 37°C, washed and chased overnight to label lysosomes. Coverslips were mounted on microscope slides using Vectashield (Vector Labs., Peterborough, UK). Confocal laser scanning microscopy was performed using the LaserSharp software in conjunction with a Bio-Rad MRC-1024, mounted on a Nikon Diaphot 200 microscope equipped with a 60:1 planapochromat NA 1.4 objective. Representative images were collected and saved in PICT format, then imported to Adobe Photoshop version 4.0 software, where the single fluorophore images were merged and processed to produce the final TIFF format of single and multiple immunostained and corresponding phase-contrast sections.

Transmission electron microscopy

MØ were incubated overnight with BSA-6-nm gold conjugate and chased for 2 hours to label the late endocytic compartments. MØ were allowed to bind *C. albicans* at 4°C for 15-30 minutes, and uptake was initiated at 37°C in medium containing 10 mg/ml horseradish peroxidase (Bio Whittaker UK Ltd, Reading). Cells were fixed in 0.5% glutaraldehyde in 0.2 M sodium cacodylate buffer at pH 7.2 at 4°C for 10 minutes, then at room temperature for 20 minutes and postfixed in 1% osmium tetroxide and 1.5% potassium ferrocyanide for 1 hour, then in 0.5% magnesium uranyl acetate overnight (all from BDH-Merck). Cells were processed for Epon embedding, ultrathin sectioning (110 nm), stained with lead citrate and analysed by electron microscopy (Zeiss EM 912 Omega).

Phagocytosis assay

Phagocytosis by adherent peritoneal Biogel MØ of live or heat-killed *C. albicans* and latex beads coated with ManLAM, C3bi or IgG was determined by immunofluorescence. The phagocytic particles*:* MØ ratio was 10:1 in the phagocytic mixture, and the assay was performed for 60 minutes. Then, cells were fixed, permeabilised, stained with FITC-phalloidin, and the localisation of the particles was determined

by confocal microscopy. Fusion of phagosomes with late endocytic organelles and lysosomes was also examined by calculating the ratio of Lamp$^+$ phagosomes. 600 infected MØ were counted in triplicate.

Inhibitor studies

MØ on glass coverslips were pre-treated for 1 hour at 37°C with 2 μM cytochalasin D (Sigma) to inhibit actin assembly; 10 μM nocodazole to disrupt the microtubule network; 0.1 μM wortmannin, a PI3-K inhibitor; 0.2 μM staurosporine, a PKC inhibitor, with 0.1 μM thapsigargin to inhibit endoplasmic reticulum (ER) Ca^{2+}-ATP-ase (all from Calbiochem-Novabiochem Ltd, Nottingham, UK) or 0.1 mM chloroquine (Sigma) to inhibit lysosomal acidification. Then, phagocytosis in the presence of inhibitors was assayed as described.

Importance of acidification in germ tube formation

C. albicans blastoconidia were cultured in different 0.05 M citrate-0.1 M phosphate buffers (pH 3.5-8.0) in the presence of 10% normal murine serum at 37°C for 90 minutes. The pH of the different culture media did not change significantly during this period (≤0.1). Cells were fixed and analysed by phase-contrast microscopy. The percentage of germ tubes was calculated by counting 600 cells in triplicate. Intracellular and extracellular germ tube formation was also determined in the presence of a weak base, chloroquine (0.1 mM), or foliomycin (0.1 μM) (Calbiochem-Novabiochem), an inhibitor of H$^+$-ATPase.

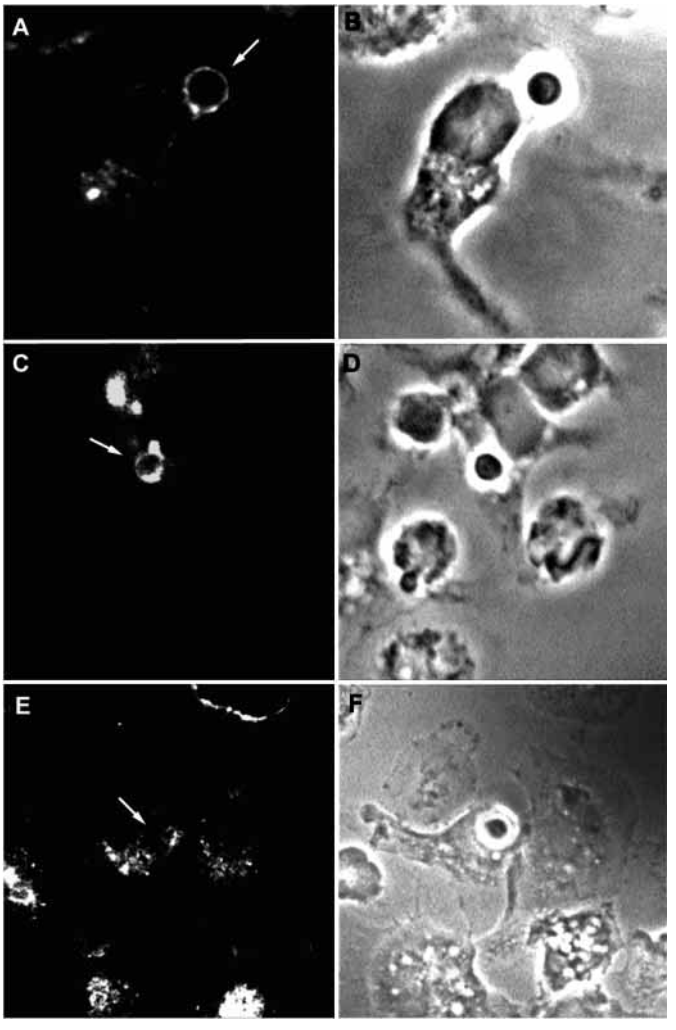


Fig. 1. *C. albicans* blastoconidia engulfed by peritoneal MØ are associated with F-actin and talin, but not with vinculin. Cells were fixed and permeabilised at 2 minutes of phagocytosis and stained with FITC-phalloidin to visualise actin (A), or with antibodies to talin (C) or vinculin (E) as indicated. Left panel is a single representative section from the confocal microscope, right panel is the corresponding phase-contrast image. The arrows point to the entering *Candida* in the confocal images. Scale: (A-D) 0.07 μM/1 pixel, and (E-F) 0.11 μM/1 pixel; the width of each picture = 425 pixels.

RESULTS

Phagocytosis assay

Adherent peritoneal MØ were infected with a virulent strain of *C. albicans* that allowed us to analyse the interactions between the pathogen and MØ in the absence of immune activation. ManLAM-, complement- and IgG-coated latex beads of similar size were used for comparison. Uptake of unopsonised *Candida* was mannan-inhibitable (>80% inhibition in the presence of 10 mg/ml Saccharomyces cerevisiae mannan), and did not result in a significant increase in superoxide release and nitrite production (data not shown). Intracellular localisation of beads and yeast was assessed by confocal microscopy after phalloidin staining, which revealed very well the MØ cortical actin and the boundaries of the cells, allowing us to determine particle position precisely. After 10 minutes incubation, 66.4±5.6% of MØ-associated yeasts were already internalised,

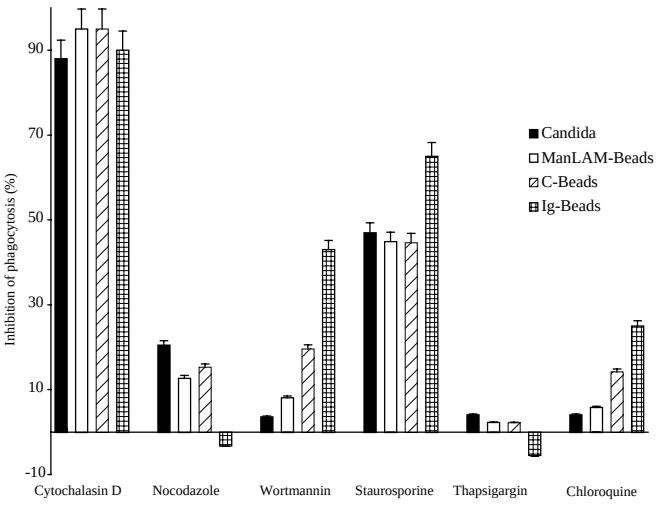


Fig. 2. Inhibition of phagocytosis of different particles by treatment with inhibitors. MØ were treated with 2 μM cytochalasin D, 10 μM nocodazole, 0.1 μM wortmannin, 0.2 μM staurosporine, 0.1 μM thapsigargin or 0.1 mM chloroquine for 60 minutes, and allowed to ingest *Candida* blastoconidia or ManLAM-, C3bi-or IgG-coated beads for 60 minutes. Then cells were fixed, permeabilised and stained with FITC-phalloidin to determine the localisation of particles using confocal microscopy. Cytochalasin D and staurosporine had the strongest effect on the phagocytosis of particles, nocodazole slightly reduced the internalisation, while treatment with wortmannin, thapsigargin and chloroquine did not result in significant inhibition of uptake of mannose-rich particles. However, wortmannin and chloroquine resulted in inhibition of Fc-receptor-mediated phagocytosis. Each bar represents the inhibition percentage of the control ± s.e. of experiments performed in triplicate; 600 infected MØ were counted for each coverslip.

reaching 98.1±1.6% at 60 minutes, but the killing of *Candida* by MØ remained <5% at 120 minutes of incubation. Killing was determined by a colony counting method (Maródi et al., 1991b). Uptake of the ManLAM-coated latex beads reached 87.2±3.2 at 60 minutes. Similarly, uptake levels of IgG- and C3Bi-coated particles were 78.3±4.1% and 82.5±4.4, respectively.

Mechanism of pathogen entry

To determine the involvement of phagocytosis versus invasion in the uptake of *C. albicans*, we studied actin assembly as an indicator of phagocytosis at the site of entry, and also the recruitment of talin and vinculin, actin-associated proteins, which have been reported to contribute to phagocytosis of IgG- or complement-opsonised particles (Allen and Aderem, 1996; Greenberg et al., 1990). F-actin and talin in association with the entering live yeasts were detected by confocal immunofluorescence 2 minutes after addition of *Candida* to MØ at 37°C (Fig. 1A-D). The condensation of actin filaments and talin largely disappeared from the phagosomes at a slightly later stage (5 minutes), after the yeasts had been fully internalised. The forming phagosomes were not associated with vinculin at any time of phagocytosis (Fig. 1E-F). Internalisation of live and heat-killed yeasts was accompanied by these same features. Contribution of F-actin and the actin-associated protein, talin, strongly suggest that phagocytosis is the main mechanism involved in entry of *Candida* yeast forms into MØ.

To gain further insight into the internalisation process, inhibitors of actin assembly (2 μM cytochalasin D), the microtubular network (10 μM nocodazole), PI3-K (0.1 μM wortmannin), PKC (staurosporine 0.2 μM), ER Ca^{2+}-ATP-ase (0.1 μM thapsigargin) and lysosomal/endosomal acidification (0.1 mM chloroquine) were used to alter the phagocytic machinery. The total number of phagocytic particles bound and ingested was 1.67±0.16 per MØ in the control, and did not change significantly in the presence of most of the inhibitors: binding was 1.5±0.05 with cytochalasin D, 1.63±0.13 with wortmannin and 1.53±0.01 with thapsigargin treatment. Staurosporine slightly reduced the binding of the particles (1.45±0.01), while nocodazole increased it (1.93±0.19).

The most potent inhibitors of uptake of *Candida* and ManLAM-coated beads were cytochalasin D and staurosporine (Fig. 2). Nocodazole slightly reduced internalisation of both

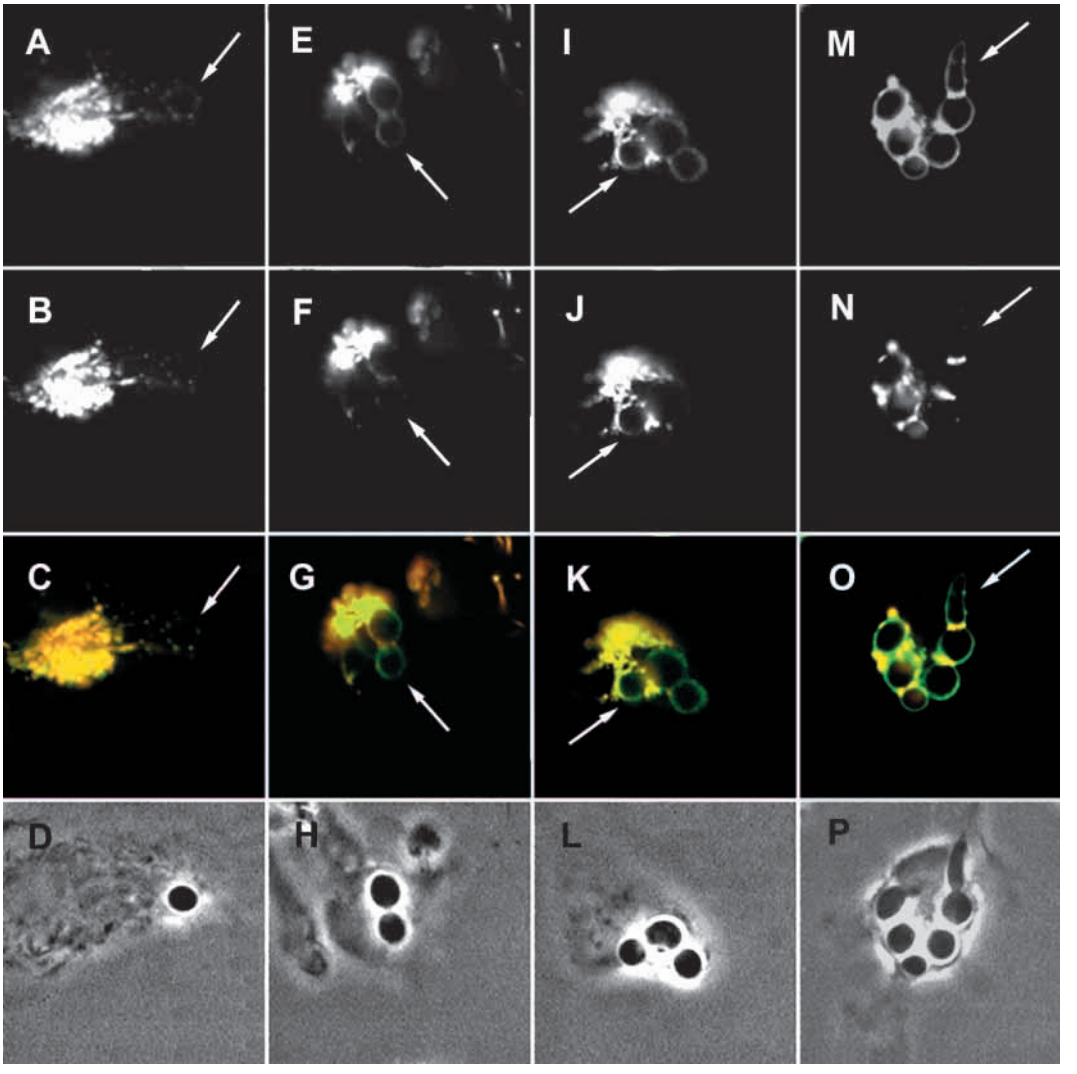


Fig. 3. Time-course of vacuolar fusion with *Candida*-phagosomes. Peritoneal MØ were loaded with Texas Red-70 kDa Dextran, chased overnight and then allowed to ingest *Candida* blastoconidia for 5 (A-D), 15 (E-H), 30 (I-L) or 60 minutes (M-P). Then cells were fixed, permeabilised and stained for Lamp, using specific mAbs and a secondary antibody conjugated with FITC. The top panel (A,E,I,M) shows Lamp staining of infected MØ at different times after binding. In the next row (B,F,J,N) the localisation of Texas Red-Dextran-labelled lysosomes in these cells can be seen, which is followed by the merge of the previous images (C,G,K,O). Colocalisation of Dextran (red) in lysosomes with Lamp (green) yields a yellow image by confocal immunofluorescence. The last panel is the corresponding phase-contrast image (D,H,L,P). Fusion of *Candida*-phagosomes (arrows) with lysosomes and late endosomes had already started at 5 minutes of phagocytosis (A-D), and at 60 minutes germ tube formation could be observed within the phagolysosomes (M-P) (arrows). Scale: 0.1 μM/1 pixel; the width of each picture = 300 pixels.

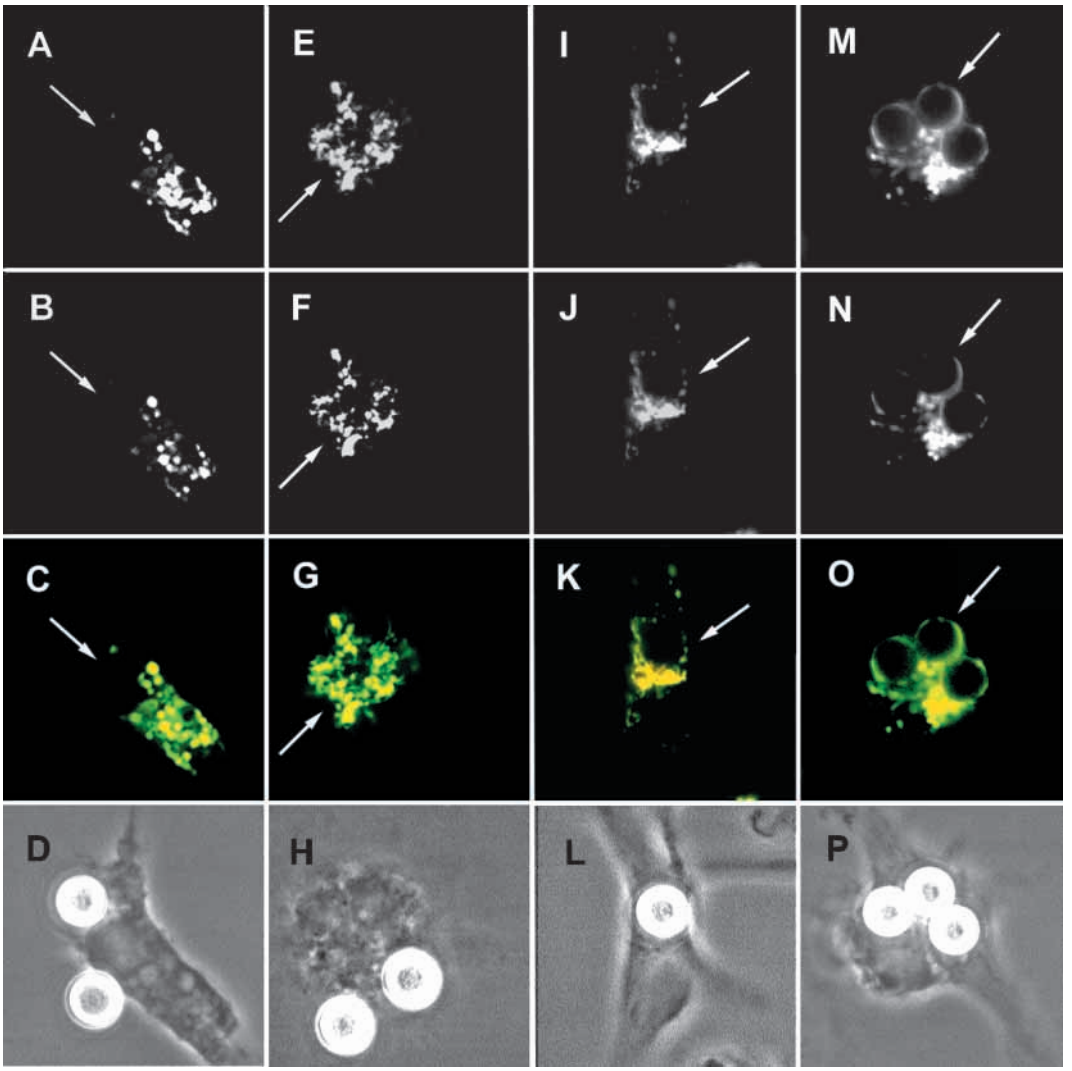


Fig. 4. Time-course of vacuolar fusion with bead-phagosomes. Peritoneal MØ were loaded with Texas Red-70 kDa Dextran, chased overnight and then allowed to ingest ManLAM-coated latex beads for 5 (A-D), 15 (E-H), 30 (I-L) or 60 minutes (M-P). Then cells were fixed, permeabilised and stained for Lamp, using specific mAbs and a secondary antibody conjugated with FITC. The top panel (A,E,I,M) shows Lamp staining of MØ at different time of phagocytosis. Localisation of Texas Red-Dextran-labelled lysosomes in these cells can be seen in the next row (B,F,J,N), which is followed by the merge of the previous images (C,G,K,O). Colocalisation of Dextran (red) in lysosomes with Lamp (green) yields a yellow image by confocal immunofluorescence. The last panel is the corresponding phase-contrast image (D,H,L,P). Fusion of lysosomes with bead-phagosomes could be observed from 30 minutes of phagocytosis (I-L). Scale: 0.1 μM/1 pixel; the width of each picture = 300 pixels.

particles, while the inhibitory effect of wortmannin, thapsigargin and chloroquine was not significant (Fig. 2). There were no differences between the uptake rates of the two mannosyl-rich particles in the presence of different inhibitors and these findings prove phagocytosis is the mechanism involved in yeast entering into MØ. To compare the effect of inhibitors on MR-independent uptake we used C3bi- and IgG-coated latex beads. The inhibitory pattern was slightly different. Similarly to mannose-rich particles, the most effective inhibitors of uptake of C3bi-and IgG-coated beads were again cytochalasin D and staurosporine. However wortmannin and chloroquine resulted in higher inhibition of Fc-receptor-mediated phagocytosis, whilst uptake of C3bi-coated beads remained largely unaffected. This is in good agreement with previously published data describing the importance of PI3-K in Fc receptor signalling (Ninomiya et al., 1994).

Maturation of *Candida* phagosomes

Characteristics of *Candida* vacuoles were studied at different times by confocal immunofluorescence to analyse their fusogenicity and maturation. First, the time-course of lysosomal and late endocytic fusion was studied. The lysosomes of MØ were loaded with Texas Red-Dextran, and cells allowed to ingest *Candida* (Fig. 3) or ManLAM-coated beads (Fig. 4) for 5, 15, 30 or 60 minutes. They were then fixed and stained for Lamps, using mAbs ABL93 and ID4B plus a FITC-labelled secondary antibody. Fusion of lysosomes and late endosomes had already started at 5 minutes of yeast phagocytosis (Fig. 3A-D), soon after depolymerisation of the surrounding actin. 10 minutes after the onset of phagocytosis 66.4±5.6% of MØ-associated *Candida* had $Lamp^+$-rings, and 79.2±8.4% of $Lamp^+$ phagosomes colocalised with Dextran-labelled lysosomes. In contrast to *Candida*-phagosomes, $Lamp^+$-rings around latex bead-phagosomes appeared only after 30 minutes of phagocytosis (Fig. 4I-L). The results relating to the latex bead-phagosomes are in good agreement with previously reported data (Desjardins et al., 1994). By 60 minutes of phagocytosis 93.1±3.3% of *Candida*-phagosomes and 59.3±4.6% of bead-phagosomes had become $Lamp^+$, and contained Dextran. *Candida*-phagosomes attracted lysosomes and late endosomes and fused with them rapidly, even if *Candida* was heat-killed (Fig. 5E-H) or serum-opsonised (Fig. 5I-L).

We next examined whether *Candida*-phagosomes were selectively fusogenic with acidic compartments, recruiting late endosomes and lysosomes, or whether early endosomes also took part in accelerated fusion. Dual labelling of lysosomes,

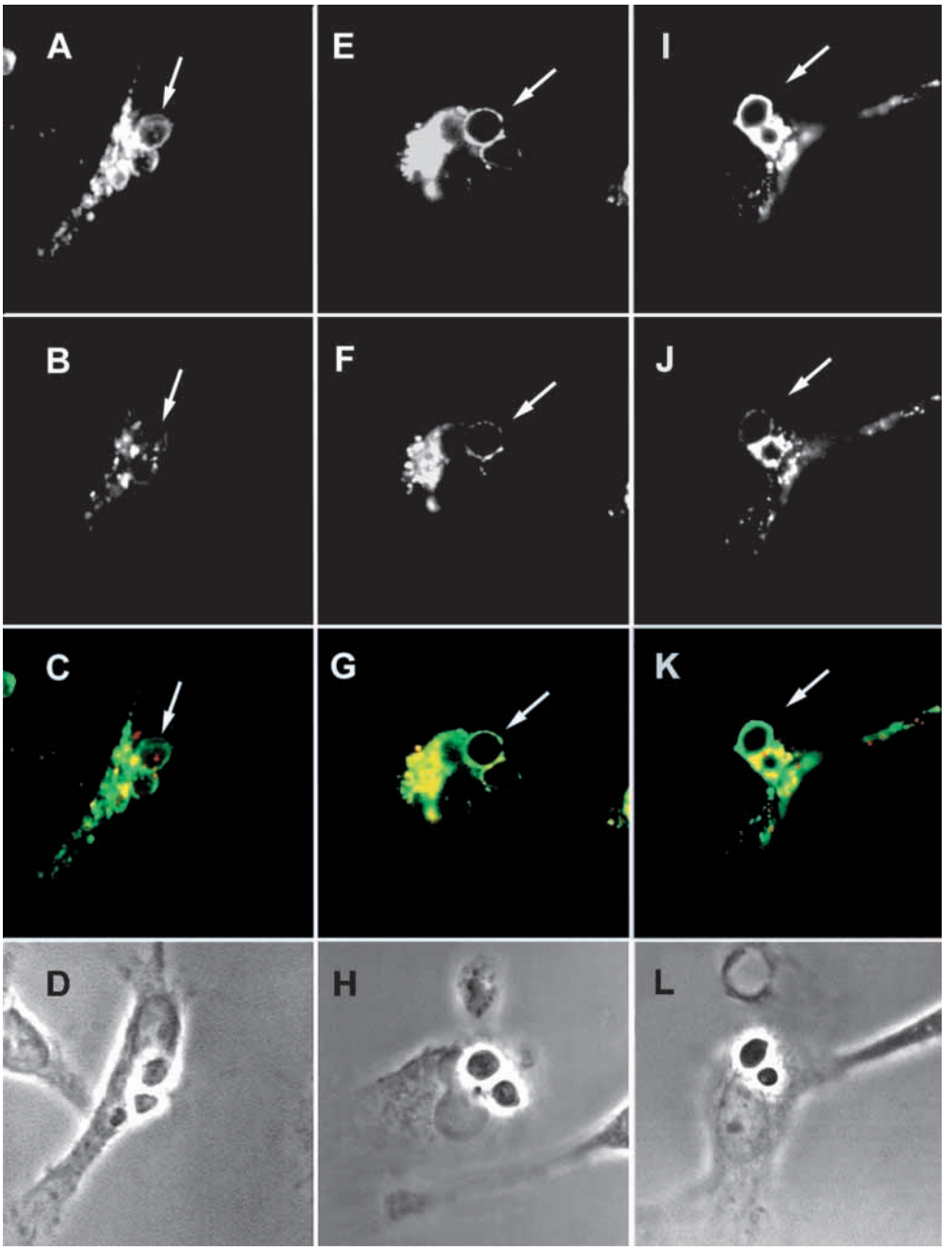
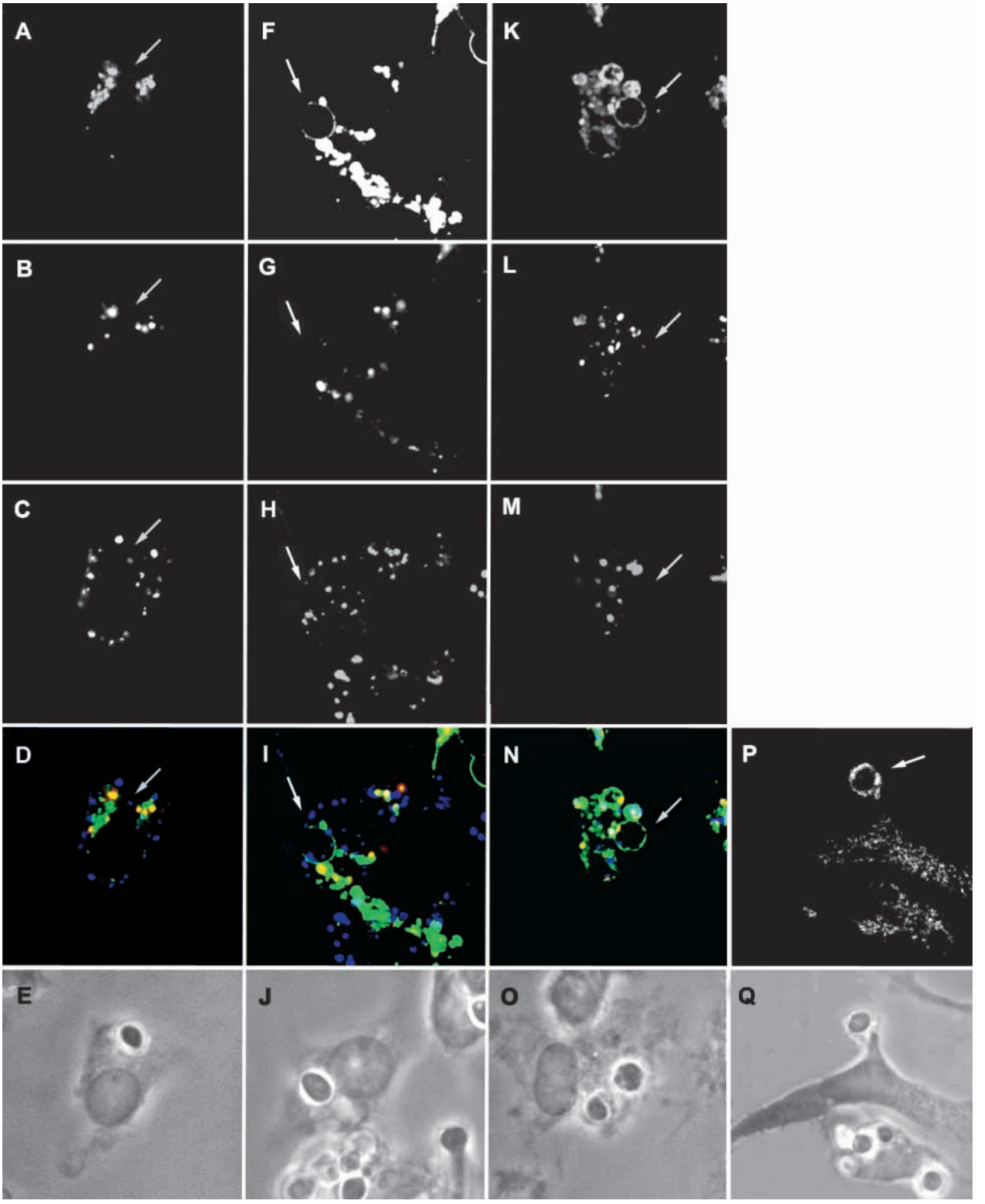


Fig. 5. *Candida* phagosomes rapidly become Lamp$^+$ and fuse with lysosomes. Peritoneal MØ were loaded with Texas Red-70 kDa Dextran, chased overnight, then allowed to ingest *Candida* blastoconidia. Cells were fixed, permeabilised at 10 minutes of phagocytosis, and stained for Lamp, using specific mAb and a secondary antibody conjugated with FITC. The top row shows Lamp staining of different MØ, which are ingesting unopsonised live (A), heat-killed (E) and opsonised live (I) *Candida*. In the next row (B,F,J) the localisation of lysosomes loaded with Dextran, can be seen in these cells. Colocalisation of Dextran (red) in lysosomes with Lamp (green) yields a yellow image by confocal immunofluorescence (C,G,K), and the bottom panel is the corresponding phase-contrast image (D,H,L). Forming phagosomes (arrows), containing unopsonised live (A-D), heat-killed (E-H), or opsonised (I-L) blastoconidia, quickly became Lamp$^+$, after fusing with lysosomes.

Fig. 6. *Candida* phagosomes recruit late compartments rapidly and also fuse with early compartments of the endocytic pathway. Lysosomes of peritoneal MØ were labelled with Texas Red-Dextran as described, cells were loaded with HRP and chased for 5 minutes to access the early endosomes (A-E) or 20 minutes for late endosomes (F-J), then allowed to ingest *C. albicans* for 5 minutes (A-E) or 10 minutes (F-J). Cells were fixed, permeabilised and stained for Lamp using specific mAbs and a FITC-conjugated secondary antibody (A,F). The second row shows lysosomes loaded with Dextran (B,G). HRP$^+$ early endosomes (C) and late endosomes (H) are shown in the next panel. The following row shows the merge of the different staining reactions: the HRP$^+$-vesicles appear in blue, the Lamp in green and colocalisation of Dextran (red) in lysosomes with Lamp (green) yields a yellow image (D,I) by confocal immunofluorescence. The arrows point to the forming phagosomes. The last panel is the corresponding phase-contrast image (E,J). At 5 minutes of phagocytosis, HRP$^+$ early compartments (C) could be observed close to the yeast phagosomes. Although the phagosome is still Lamp$^-$ at this stage, Lamp$^+$ organelles and Texas Red-Dextran-loaded vesicles could also be observed in its proximity (A-B). At 10 minutes the phagosomes are already strongly Lamp$^+$ (F), and Texas Red-Dextran-loaded lysosomes and HRP$^+$ late endosomes are also seen in association with the vacuole (G-H). Fusion of the *Candida*-phagosomes with early endosomes was confirmed by positive immunostaining of MØ for transferrin receptor at 10 minutes of phagocytosis (K-L). Scale: 0.1 μM/1 pixel; the width of each picture = 320 pixels.

early or late endosomes with Texas Red-Dextran, or of lysosomes with FITC-HRP, was used in different experiments to determine localisation of these compartments. At 5 minutes of phagocytosis, HRP-labelled early compartments (Fig. 6C), could be observed close to the yeast phagosomes. Although still Lamp$^-$ at this stage, Lamp$^+$ organelles and Texas Red-Dextran-loaded vesicles could also be observed in the proximity of the phagosome (Fig. 6A-B). At 10 minutes the phagosomes were already strongly Lamp$^+$ (Fig. 6F), and Texas Red-Dextran-loaded lysosomes and HRP-loaded late endosomes were also seen in association with the vacuole (Fig. 6G-H). Positive immunostaining for transferrin receptor also suggested fusion of early endosomes with the *Candida*-phagosomes at 10 minutes of phagocytosis (Fig. 6K-L).



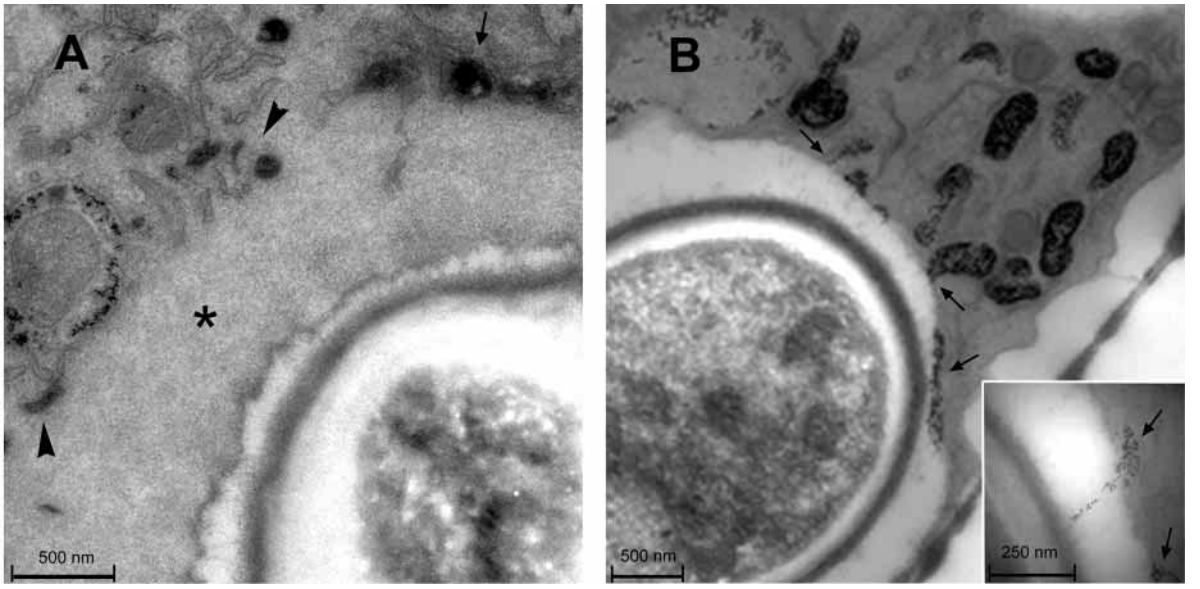


Fig. 7. Electron micrographs of *Candida*-phagosomes at different stages of maturation. 6 nm gold-BSA had been internalised by MØ overnight and chased into late endocytic structures: lysosomes and late endosomes (arrows) for 2 hours. MØ were allowed to bind *C. albicans* at 4°C for 15-30 minutes, then to ingest at 37°C for 15 minutes with HRP (10 mg/ml) in the medium to label early endosomes (arrowheads). At the beginning of phagocytosis, a thick actin-rich phagocytic coat, shown as an electron-transparent zone (asterisk), appears around the engulfed yeast (A). In proximity to the actin-coat, HRP^+ early endosomes, predominantly tubular-vesicular structures (arrowheads), can be observed. The late endocytic vesicles are rapidly recruited to the phagosome, and appear as spherical, electron-dense organelles which have accumulated BSA-gold (arrows) (A). The yeast is not covered tightly by the phagosome membrane (B), and fusion with the late endocytic structures follows soon after depolymerisation of the actin coat (B, inset).

Transmission EM provided further evidence of engulfment and vacuolar fusion. Binding of *Candida* induced membrane ruffling in the MØ, with extended pseudopodia enveloping the relatively large particles (data not shown). A thick actin-rich phagocytic coat could be observed around the yeast and was surrounded by early and late endocytic structures (Fig. 7A). Again, fusion of the lysosomes and delivery of preloaded gold tracer to *Candida*-phagosomes was soon observed after depolymerisation of actin from the phagosomes (Fig. 7B).

Germ tube formation and *Candida* escape from macrophages

In our model, *C. albicans*-MØ interaction ended with >95% survival of ingested fungi, destroying the phagocytes. *Candida* survival was calculated by using colony counting and also by assessing *Candida* viability with 1% eosin staining (Maródi et al., 1991b; Costantino et al., 1995). After 60 minutes of incubation at 37°C, both extracellular and ingested *C. albicans* had started to form germ tubes, which elongated further and became more easily observed by 90 minutes. The intracellular ones grew inside the phagolysosomes at the beginning, distending the $Lamp^+$ vacuolar membrane (Fig. 8C-D), before rupture and escape from the MØ (Fig. 8A-B). The culture conditions (37°C, 5% CO_2, L-glutamine supplemented media) were suitable for germ tube formation: 93±2.9% of extracellular, non-MØ-associated yeasts also formed germ tubes by 90 minutes of the phagocytosis assay, and similar results were obtained in MØ-free cultures. However, the frequency of germ tube formation inside the intact MØ was much lower (26.6±9.0%), and it could be further reduced by neutralisation of lysosomal pH or inhibition of lysosomal H^+-ATP-ase (Fig. 8E-F and Fig. 9).

In order to study the potential role of acidic pH in promoting dimorphic differentiation of *C. albicans*, the frequency and morphology of germ tube formation were examined at different pH in MØ-free cultures. To exclude the possible influence of CO_2 on pH, germ tube formation was induced with 10% normal murine serum in phosphate or citrate buffers. Germ tube formation was more frequent in acidic conditions, and they became moderately longer at pH 5.0-6.5 (Fig. 10). Our findings indicated that, whilst acidic pH is not essential for germ tube formation, it may be one of the factors that promote this process. However, we did not observe any differences in germ tube formation when the pH was lowered below 6.0, suggesting that other factors during lysosomal fusion to the vacuole were involved in intracellular germ tube formation.

Penetration of *Candida* germ tubes into macrophages

To support our hypothesis that germ tube formation is responsible for the escape of ingested fungus from infected MØ, invasivity of this form was examined directly. Since cytochalasin D, nocodazole, wortmannin and staurosporine did not inhibit germ tube formation, MØ were first incubated with blastoconidia in the presence of these drugs. After 60 minutes of incubation, the non-phagocytosed, MØ-bound *Candida* developed germ tubes that penetrated the phagocyte. The invading germ tubes also recruited late endocytic/lysosomal structures and soon became $Lamp^+$. This indicates that intracellular germ tubes grew in membrane-bound structures

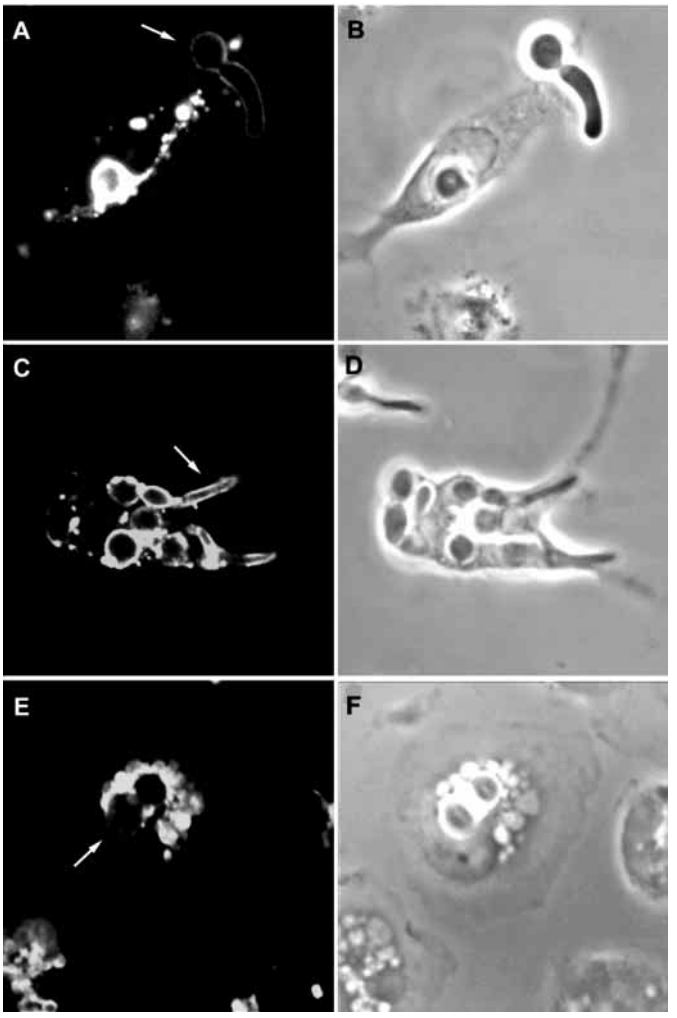


Fig. 8. Dimorphic transformation of *C. albicans* in MØ phagolysosomes. MØ were allowed to ingest unopsonised (A,B,E,F) and opsonised (C-D) *Candida* blastoconidia for 90 minutes, then fixed, permeabilised and processed for Lamp immunostaining with specific mAbs and a FITC-conjugated secondary antibody. MØ were pretreated with chloroquine before ingestion(E-F) to neutralise the vacuolar pH. *Candida* blastoconidia generated germ tubes (arrows) in the phagolysosomes from 60 minutes of ingestion, distending the $Lamp^+$ phagolysosome membrane (C), then escaped from the MØ and remained weakly $Lamp^+$ (A). When cells were treated with chloroquine (0.1 mM) (E-F) MØ became vacuolated, and the frequency of germ tube formation, as well as fusion of phagosomes with $Lamp^+$ organelles was reduced. The arrow (E) points to $Lamp^-$ phagosomes, which contain *Candida* blastoconidia. The left panel shows a single representative image of confocal microscopy, the right the corresponding phase-contrast image. Scale: 0.07 μM/1 pixel; the width of each picture = 425 pixels.

and not freely in the cytosol (Fig. 11). A similar phenotype was observed when preformed germ tubes were incubated with MØ for 15 minutes with or without these inhibitors.

Since inhibition of Ca^{2+}-ATP-ase also blocks the dimorphic change of fungus, the effect of thapsigargin was tested by using preformed germ tubes. Depletion of intracellular calcium stores by thapsigargin did not inhibit penetration, but blocked lysosomal movement and fusion to the germ tube compartment (Fig. 11I-J). Inhibition of PKC by staurosporine (Fig. 11G-H),

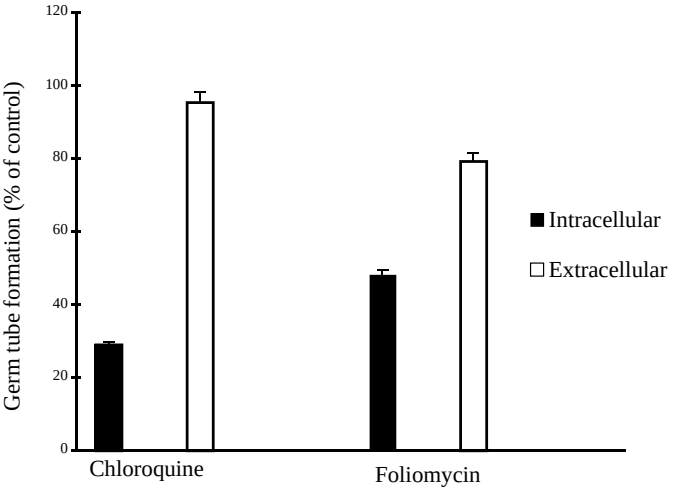


Fig. 9. Neutralisation of lysosomal pH decreases germ tube formation of ingested *C. albicans*. MØ were allowed to ingest *C. albicans* for 60 minutes, then fixed, permeabilised and processed for Lamp immunostaining. The percentage of germ tubes that grew intracellularly ($Lamp^+$) and extracellularly (non-MØ-associated) was compared with the control (without treatment). Each bar represents the mean ± s.e. of experiments performed in triplicate; for each coverslip 600 *Candida* were counted. Chloroquine (0.1 mM) inhibited germ tube formation of *Candida* within MØ, while inhibitory effect of foliomycin (0.1 μM) was weaker and could also be observed when the pathogen grew extracellularly.

disruption of actin polymerisation by cytochalasin D (A-B) and of the microtubule network by nocodazole (C-D), and blockade of PI3-K activity by wortmannin (E-F) did not inhibit lysosomal fusion to the germ tube vacuole.

DISCUSSION

This detailed morphological analysis of interactions between MØ and *Candida* as summarised schematically in Fig. 12, provides new insights into the mechanisms of infection and virulence. Phagocytic uptake of yeast by peritoneal MØ is relatively rapid and involves immediate recruitment of late endocytic/lysosomal organelles. Lysosomal fusion to the vacuoles in MØ promoted germ tube formation of *Candida*, although sprouting in extracellular yeast is generally more efficient. Germ tubes were invasive, could escape from phagolysosomes and penetrate intact MØ, even when phagocytosis was blocked by various inhibitors. Invading germ tubes also recruited $Lamp^+$ organelles, and grew membrane-bound within the MØ. Therefore rapid recruitment of late endocytic/lysosomal organelles and vacuolar fusion might be part of the survival strategy and pathogenicity of both forms of *C. albicans*.

We have demonstrated that the key mechanism responsible for the uptake of unopsonised *C. albicans* yeast is phagocytosis. As reported in the literature (Maródi et al., 1991a) the unopsonised yeast binds preferentially to the MR. This interaction initiates an intracellular signalling pathway leading to membrane ruffling and envelopment of the relatively large particle by pseudopodia and actin assembly, followed by recruitment of talin, but not vinculin at the phagosomal coat.

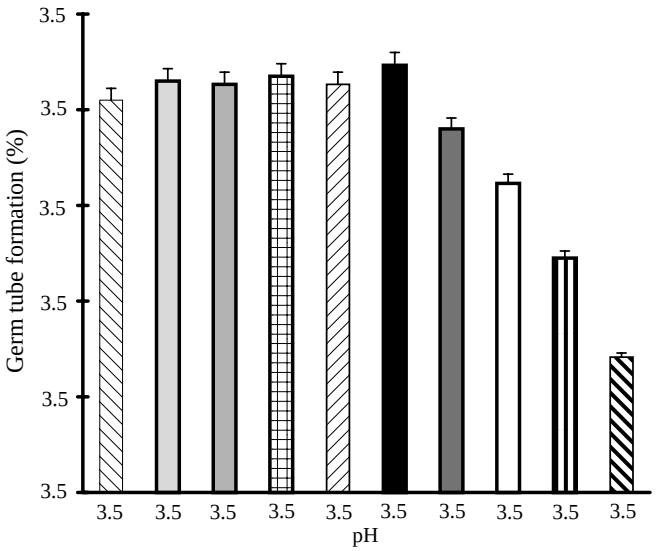


Fig. 10. Acidic conditions (pH ≤7.0) promote germ tube formation of *C. albicans* in the absence of MØ. *Candida* yeasts were cultured in 0.05 M citrate/0.1 M phosphate buffers in the presence of 10% normal murine serum at 37°C. The percentage of germ tubes formed was calculated by phase contrast microscopy. Each bar represents the mean ± s.e. of experiments performed in triplicate; 600 *Candida* were counted for each coverslip.

Our findings show that MR-mediated ingestion leads to talin association with the phagosome, but not vinculin, in contrast to Fc- and complement receptor-mediated ingestion (Allen and Aderem, 1996). Similarly to *C. albicans*, unopsonised zymosan entering MØ via mannose and β-glucan receptors also recruits talin, but not vinculin around the phagosomes (Greenberg, 1995).

The signal transduction pathway responsible for phagocytosis via the MR is not well understood. We have shown that PKC activity is essential for phagocytosis both of *Candida* and ManLAM-coated beads. Localisation of PKC has been demonstrated in phagosomes of unopsonised zymosan (Allen and Aderem, 1995). The inhibitory effect of staurosporine on *Candida* internalisation might reflect the role of PKC in actin polymerisation and in recruitment of cytoskeletal proteins to the binding sites of particles (Allen and Aderem, 1995, 1996). Inhibition of ER Ca^{2+}-ATP-ase with thapsigargin did not reduce the uptake of mannosyl-rich particles. Data in the literature about the role of Ca^{2+} in the

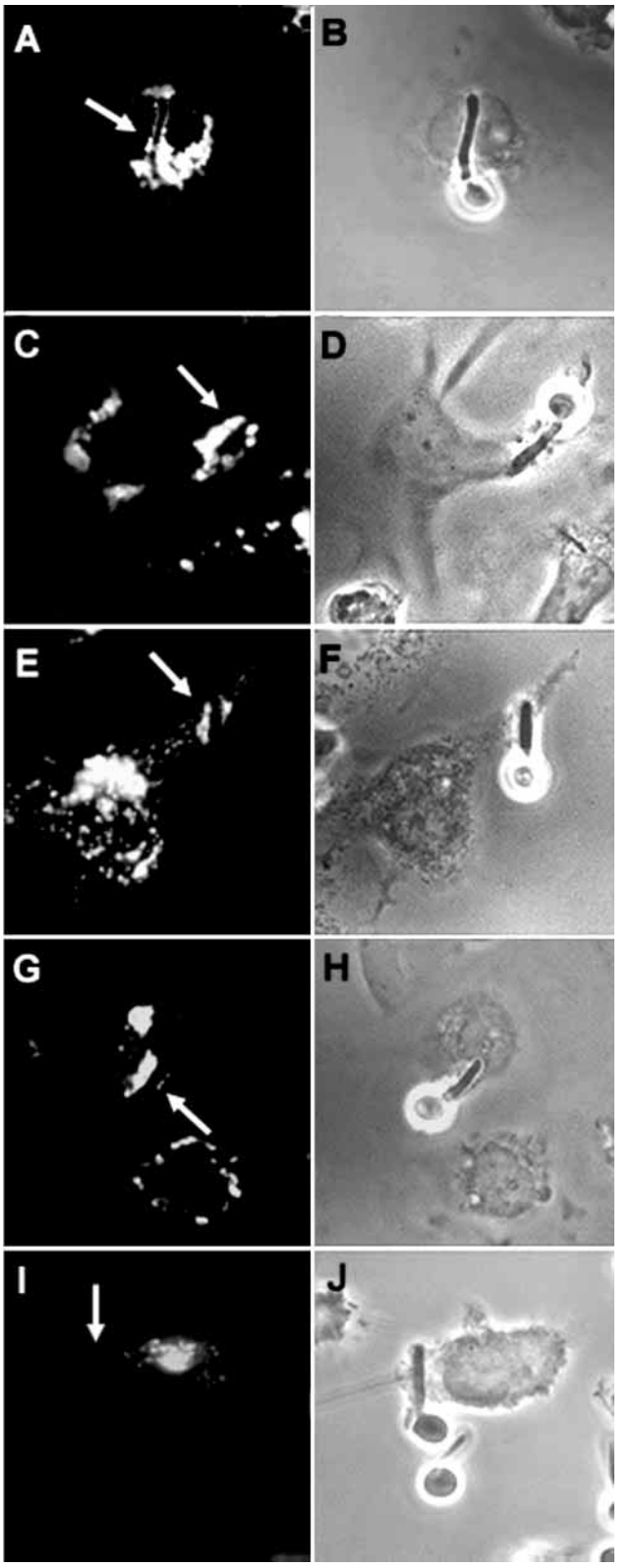


Fig. 11. Germ tubes of *C. albicans* penetrate MØ and attract $Lamp^+$ compartments. Peritoneal MØ were treated with inhibitors of phagocytosis (0.2 μM cytochalasin D: A-B; 10 μM nocodazole: C-D; 0.1 μM wortmannin: E-F; 0.2 μM staurosporine: G,H; 0.1 μM thapsigargin: I-J) and were incubated with *Candida* blastoconidia for 60 minutes (A-H) or with preformed germ tubes for 15 minutes (I-J) (thapsigargin inhibited germ tube formation). Cells were fixed, permeabilised and processed for Lamp immunostaining using specific mAbs and a FITC-labelled secondary antibody. When phagocytosis was blocked the yeast remained outside, but bound to the MØ and by 60 minutes of incubation the *Candida* formed germ tubes that penetrated the cell, and fused with $Lamp^+$ organelles. Cytochalasin, nocodazole, wortmannin and staurosporine did not block the attraction of $Lamp^+$ compartments, but in the presence of thapsigargin germ tubes grew into the cytosol and failed to recruit lysosomes. The left panel is a single representative image of confocal microscopy, the right, the corresponding phase-contrast image. Arrows point to the penetrating germ tubes in the immunofluorescence images. Scale: 0.09 μM/1 pixel; the width of each picture = 384 pixels.

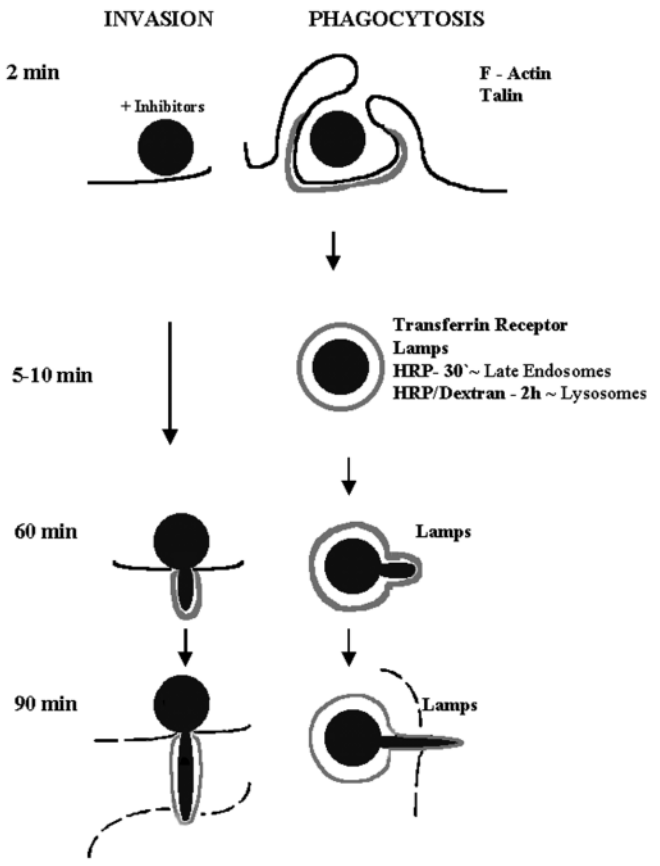


Fig. 12. Proposed model for internalisation of *C. albicans* by MØ cultured with or without phagocytosis inhibitors, and for maturation of the *Candida*-phagosome. In the presence of inhibitors, the yeast remains extracellular, bound to the MØ surface and forms germ tubes penetrating the cell within a Lamp$^+$-compartment after 60 minutes of incubation. When phagocytosis is not inhibited, the MØ-bound yeast induces membrane ruffling and an F-actin-rich, talin$^+$-phagocytic coat appears around the particle in the submembranous area. By 5 minutes of phagocytosis, the phagosome has become F-actin$^-$, but still talin$^+$, and has been surrounded by HRP$^+$-organelles (chased for 10 minutes), corresponding to early endosomes. From that time the proportion of transferrin receptor$^+$ and Lamp$^+$ phagosomes has increased, and they fuse with HRP$^+$-vacuoles (chased for 30 minutes), corresponding to late endosomes, as well as HRP$^+$ and/or Dextran$^+$-structures (chased for 2 hours), corresponding to lysosomes. In the phagolysosome, *Candida* forms germ tubes distending the Lamp$^+$-membrane, then escapes from the cell in a thin Lamp$^+$-coat.

phagocytic process are controversial (Greenberg et al., 1991; Hishikawa et al., 1991). Previous study has demonstrated that *C. albicans* could evoke Ca^{2+} transients in the first few minutes of uptake by human monocyte-derived MØ only if they were serum-opsonised or the cells had been activated by IFN-γ (Maródi et al., 1991a, 1993).

Our data show that the newly formed phagosomes attracted late endosomes and lysosomes, fusing with them soon after depolymerisation of the surrounding actin, regardless of heat-inactivation or serum-opsonisation. These findings suggest the rapid recruitment of late endocytic organelles and their fusion with *Candida*-phagosomes are not MR-specific, and might be provoked by the pathogen.

One possibility is that *Candida* might have developed mechanisms to exploit pre-established cellular mechanisms for regulated lysosomal secretion (Rodriguez et al., 1997) in order to favour its own survival. Although extracellular germ tube formation was more efficient, *Candida* yeasts also formed germ tubes within the phagolysosomes of MØ. We have no explanation for the host inhibitory mechanisms involved, but our data argue in favour of a further level of regulation dependent on the phagolysosomal localisation of the ingested yeast. Our results showed that the late endosomal/lysosomal milieu is relatively favourable for sprouting, since neutralisation of pH and inhibition of lysosomal H^+-ATP-ase with concomitant effects on lysosomal movement and fusion, further decreased the frequency of germ tube formation. Accordingly, the frequency of germ tube formation in MØ-free culture was higher at acidic pH, and this could be one of the late endosomal/lysosomal factors which promotes sprouting. Polarised growth of fungal cells has been associated with extra- and intracellular H^+ and Ca^{2+} gradients (McGillivray and Gow, 1987). We have demonstrated that germ tubes of *Candida* do not enter passively into MO, but actively attract late endocytic and lysosomal organelles for fusion, and that this could be blocked by inhibition of ER Ca^{2+}-ATP-ase with thapsigargin. The importance of Ca^{2+} in fusion of phagosomes with specific granules in neutrophils and in regulated lysosomal secretion has been demonstrated (Jaconi et al., 1990; Rodriguez et al., 1997). Dependency on Ca^{2+} gradients could also be one of the factors accounting for the inhibitory effect of thapsigargin on germ tube formation in MØ-free cultures of *C. albicans*.

C. albicans secretes acid phosphatase, phospholipase and hemolytic factors. In terms of virulence, the secreted aspartyl acid proteinase with pH optimum of 2.2-4.5 has received the most attention (Lerner and Goldman, 1993; Ibrahim et al., 1995; Manns et al., 1994; Shepherd et al., 1985). Although the connection between dimorphism and enzyme secretion is not clearly defined it is conceivable that lysosomal fusion and acidification of the vacuole might induce metabolic changes, including enzyme secretion and activation, which are consecutive or coincidental with the morphologic change, and that these together contribute to escape and survival of pathogen.

So far two microorganisms, *Trypanosoma cruzi* and *Salmonella typhimurium*, have been reported to attract late endosomal/lysosomal structures, both of them requiring an acidic environment for survival and virulence (Andrews, 1995; Garcia-del Portillo and Finlay, 1995). Lysosomal recruitment during Trypanosoma cruzi invasion may be regulated by a serine peptidase produced by the pathogen, generating factors that induce Ca^{2+}-signalling (Burleigh, 1997). *Salmonella typhimurium* fuses rapidly with selected Lamp$^+$ vesicles avoiding the mature lysosomes. Intracellular Ca^{2+}-flux is also involved in this process, but its signal transduction pathway is unknown (Joiner, 1997; Garcia-del Portillo and Finlay, 1995; Pace et al., 1993). The maturation of *Candida* phagosomes differs from that of these two microorganisms, but shares common features. The entering *Candida* recruited both late endosomes and lysosomes, but might also fuses with early endosomes; its phagosome is highly fusogenic and becomes Lamp$^+$and also transferrin receptor$^+$.

Apart from its role in infection, *C. albicans* provides a useful experimental tool to explore cellular mechanisms involved in endocytic organelle movement and fusion. Better

understanding of this process should clarify pathogenic properties of the fungus and help understand the biology of the endocytic pathway.

This work was supported by grants from the Medical Research Council, the National Science Foundation of Hungary (OTKA T 017 100 and 025 780) and European Commission (PECO No CIPD CT 9400303). Facilities for confocal and electron microscopy were funded by the Wellcome Trust. The first author is a participant in the UK-Hungary Health Scientist Exchange Program jointly funded by the British Council, Hungarian Ministry of Education and Soros Foundation. We thank Dr Philip D. Stahl, Dr Norma W. Andrews and Dr Gillian Griffiths for critical reading and comments, and thank Peter Tree, Lance Tomlinson and Jeremy Sanderson for technical assistance.

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