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Acanthamoeba polyphaga Trophozoite Binding of Representative Fungal Single Cell Forms

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Summary. *Acanthamoeba polyphaga* trophozoites bind yeast cells of *Candida albicans* isolates within a few hours, leaving few cells in suspension or still attached to trophozoite surfaces. The nature of yeast cell recognition, mediated by an acanthamoebal trophozoite mannose binding protein is confirmed by experiments utilizing concentration dependent mannose hapten blocking. Similarly, acapsulate cells of *Cryptococcus neoformans* are also bound within a relatively short timescale. However, even after protracted incubation many capsulate cells of *Cryptococcus* remain in suspension, suggesting that the capsulate cell form of this species is not predated by acanthamoebal trophozoites. Further aspects of the association of *Acanthamoeba* and fungi are apparent when studying their interaction with conidia of the biocontrol agent *Coniothyrium minitans*. Conidia which readily bind with increasing maturity of up to 42 days, were little endocytosed and even released. Cell and conidial surface mannose as determined by FITC-lectin binding, flow cytometry with associated ligand binding analysis and hapten blocking studies demonstrates the following phenomena. *Candida* isolates and acapsulate *Cryptococcus* expose most mannose, while capsulate *Cryptococcus* cells exhibit least exposure commensurate with yeast cellular binding or lack of trophozoites. Conidia of *Coniothyrium, albeit in a localized fashion,* also manifest surface mannose exposure but as shown by Bmax values, in decreasing amounts with increasing maturity. Contrastingly such conidia experience greater trophozoite binding with maturation, thereby questioning the primacy of a trophozoite mannose-binding-protein recognition model.

Key words: Conidia, Endocytosis, Mannose, Trophozoite, Yeast.

INTRODUCTION

Acanthamoebae, which are free-living small protozoa, occur both in an actively motile trophozoite form and a resistant double walled cyst form, the latter often of great longevity. These organisms are commonly found in moist soils and are ubiquitous in a wide range of natural and man-made aquatic habitats (Biddick *et al.* 1984, Visvesvara and Stehr-Green 1990, Rodriguez-Zaragoza *et al*. 1994). In line with such widespread distribution, human contact is commonplace, in turn these organisms may be considered opportunistic human pathogens and are associated with life threatening acanthamoebal keratitis or encephalitis (Khan 2009).

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Mannose or mannosylated entities have been long recognized to be important in acanthamoebal attachment or surface recognition induction of phagocytosis by trophozoites (Allen and Dawidowicz 1990) and the progression of acanthamoebal keratitis (Yang *et al*. 1997, Leher *et al.* 1998, Garate *et al.* 2004, Garate *et al.* 2005, Khan 2009). These authors have also demonstrated that trophozoites express a specific cell surface mannose receptor, which supports trophozoite binding of mannose rich entities, as well as initiating the onset of phagocytosis and lysosomal formation, a process similar to that employed by phagocytic cells associated with innate immunity (Gordon 2002). In turn trophozoites, through a 136 kDa protein, also specifically bind to corneal epithelium mannose-glycoproteins, which results in cytolytic factor release by trophozoites and corneal degradation.

Fungal cell walls are rich in mannose (Gooday 1995), where mannose takes the form of an outer layer matrix of glycoproteins overlaying an inner fungal cell wall layer of more fibrous chitin and glucan moieties. Using a combination of mannose specific fluorescent lectins, flow cytometry and confocal microscopy, Elloway *et al.* (2004), Smith *et al.* (2001), Foster *et al.* (2004) and Smith *et al.* (1999), have respectively studied acanthamoebal cyst and trophozoite, fungal cell and conidial surface mannose exposure, thereby demonstrating that total mannose exposure varies amongst species and cell types.

This study further investigates the surface interaction and associated recognition phenomena between various single cell fungal forms and trophozoites of *Acanthamoeba polyphaga.* Single cell entities of pathogenic fungi of varying morphology including those of yeasts *Candida albicans* (Odds *et al.* 2006), *Cryptococcus neoformans* both capsulate and acapsulate (Perfect 2006), as well as conidia of *Coniothyrium minitans,* the latter a well characterized mycopathogen and commercial biocontrol agent (Whipps and Gerlagh 1992, McQuilken *et al.* 1995, Whipps and Davies 2000, Whipps and McQuilken 2009) with a worldwide natural distribution (Sandys-Winsch *et al*. 1993) were selected. Trophozoite interaction with stationary phase cells of *Candida* and *Cryptococcus* as well as *Coniothyrium* conidia of varying maturation is assessed, together with the specificity of such interaction through the use of methyl-α-D-mannopyranoside. Further experiments determine cell surface mannose exposure through use of fluorescein isothiocyanate (FITC) complexed lectins of known mannose specificity (Van Damme *et al.* 1998), flow cytometry, ligand-receptor binding analysis (Wingard *et al.* 1991) and localization of conidial surface mannose using confocal microscopy.

MATERIALS AND METHODS

Organisms, isolates and culture methods

Organisms employed and associated culture conditions are well defined. Stock cultures of *Acanthamoeba polyphaga*, originally associated with an outbreak of Legionnaire's disease were obtained from Leeds Public health Laboratory, Leeds, UK and maintained in the manner outlined by Barker *et al.* (1992, 1993, 1999).

Candida albicans isolates TP7, 630G, 017J and 398M as characterized by Smith *et al.* (2001), three capsulated isolates of *Cryptococcus neoformans* designated 5815, 5854, 45922 and the acapsulate isolate 52817(Cap 67) as characterized by Foster *et al.* (2004) were cultured and maintained as outlined by said authorities.

Four dark isolates of *Coniothyrium minitans* (Sandys-Winsch *et al.* 1993) namely A2 960, B1300/2, CH1 and CH2 were maintained as outlined by Smith *et al.* (1998).

Trophozoite – yeast cell and conidial association

Acanthamoebal trophozoites – replicate 10-ml-five-day-old cultures of *Acanthamoeba* were employed, as these yielded the desired number of active trophozoites. In turn trophozoites were harvested by centrifugation at 1000 g for 10 min., washed twice in amoebal saline as defined by Rowbotham (1983), resuspended in fresh amoebal saline and their numbers adjusted to 0.5×10^6 cells ml^{-1}, prior to being incubated under starvation conditions for a further 24 hour. In turn 1 ml aliquots of starved *Acanthamoeba* were dispensed into sterile microcentrifuge tubes and pelleted by centrifugation at 1000 g for 10 min. prior to near immediate addition of fungal cells or conidia.

Yeast phase cells – were obtained by inoculating a single colony from subcultures into Sabouraud liquid medium and incubating overnight at 37°C, 250 rpm. Subsequently 100 µl aliquots of culture were inoculated into fresh Sabouraud dextrose liquid medium (Oxoid) and incubated for 20 hours at 37°C and 250 rpm. Aliquots of 1 ml after repeated centrifugation at 10,000 g, resuspension of resultant cell pellets in amoebal saline and adjustment to a cell concentration of 1.0×10^7 cells ml^{-1}, to ensure a subsequent trophozoite/cell ratio of 1 to 20, were added to acanthamoebal pellets already present in microcentrifuge tubes and the pellets gently dispersed. All samples were subsequently incubated on a rollermixer (40 rpm) at 25°C for up to 24 hours in darkness. Free cells in each replicate sample were determined over the experimental period by removal of 10 µl and their numbers/concentration determined by means of haemocytometer based assessment.

Similarly, yeast cells of *Candida* isolates TP7, 017J and the acapsulate *Cryptococcus* isolate 52817 were adjusted to a concentration of 1.0×10^7 cells ml^{-1} but resuspended in a range of methyl-α-D-mannopyranoside concentrations up to 200 mM in amoebal saline and then added to acanthamoebal pellets, which were gently dispersed. Samples were incubated as described above and the numbers of free cells again determined.

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The time course of binding of *Candida* to acanthamoebal trophozoites in the presence of different mannose concentrations was fitted by a mono-exponential decay function, constrained to reach 0, to estimate the first-order rate constant for the process and also $T_{1/2}$, the time needed to achieve half the binding, which is reciprocally related to the rate constant. Each binding curve was normalised by setting the initial value to 1. Subsequently, relationship between the first order rate constants obtained above and the mannose concentration was fitted to either a mono- or biexponential function. Curve fitting was by PRISM Graphpad 4 (Graphpad Software Inc.).

Coniothyrium conidia – of varying age were produced and harvested in a similar manner to that outlined by Smith *et al.* (1998, 1999) but suspended in amoebal saline. Replicate suspensions were individually adjusted by extensive dilution to ensure 1.0×10^7 conidia ml^{-1} would eventually be mixed with *Acanthamoeba*, a trophozoite/conidia ratio of 1 to 20. Aliquots of 1 ml, after repeated centrifugation at 10,000 *g*, removal of resultant superanatants and resuspension of conidia in amoebal saline, were added to acanthamoebal pellets already present in microcentrifuge tubes and said pellets gently dispersed in conidial suspensions. All samples were subsequently incubated on a rollermixer as described above and the numbers of free conidia determined over 24 hours.

Similarly, conidia of strain B1300 and CH2 were harvested from 18-day-old-cultures and adjusted to a concentration of 1.0×10^7 conidia ml^{-1}. After repeated washing, conidia were finally resuspended in a range of methyl-α-D-mannopyranoside concentrations up to 200 mM in amoebal saline and as above added to acanthamoebal pellets, which were again gently dispersed. Samples were incubated and the numbers of free conidia determined over eight hours.

Yeast cell and conidial surface mannose exposure

Flow-cytometry determination of FITC-lectin binding – replicate 20 hours yeast cell samples from *Candida* isolates TP7, 017J and acapsulate *Cryptococcus* 52517 were obtained as described above but suspended in 10 mM Hepes pH 7.5 buffer and adjusted to 1.0×10^7 ml^{-1}. In turn conidial samples of varying age (12–42 days) from B1300, CH1 test *Coniothyrium* strains were initially suspended in 10 mM Hepes pH 7.5 buffer then adjusted to 1.0×10^7 ml^{-1}. Subsequent determination of fungal FITC-lectin binding followed protocols outlined by Smith *et al.* (1999, 2001), Foster *et al.* (2004). FITC-lectins employed were concanavalin A (Con A), 5.7 FITC mol^{-1} lectin and *Galanthus nivalis* (GNA), 5.2 FITC mol^{-1} lectin (Vector Laboratories). Relative cell and conidial population fluorescence was determined with a Beckman Coulter, Cell Lab Quanta SC flowcytometer employing an argon laser (22 mW), excitation wavelength 488 nm, gated and optimized around a range of FITC-microspheres of similar size to test cells and conidia (Polysciences, Warrington, USA).

Fluorescence values, which represent specific lectin concentration binding, were further assessed as in Smith *et al.* (2001) and Foster *et al.* (2004) to derive Bmax values, which indicate the total number of specific ligand or lectin binding sites and KD values, and therefore estimate the concentration of ligand or lectin required to occupy half the total number of binding sites, from binding curves of lectin concentration, relative fluorescence and conidial strain age.

Similarly, the specificity of lectin binding to yeast cells of representative *Candida* isolates TP7, 017J, *Cryptococcus* 52817 and *Coniothyrium* isolates B1300, CH1 was also determined. Yeast cells and conidia from 18 day old cultures at a concentration of 1.0×10^7 ml^{-1} were obtained as described above however, cell and conidial samples were finally suspended in 200 mM methyl-α-D-mannopyranoside in 10 mM Hepes pH 7.5. Such suspensions were incubated with varying FITC-lectin concentrations, washed and their fluorescence determined as before.

Confocal microscopy imaging of conidial FITC-lectin binding – conidia were harvested as noted above into 10 mM Hepes pH 7.5 and in a similar fashion exposed to FITC-Con A (100 µg ml^{-1}) and subsequently suspended in Hepes/paraformaldehyde. Aliquots of 5 µl were dispensed into the four central wells of multisport microscope slides (Hendley) and gently air-dried. Wells were then sealed with 5 µl Vectashield (Vector Laboratories, USA) and stored at 5°C, in the dark, until viewed with a Zeiss LSM 510 Meta/Zeiss Plan-Apochromat 63 × objective (Carl Zeiss) facility. In turn through a combination of filters, laser intensity (488 nm/633 nm), pin-hole dimensions, gain and offset functions, image material (512 × 512 pixels) was captured and manipulated in associated software (Laser Scanning Microscope LSM510 Version 3.2 SP2), exported into Adobe Photoshop Elements (Adobe Systems Incorporated, USA).

Statistical analysis – data analysis was carried out using STATISTICA software (Statsoft Inc., 2300 East 14th St, Tulsa, Ok, 74104, USA). The effect of fungal species and strain, acanthamoebal/yeast cell/conidial association period (0–24 hours), conidial age and hapten blocking was analysed using three factor analysis of variance (ANOVA) with association period as a repeated measure where appropriate. In turn the levels of FITC-lectin mediate fluorescence, their concentrations, fungal species, conidial age for each test strain was analysed using three factor repeated measure ANOVA. These analyses enabled both the main effects of each variable to be tested but also their interaction with time. Finally the effect of blocking, lectin concentration and fungal species and strain were analysed using two-factor and three factor ANOVA. Where appropriate 95% confidence limits were calculated.

RESULTS

Trophozoite-yeast cell association (Fig. 1) demonstrates that cells of all test strains of *Candida* are significantly bound to trophozoites within a relatively short timescale ($F = 1812.1$; df 5,15; $P < 0.001$). Similarly microscopic observation of *Candida* cells shows that they are readily bound to the surfaces of trophozoites and after an extended incubation period neither surface bound nor trophozoite internalised *Candida* cells were observed. By contrast, Fig. 2 shows that *Cryptococcus* trophozoite interaction varies significantly with test strain ($F = 13.1$; df 3,8; $P < 0.01$). Numbers of free acapsulate (52817) cryptococcal cells are greatly reduced suggestive of their ready binding to trophozoites. All test capsulate *Cryptococcus* strains are not readily bound by trophozoites and large numbers of free cells were present over the incubation period. Observation of mixed trophozoites/cryptococcal populations demonstrates frequent extension and near contact between

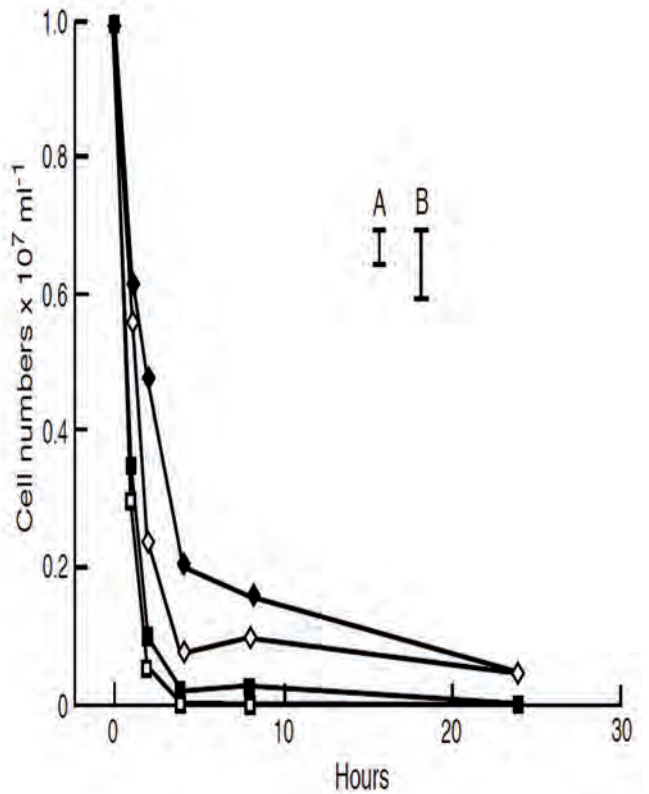

Fig. 1. Binding and potential phagocytosis of *Candida albicans* strains, □ 017J; ◆ 398M; ■ 630G; ◇ TP7; by *Acanthamoeba polyphaga* trophozoites over 24 hours as reflected in numbers of free yeast like cells remaining in solution. **A** – 95% confidence limits for differences between times within a strain and **B** – 95% confidence limits between two strains at the same or different times.

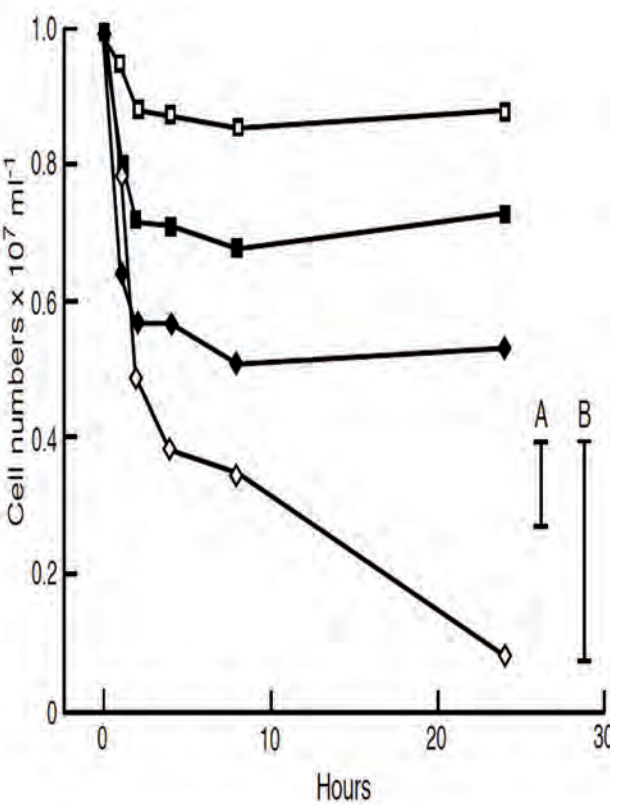

Fig. 2. Binding and potential phagocytosis of capsulate and acapsulate *Cryptococcus neoformans* strains, □ 5815; ◆ 5854; ■ 45922; ◇ 52817 (acapsulate); by *Acanthamoeba polyphaga* trophozoites over 24 hours as reflected in numbers of free yeast like cells remaining in solution. **A** – 95% confidence limits for differences between times within a strain and **B** – 95% confidence limits between two strains at the same or different times.

threadlike psuedopodia and capsulate cryptococcal cells but no obvious internalised cells.

The specificity of the binding process to yeast like cells, is shown in Fig. 3a and Fig. 3b. There is binding of *Candida* ($F = 255.1$; $df = 6,14$; $P < 0.001$) and acapsulate *Cryptococcus* ($F = 76.1$; $df = 6,14$; $P < 0.001$) cells to trophozoites, which is significantly inhibited by increasing concentrations of the inhibitory hapten methyl-α-D-mannopyranoside. The rate of cellular binding, as assessed by either the first-order rate constant, or the $T_{1/2}$ (the time for half binding to occur), is progressively reduced by increasing inhibitor concentrations. Trophozoite binding of isolate TP7 is more sensitive to methyl-α-D-mannopyranoside presence than 017J, with an almost complete block being achieved by 200 mM methyl-α-D-mannopyranoside. The dependence of the rate constant on inhibitor concentration is shown in Fig. 3c. For isolate TP7, these data are fitted by a mono-exponential decay function with a concentration of 8.8 mM methyl-α-D-mannopyranoside inhibiting binding by 50%. By contrast, the relationship of isolate 017J and trophozoite binding requires a more complex biexponential function, indicating that the effects of methyl-α-D-mannopyranoside are not the result of simple blocking.

Trophozoite-Coniothyrium conidia association – representative results for 18-day-old-conidia from test strains are shown in Fig. 4a. Significant numbers of conidia, irrespective of strain, are bound to trophozoites within one hour of incubation. In addition, there are further reductions in free conidia after one hour, which reach their lowest numbers between two and four hours. Numbers of free conidia subsequently rise irrespective

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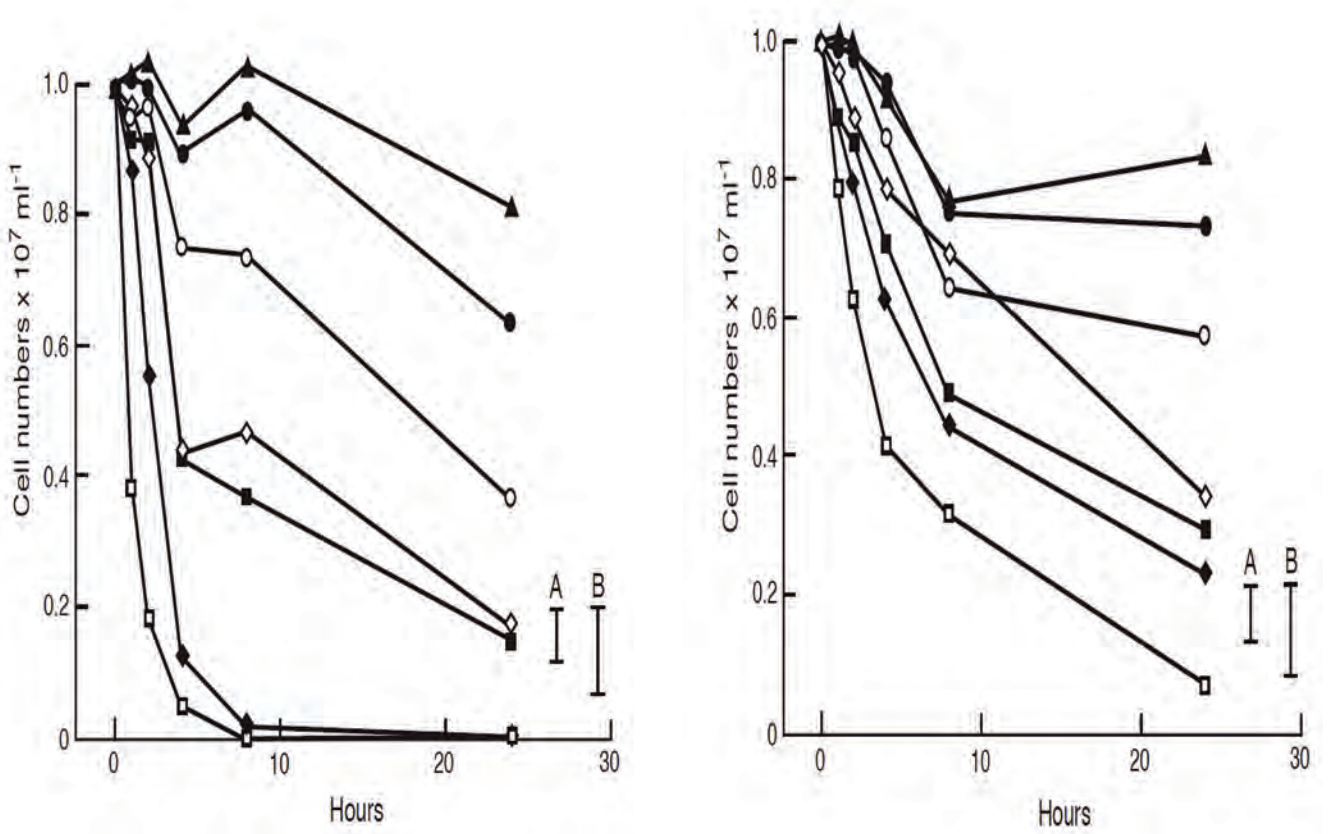

Figs 3a, b. Specificity of representative *Candida albicans* strain TP 7 (Fig. 3a) and acapsulate *Cryptococcus neoformans* strain 52817 (Fig. 3b) binding to acanthamoebal trophozoites as demonstrated through concentration dependent hapten methyl-α-D-mannopyranoside blocking. Concentrations of methyl-α-D-mannopyranoside, □ control lacking hapten; ◆ 10 mM; ■ 25 mM; ◇ 50 mM; ○ 100 mM; ● 150 mM; ▲ 200 mM. **A** – 95% confidence limits for differences between times within a methyl-α-D-mannopyranoside concentration and **B** – 95% confidence limits between two methyl-α-D-mannopyranoside concentrations at the same or different times.

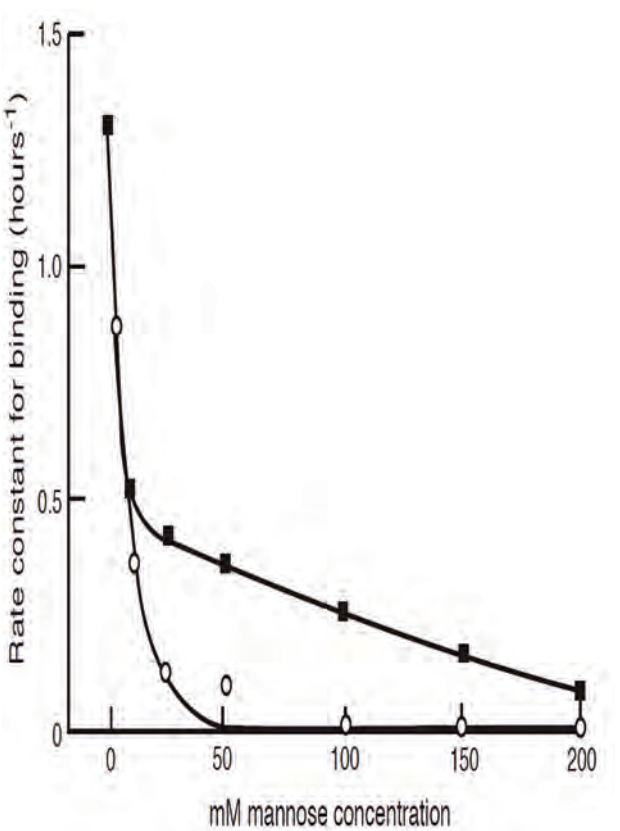

Fig. 3c. Dependence of rate constant for binding with respect to methyl-α-D-mannopyranoside concentration (⑩ TP 7, 017J). Rate constants were computed from the data associated with Fig. 3a for TP 7 and corresponding data for 017J.

of strain until the end of the experiment, a time trend that is highly significant ($F = 758.93$, $df = 5,250$; $P < 0.001$) but with some significant difference between strains ($F = 2.227$; $df = 20,50$; $P < 0.01$). In addition, the pattern varies with conidial age ($F = 16.052$, $df = 20,80$; $P < 0.001$), but the general trend shown in Fig. 4a is little altered over 42 days maturation. The avidity of trophozoites for conidia results in considerable numbers appearing to surface bind conidia, thereby forming large conglomerates of cross linked trophozoites and conidia. Observations also suggest that bound or adherent conidia are not readily phagocytosed, even after 24 hours, hence their release in small numbers from a bound state rather than their endocytosis.

Ageing and maturation of conidia over 42 days, markedly affects the extent of binding to acanthamoebal trophozoites. This phenomenon is demonstrated in Fig. 4b, which shows free conidial numbers derived from cultures of varying age, after four hours incubation with trophozoites, which as indicated in Fig. 4a are at or near their lowest values. There is marked variation with fungal strain/conidial maturation periods and association over 24 hours with trophozoites ($F = 3.27$; $df = 4,50$; $P < 0.05$; $F = 16.05$; $Df 20,80$; $P < 0.001$). In particular, young conidia of 12 days, the minimum incubation period supporting sufficient conidial recovery, are less readily bound by acanthamoebal trophozoites. After a further six days or longer maturation periods, significantly greater numbers are bound and fewer free conidia observed ($F = 11.61$; $df 4,50$; $P < 0.001$).

The specificity of the binding process is shown in Fig. 5, which shows that binding of conidia to tropho-

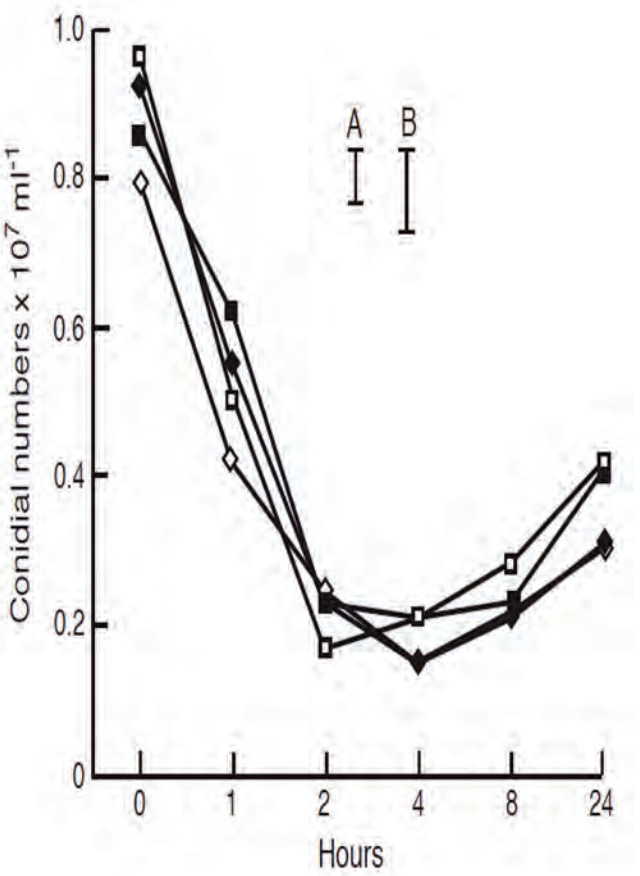

Fig. 4a. Binding and potential release of *Coniothyrium* conidia strains, □ A2960; ◆ B1300; ■ CH1; ◇ CH2; by *Acanthamoeba polyphaga* trophozoites over 24 hours as reflected in numbers of free conidia remaining in solution. **A** – 95% confidence limits for differences between times within a strain and **B** – 95% confidence limits between two strains at the same or different times.

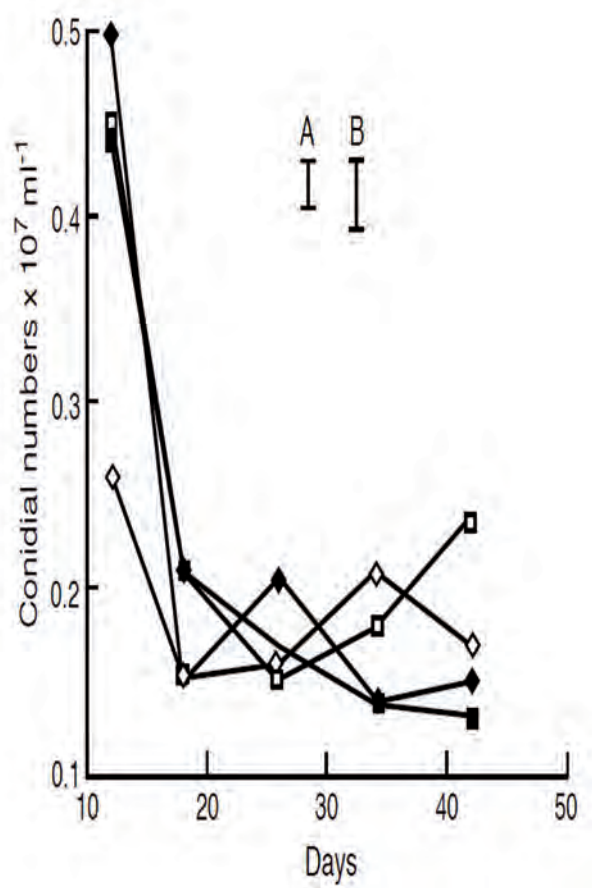

Fig. 4b. Binding of *Coniothyrium* conidia of varying maturity from strains, □ A2960; ◆ B1300; ■ CH1; ◇ CH2; by *Acanthamoeba polyphaga* trophozoites as reflected in numbers of free conidia remaining in solution. **A** – 95% confidence limits for differences between times within a strain and **B** – 95% confidence limits between two strains at the same or different times.

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zoites is reduced by an inhibitory hapten. Increasing concentration of methyl-α-D-mannopyranoside, irrespective of incubation period, reduces the numbers of conidia that are bound to trophozoites ($F = 8.67$, $df = 7,80$; $P < 0.01$), and hence the greater numbers of free conidia (Fig. 5), a trend significantly ($F = 6.44$, $df = 6,44$; $P < 0.01$) maintained throughout the assessment period. However, the 95% confidence limits suggest that inhibition by methyl-α-D-mannopyranoside of conidial binding compared to that of *Candida* strains (Fig. 3a) is not as marked and appears less graduated with increasing methyl-α-D-mannopyranoside concentrations.

Yeast cell fluorescent lectin avidity – as determined by flowcytometry, increasing concentration of FITC-ConA and –GNL (Fig. 6a) resulted in progressively and significantly greater cell fluorescence ($F = 124.19$; $df = 5,144$; $P < 0.001$) with respective FITC-lectin usage ($F= 4.23$; $df = 1,144$; $P < 0.05$), which also differed significantly amongst test species and isolates ($F = 212.07$; $df = 2,144$; $P < 0.001$). *Candida* cells, irrespective of isolate and FITC-lectin, demonstrated the greatest fluorescence values. A similar pattern may be observed when acapsulate *Cryptococcus* 52817 is incubated with increasing concentrations of FITC-ConA, but it shows markedly less fluorescence when incubated with FITC-GNL indicating that the nature of cell surface mannose exposure may differ between *Candida* and *Cryptococcus* isolates. Furthermore capsulate *Cryptococcus* 5815 exhibits significantly less fluorescence irrespective of test FITC-lectin, which reflects the paucity of lectin accessible cell surface mannose. While the paucity of mannose exposure to test FITC-lectin precludes the use of *Cryptococcus* 5815, specificity of lectin binding is demonstrated in Fig. 6b, and when compared with Fig. 6a shows a significant reduction in fluorescence when cells and FITC-lectins are incubated in the presence of 200 mM methyl-α-D-mannopyranoside (*Candida* TP7, ConA/GNL, $F = 599.06/67.7$; both $df = 1,28$, $P < 0.001$ *Candida* 017J, ConA/GNL, $F = 458.5/53.03$; both $df = 1,28$; $P < 0.001$ *Cryptococcus* 52817, ConA/GNL, $F = 1020.5/465.5$, both $df = 1,28$; $P < 0.001$). The progressive increase in fluorescence with increasing FITC-lectin concentration (Fig. 6a) supports determination of Bmax and KD values. With the exception of acapsulate *Cryptococcus* where KD values obtained are compromised by the paucity of binding sites, KD values irrespective of lectin and isolate are relatively low, indicative of tight affinity and common binding sites. By contrast, Bmax values differ markedly amongst species and isolates (Table 1). Greatest values are obtained for FITC-ConA, while those of a lesser extent occur with the more mannose specific FITC-GNL irrespective of species. Corresponding Bmax values suggest the following order: *Candida* isolates > acapsulate *Cryptococcus*, > capsulate *Cryptococcus*.

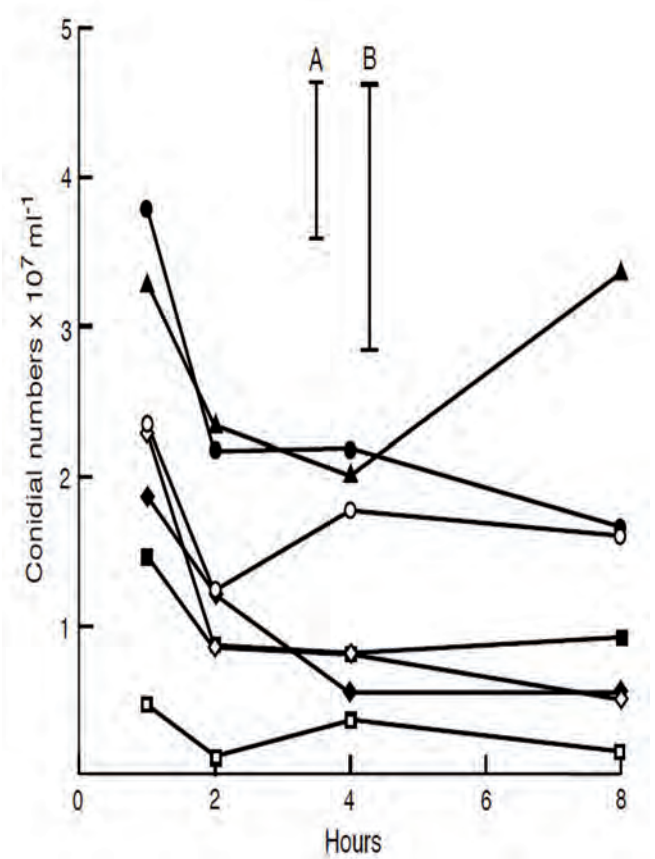
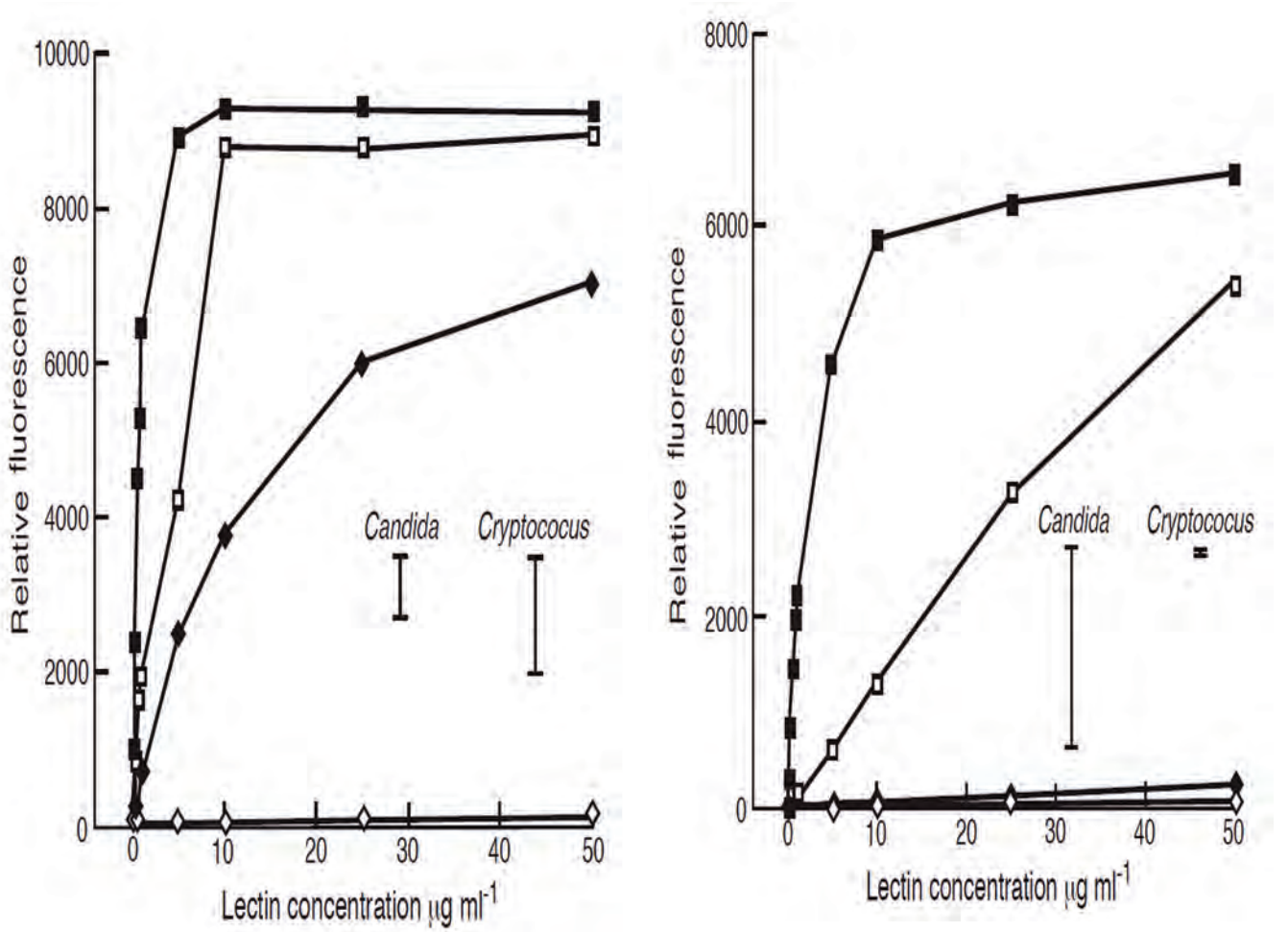


Fig. 5. Specificity of representative *Coniothryrium minitans* strain CH2 binding to acanthamoebal trophozoites as demonstrated through concentration dependent hapten methyl-α-D-mannopyranoside blocking. Concentrations of methyl-α-D-mannopyranoside, □ control lacking hapten; ◆ 10 mM; ■ 25 mM; ○ 50 mM; O 100 mM; ● 150 mM; ▲ 200 mM. **A** – 95% confidence limits for differences between times within a methyl-α-D-mannopyranoside concentration and **B** – 95% confidence limits between two methyl-α-D-mannopyranoside concentrations at the same or different times.

Table 1. *Candida* and *Cryptococcus* cellular Bmax values calculated from FITC-lectin fluorescence.

	FITC-Lectin	
Species/Isolate	Con A	GNL
Candida TP7	10346	9731
Candida 017J	9523	8004
Cryptococcus 52817	8979	95.13
Cryptococcus 5815	1441	36.05



Figs 6a, b. Fluorescence as determined by flow cytometry of representative yeast cells from *Candida albicans* strain TP7 and acapsulate *Cryptococcus neoformans* strain 52817 after incubation with increasing concentrations of FITC-conjugated lectins alone and in the presence of 200 mM methyl-α-D-mannopyranoside (Fig. 6b). ■ *Candida albicans* TP7, FITC-Con A; □ *Candida albicans* TP7, FITC-GNL; ◆ *Cryptococcus neoformans* 52817, FITC-Con A; ◇ FITC-GNL. Bars denote 95% confidence limits for differences within a treatment

Conidial fluorescent lectin avidity – relative fluorescence results for 18-day-old-strain CH1 conidia are shown by Fig. 7a. Increasing concentrations of FITC-ConA and GNL resulted in significantly greater conidial fluorescence (CH1 $F = 20.76$, B1300 $F = 187.24$; both df = 1,112, $P < 0.001$). In turn, the specificity of lectin binding is confirmed (Fig. 7b), as significantly less conidial fluorescence is shown in the presence of 200 mM methyl-α-D-mannopyranoside irrespective of test lectin (CH1 $F = 101.63$, B1300 $F = 830.568$; both df = 1,112; $P < 0.001$). KD values (Fig 7a) remain relatively unchanged at greater than 10 nM throughout the 42 days maturation period, suggesting the concentration of lectin required to occupy half the total number of binding sites alters little with conidial maturation. By contrast, Bmax values (Fig. 7c), fall significantly with increasing conidial maturity (CH1 $F = 733.73$, B1300 $F = 1253.26$; both df = 1,112; $P < 0.001$), suggesting that the total number of ligand or lectin binding sites is modified by conidial aging. Confocal imaging as represented in Fig. 8, reveals that the distribution of fluorescence is not uniform. Z section imaging, which as a result of combining multiple section images reveals rounded entities, suggests the surface topography of conidia is markedly irregular. Hence, fluorescence exposure and lectin binding and associated mannose moiety

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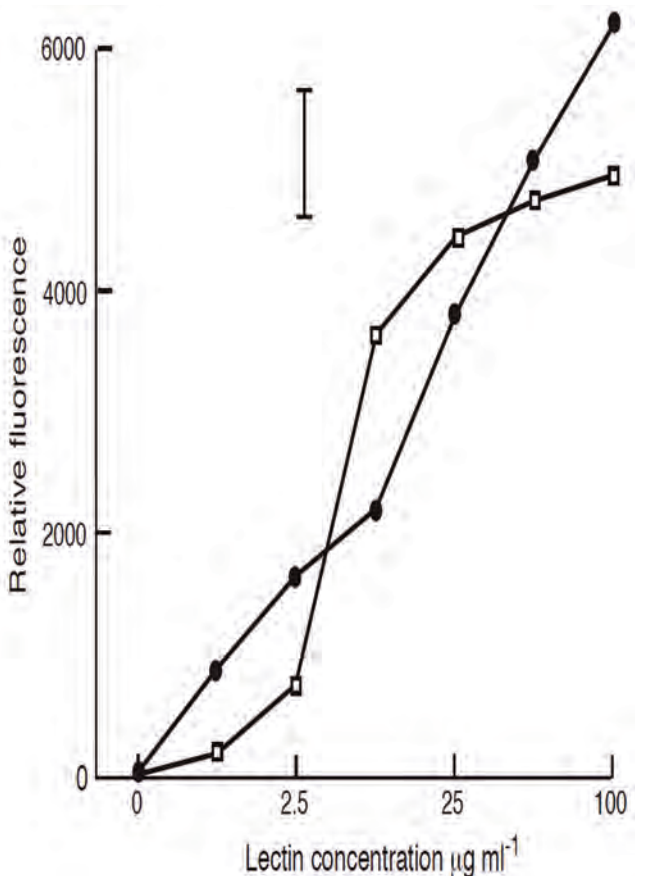

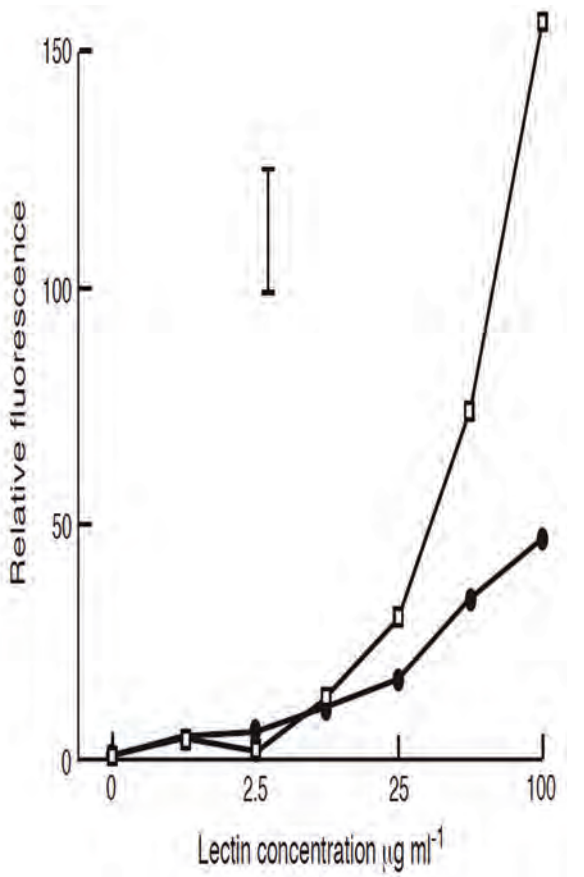

Figs 7a, b. Fluorescence as determined by flow cytometry of representative *Coniothyrium minitans* strain CH1 conidia after incubation with increasing concentrations of FITC-conjugated lectins alone and in the presence of 200 mM methyl-α-D-mannopyranoside (Fig. 7b). *Coniothyrium minitans* CH1, □ FITC-Con A; ● FITC-GNL. Bars denote 95% confidence limits for differences within a treatment.

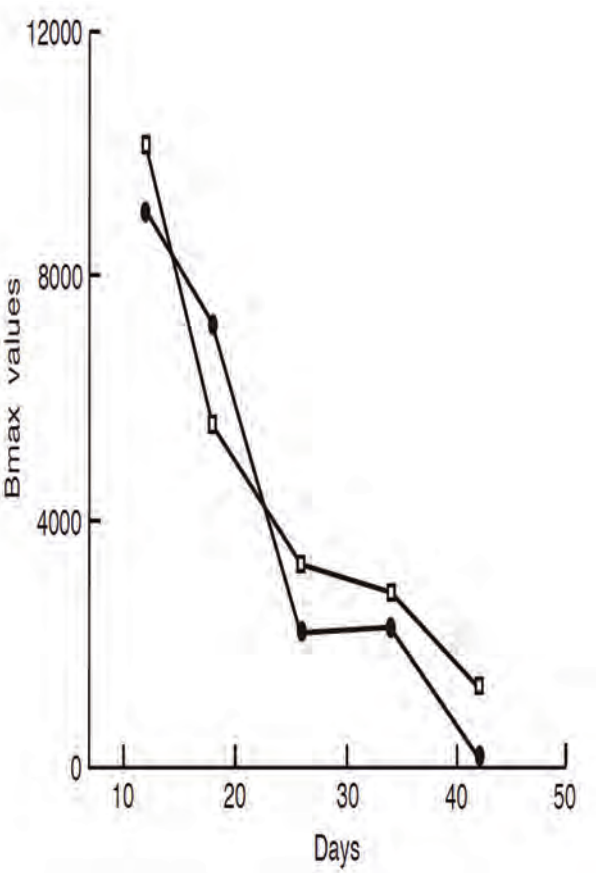

Fig. 7c. Representative Bmax values from strain CH1 *Coniothyrium minitans* conidia of increasing maturity (□ FITC-Con A; ● FITC-GNL).

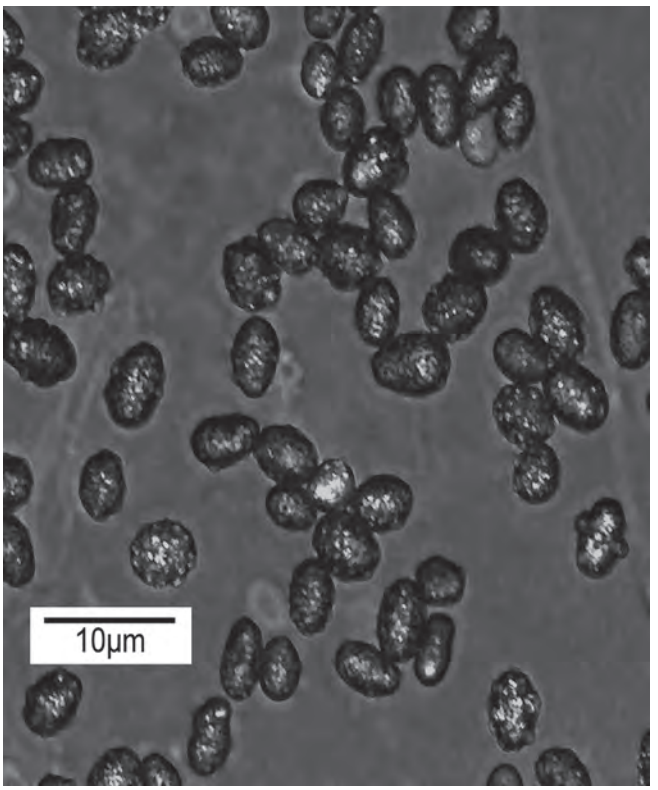

Fig. 8. Representative Z section confocal image of *Coniothyrium minitans* strain B1300 conidia after incubation with 100 µg FITC-Con A ml^{-1}.

exposure is likely to be greatly localised as a result of the irregular topography.

DISCUSSION

As previously demonstrated (Allen and Dawidowicz 1990), *Candida* isolates, which have been shown by Smith *et al.* (2001) to extensively expose mannose moieties in a uniform manner, were readily bound and phagocytosed by acanthamoebal trophozoites. As in previous studies, binding, and hence phagocytosis, was inhibited by a mannose hapten, confirming the involvement of mannose-receptor mediated interaction and recognition amongst acanthamoebal trophozoites (Khan 2009). As a result, kinetic analyses, normally reserved for free ligand binding determinations, were possible. These analyses have demonstrated that cell surface recognition is a subtle and non-homogeneous phenomenon, with differing kinetics which depend on *Candida* isolate.

Comparison of *Cryptococcus* capsulate and acapsulate surface mannose exposure, trophozoite binding and associated specificity, further demonstrates the critical nature of associated interactions in relation to trophozoite predation of cellular yeasts. As noted by Foster *el al.* (2004), acapsulate cryptococcal cells, in common with those of *Candida*, expose more cell surface mannose, than capsulate counterparts. In turn, these cells are more readily bound and potentially phagocytosed by trophozoites compared to their capsulate counterparts. *Cryptococcus* demonstrates many virulence factors (Casadevall and Perfect 1998), which may compromise phagocytosis. However, the presence of a capsule, around a mannose backbone (Doering 1999) and orientated to avoid terminal exposure of mannose, further reduces trophozoite access to the relatively low levels of cell surface mannose of cryptococcal cells in general (Foster *et al.* 2004), thus protecting such cells from widespread protozoan predation.

Studies of acanthamoebal trophozoite and fungal dispersal agents, demonstrates further aspects of conidial surface mannose exposure and trophozoite mannose binding. Although acanthamoebal trophozoites will bind *Coniothyrium* conidia within a few hours, prolonged observation of trophozoite interaction with *Coniothyrium* shows some conidial release from trophozoites. Furthermore, the blastospores of *Cladosporium* and *Alternaria* porospores (personal communication), manifest little trophozoite endocytosis or phagocytosis. These results suggest that the use of fungal spores, especially those of *Coniothyrium* as dispersal agents, should not compromise biocontrol efficacy through marked protozoan predation but some minor loss of efficacy through agglomerations of conidia and protozoa may occur.

The resistance of spores to predation or phagocytosis by macrophages associated with vertebrate fungal disease such as *Aspergillus fumigatus*, has been studied. While conidia of *Aspergillus* species may become attached to neutrophils and phagocytic cells, diffusates from conidia reduce phagocytosis by neutrophils and trophozoites (Hobson, 2000) and suppress respiratory burst biochemistry (Robertson 1991). Contrastingly, *Coniothyrium* has been shown to produce at least one toxic metabolite (McQuilken *et al.* 2003) and airborne fungal spores are associated with a marked range of toxic metabolites from specific haemolysins such as stachylysins (Vesper and Vesper 2002) to generic microbial volatile organic compounds (Fischer *et al.* 1999). Hence, although the complexity of metabolites derived from *Coniothyrium* conidia may preclude identification of specific causal agents,

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such agents may compromise phagocytosis. However, the presence of melanin amongst dark isolates of *Coniothyrium* is potentially of greater importance. Melanin, through studies associated with pathogenic fungi (Haase and Brakhage 2004) has been defined as a virulence factor (Perfect 2006). Incorporation of melanin into the fungal cell wall may ameliorate UV disruption, enhance cell wall strength and integrity, and increase antioxidant capacity. The latter is considered to reduce phagocytosis of melanin rich entities and to compromise macrophage killing and therefore, trophozoite predation of *Coniothyrium* conidia.

A significant finding of this study is the uniform decrease in numbers of free conidia when incubated with active trophozoites with increasing age of conidia. Hence, there is an increase in binding of mature conidia to trophozoites suggesting marked conidial surface change and concomitant recognition by trophozoites. Nonsanchuk *et al*. (1999) noted an association between greater macrophage phagocytosis of *Cryptococcus* and related reduced cellular charge. In turn, Smith *et al*. (1998) demonstrated that non-specific microbial adhesion forces also varied with conidial maturation. Conidial hydrophobicity varied with *Conothyrium* strain and cell surface electrostatic charge, which may be influenced by melanin (Casadevall and Perfect 1998), increased with conidial maturation. A marked contribution by non specific adhesion forces to trophozoite and *Coniothyrium* conidial interaction, in contrast to yeast cells, should therefore be dismissed.

Specificity of acanthamoebal conidial binding, via a trophozoite surface mannose-binding protein (Garate *et al.* 2004, Garate *et al.* 2005), is demonstrated by methyl-α-D-mannopyranoside blocking of conidial trophozoite binding (Fig. 5). In turn, the progressive nature of the blocking, with increasing concentrations of methyl-α-D-mannopyranoside, supports a cell-cell binding kinetic analysis. By contrast, results from *Coniothyrium* test strain B1300, while demonstrating a significant difference between samples containing methyl-α-D-mannopyranoside and those lacking methyl-α-D-mannopyranoside (Fisher protected least significant difference, $P = 0.05$), did not demonstrate a sufficiently progressive inhibition to support such analysis. These results suggest that the relationship of trophozoite mannose binding protein and conidial surface mannose moieties, is not as robust as shown by *Candida* cells or by the acanthamoebal keratitis model, where the *Acanthamoeba* mannose-binding protein is even cited as a major virulence factor (Garate *et al.* 2005).

Similarly, the weaker *Coniothyrium* conidial trophozoite interaction, mediated by mannose and associated receptor entity or entities, is demonstrated by the quantification of fluorescent mannose specific lectin binding by flow cytometry and associated determination of KD and Bmax values. The uniformity of KD values indicates that the surface mannose binding sites specific to test FITC-lectins alter little with conidial maturation. Paradoxically, conidial B max values and hence exposure of mannose moieties, significantly falls irrespective of strain. This result contrasts with that of reduced numbers of free conidia with their maturation, when incubated in the presence of fresh trophozoites. Such an observation might suggest the hypothesis that the extent of mannose exposure does not influence the frequency of mannose-binding-protein contact over and above a certain threshold. Furthermore, the rugulose conidial surface and marked localisation of mannose may compromise any association between the extent of mannose entity exposure and the frequency of trophozoite mannose-binding protein contact. In conclusion, while *Coniothyrium* conidia of increasing maturity are readily bound with at least some degree of specificity, unlike long established convention (Fraser and Ezekowitz 1999), such binding does not induce endocytosis, nor in the case of conidia, does the extent of specific mannose exposure influence the frequency of conidial trophozoite binding. These ambivalent findings question the nature of a trophozoite mannose binding protein model when considering fungal morphological entities as a whole but indicates the complexity of protozoan and macrophage spore predation phenomena.

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