Monoclonal antibodies as probes for fungal wall structure during morphogenesis

Margaret Marshall,¹ Keith Gull² and Peter Jeffries¹

Author for correspondence: Peter Jeffries. Tel: +44 1227 823478. Fax: +44 1227 787465.
e-mail: P.Jeffries@ukc.ac.uk

¹ Research School of Biosciences, The University of Kent, Canterbury, Kent CT2 7NJ, UK

² School of Biological Sciences, 2.205 Stopford Building, University of Manchester, Oxford Road, Manchester M13 9PT, UK

Three monoclonal antibodies (mAbs), S4D1, S3B3 and S1E5, were produced from hybridoma cell lines raised from mice immunized with hyphal walls of *Neurospora crassa* and one (Pax-1) from mice immunized with hyphal walls of *Paxillus involutus*. In immunofluorescence studies, the three *N. crassa* mAbs recognized epitopes with different patterns of distribution at the hyphal surface of *N. crassa*. S4D1 recognized an epitope which was present on the surface of both conidia and hyphae; S3B3 recognized an epitope seen only at the ends of conidia or in the septal region of hyphae and conidial chains; and S1E5 recognized an epitope present on the surface of hyphae, but not on mature conidia. mAb Pax-1 reacted with hyphal wall fragments of *Pax. involutus* and with *N. crassa* conidia in a similar way to S3B3. S4D1 reacted with an epitope found in 1,3- α -glucan preparations from hyphal walls of different fungi. The surface distribution of this epitope varied: it was found on the surface of both conidia and hyphae of *N. crassa* and *Aspergillus nidulans*, on the basidiospore surface only of *Amanita muscaria*, and on the hyphae but not the conidia of *Penicillium chrysogenum*. Immunogold studies revealed that the epitope was present throughout the wall of conidia and hyphae of *N. crassa*. mAbs S3B3, S1E5 and Pax-1 also reacted with other fungi: for example Pax-1 cross-reacted with all fungi tested except for a member of the Zygomycota. Immunogold studies revealed that epitopes of these three mAbs were present within the inner layers of the walls of conidia and hyphae of *N. crassa*.

Keywords: monoclonal antibodies, immunofluorescence, hyphal structure, *Neurospora crassa*, *Paxillus involutus*

INTRODUCTION

The use of mAbs as specific probes for the presence of certain fungi has received considerable attention in mycology, particularly for rapid diagnosis of plant disease (Miller & Martin, 1988; Schots *et al.*, 1994). There is also, however, the potential to use mAbs as probes to study the differential distribution of specific wall components within different fungal structures, or to monitor very local changes of wall architecture and biochemistry during hyphal and spore morphogenesis. The fungal wall is responsible for the characteristic shape of fungal structures, and information about the wall components and their distribution is important in our understanding of their role in cell integrity and

morphogenesis. Fungal walls comprise of numerous insoluble and soluble components, many of which are immunogenic, and the latter in particular have been chosen for the development of species-specific probes in diagnosis. Wall-bound antigens, however, lend themselves readily to adaptations of microscopic quantification techniques, and the durability of the walls themselves minimizes the loss of antigen during extraction procedures. Such antigens, however, may not be useful as specific tools to distinguish individual species as many epitopes within hyphal walls are common to a range of fungi and some may be immunodominant. The use of purified, insoluble hyphal wall fragments as immunogens has been less frequent than the use of soluble immunogens, but several studies (e.g. Breuil *et al.*, 1992; Thornton *et al.*, 1993) have been successful especially in medical mycology (Ste-Marie *et al.*, 1990).

Abbreviations: IF, immunofluorescence; PC, phase-contrast.

The molecular architecture of hyphal walls remains poorly documented, and most information has been derived from electron microscopy of sections of intact walls or from shadow-cast residues from drastic chemical treatments (Hunsley & Burnett, 1970). Immunofluorescent probes have been less frequently used, although Hunsley & Kay (1976) pioneered the use of polyclonal antisera to correlate immunological observations with previous ultrastructural studies. More recently, immunoconjugates such as chitin-specific lectins (Pain *et al.*, 1994a), hyphal-wall-degrading enzymes (Benhamou *et al.*, 1990), and polyclonal and monoclonal antibodies raised against specific glucans (Wycoff & Ayers, 1990; Gianinazzi-Pearson *et al.*, 1994) have been used to localize these epitopes within specific regions of ultrathin sections of fungal walls. The importance of the need for ultrastructural localization of antigens, as well as immunofluorescence (IF) labelling methods, was emphasized by Cole *et al.* (1991) who demonstrated the differential localization of a specific epitope within a range of structures formed by *Penicillium islandicum*.

In the past there have been problems in devising methods to stick insoluble hyphal fragments to microtitre plates for screening of hybridoma supernatants, but the use of ELISA screening assays in microtitre plates coated with hyphal fragments has now been developed (Banks & Cox, 1992; Breuil *et al.*, 1992) which enables these targets to be used in the screening procedures. The work described here was initiated using *Neurospora crassa*, a fungus with a well-characterized wall biochemistry, to develop procedures for the isolation of mAbs specific for insoluble antigens, and to determine their usefulness in investigations of wall architecture. It was extended to include *Paxillus involutus*, an ectomycorrhizal fungus, as a subsequent aim of the work is to use these probes to study wall morphogenesis within the developing plant-fungus interface during initiation of this ecologically important symbiosis.

METHODS

Fungi. *N. crassa* IMI 142819 was used to raise antibodies and was maintained on *N. crassa* minimal medium (NCMM; Difco) in Petri dishes. Stock cultures were stored at 4 °C on media slopes in Universal bottles. *Neurospora crassa* IMI 53240, *Neurospora sitophila* IMI 63920, and *Neurospora tetrasperma* IMI 34454 were also grown in the same way and used to screen hybridoma supernatants. Large quantities of conidia from *Neurospora* spp. were obtained from cultures grown on NCMM slopes in 250 and 500 ml medical flat bottles. *Pax. involutus* ITE16 was also used to raise antibodies and was obtained from the Institute of Terrestrial Ecology (ITE), Penicuik, UK, and grown on Modified Melin-Norkrans medium (MMN; Marx, 1969) as above. *Pax. involutus* ITE32 and *Pax. involutus* ITE35 were also obtained from the ITE and grown on this medium and used to screen hybridoma supernatants. Other fungi used for screening of hybridoma supernatants were grown on malt extract agar (Oxoid) at 25 °C and included *Aspergillus nidulans* IMI 16040, *Colletotrichum gloeosporioides* UKC24, *Geotrichum candidum* UKC50, *Mucor hiemalis* IMI 21216, *Penicillium chrysogenum* IMI 37767, *Podospora pauciceta* CBS29356, *Schizophyllum commune* UKC17, *Sordaria fimicola* IMI 105391. Basidiomata

of *Amanita muscaria* along with another 20 species of Basidiomycota were collected from the campus of the University of Kent and identified, whereas basidiomata of *Agaricus bisporus* were purchased from a local shop. In both cases, spore and hyphal samples were used in screening studies.

Germination of *N. crassa* conidia in batch culture. Conidia were harvested from 20–30-d-old slope cultures by washing the surface of the culture with sterile 0.1% (v/v) Tween 80 solution. The suspension of conidia was filtered through sterile glass wool and the number of conidia per ml in the resulting suspension counted. Erlenmeyer flasks (250 ml) containing 25 ml *Neurospora* germination medium (Chang & Trevithick, 1972) or *Neurospora* culture broth (NCB; 5 g Difco yeast extract l⁻¹, 5 g Difco Proteose peptone l⁻¹, 40 g maltose l⁻¹) were inoculated with 1×10^6 conidia ml⁻¹. Duplicate flasks were prepared for each time point (up to 12 h). The cultures were incubated at 30 °C and agitated at 200 r.p.m. At hourly intervals, duplicate flasks were removed from the shaker and 25 ml freshly prepared formaldehyde solution (7.4%, w/v) was added, giving a final formaldehyde concentration of 3.7% (w/v). After 30 min fixation, germinated conidia were washed and resuspended in sterile distilled water.

Mini-cultures of *N. crassa*. Mini-cultures of *N. crassa* were grown to obtain intact conidiophores for IF studies of epitope distribution. Aliquots (100 µl) of NCMM were placed into the wells of a 96-well microtitre plate. Each well was seeded with approximately 100 *N. crassa* conidia and incubated at 30 °C, in the light, for 3 d until the fungus formed a thin mycelial mat on the surface of the medium. The medium was then removed by aspiration and the culture fixed for 30 min by addition of 3.7% (w/v) freshly prepared formaldehyde solution.

Culture of fungi for hyphal wall isolation. For the preparation of hyphal walls of *N. crassa* IMI 142819, a 10 l carboy containing 9 l NCMM minus sucrose was autoclaved for 40 min at 121 °C, 15 p.s.i. Following sterilization, 200 g sucrose in 750 ml distilled water was added and the final medium allowed to equilibrate to 30 °C. A conidial suspension of *N. crassa* was prepared from a 20–30-d-old culture maintained on an NCMM agar slope in a 500 ml medical flat bottle by harvesting conidia in sterile 0.1% Tween 80 and filtering it through sterile glass wool. The volume of the suspension was adjusted so that the final concentration of conidia in the carboy would be 1×10^5 conidia ml⁻¹. After inoculation the carboy was incubated at 30 °C for 3 d on a magnetic stirrer and was connected to an air supply via an air inlet pipe. The air passed through a 0.2 µm filter before being dispersed throughout the culture by a glass sparger. An air outlet pipe fitted with a 0.2 µm filter allowed release of gas from the culture vessel. The medium was agitated by means of a large stirrer bar. For preparation of hyphae of *Pax. involutus*, five 2 l Erlenmeyer flasks containing 200 ml MMN broth were inoculated with *Pax. involutus* ITE16 using a plug from a Petri dish culture. The flasks were incubated statically at 20 °C, in the dark. The mycelium was collected when the mycelial mat covered the surface of the medium, usually after 1–2 months.

Isolation of hyphal walls. Hyphal walls of *N. crassa* and *Pax. involutus* were isolated for use in immunization, ELISA and IF protocols as follows. Mycelium was removed from culture media by filtering through four layers of muslin. It was then split into aliquots of approximately 25 g (wet weight). Aliquots not used immediately were stored at –80 °C. All other aliquots were subjected to the following breakage and cleaning procedure: mycelium was passed three times through

a French pressure cell, at a pressure of 14087 tonnes m^{-2} . The resulting mixture of wall fragments and cytoplasm was then spun at 4500 r.p.m. in an MSE Centaur bench-top centrifuge (2250 g) for 5 min. The supernatant was resuspended in sterile distilled water, before being sonicated in an MSE PG100 sonicator (16 μm amplitude peak-to-peak, 5 min in total, in 10 s bursts with 10 s intervals). The suspension was then re-centrifuged and the sonication repeated until cytoplasmic contamination [checked using phase-contrast (PC) microscopy and by a negative result to the orcinol test for RNA] was removed. The isolated walls were then lyophilized for storage.

Fractionation of hyphal walls of *N. crassa*. Isolated *N. crassa* cell walls were fractionated into four crude cell wall fractions according to the method of Mahadevan & Tatum (1965). This involves sequential acid and alkali hydrolysis and the four fractions comprise galactosamine-peptide-glucan (Fraction I), monosaccharide sugars (Fraction II), glucan (Fraction III), and chitin (Fraction IV), respectively. The fractions obtained were lyophilized for storage. Samples of pure chitin, *N*-acetylglucosamine and galactosamine (Sigma), 1,3- α -glucan from *Aspergillus nidulans* (from Professor B. J. M. Zonneveld, Leiden, The Netherlands), and R- and S-glucan from *Schiz. commune* (from Professor J. G. H. Wessels, Haren, The Netherlands) were similarly lyophilized and prepared for ELISA for comparative binding assays.

Development of an immunization schedule for mAb production. To determine the appropriate level of immunogen to be used, two sets of three BALB/c mice were given intraperitoneal injections with 1 and 0.5 mg (freeze-dried weight) of hyphal walls, respectively. These amounts were chosen because larger amounts of material are known sometimes to be fatal. Prior to injection the walls were suspended in sterile distilled water and sonicated to produce a homogeneous suspension. The timing of the injection was such that each mouse was given one injection of the appropriate dose on days 1, 7, 14, 28 and 49, five injections in total. All of the mice were bled from the tail vein on day 59, 10 d after the fifth injection. Plasma from each mouse was tested for its reaction with hyphal walls of *N. crassa* using ELISA. The 1 mg dose produced a much higher end-point titre ($> 1:18000$) than the 0.5 mg dose and injections of 1 mg were subsequently used for all injections, with approximately 14 d between each. A booster injection (1 mg) was given 4 d before a mouse was killed for fusion.

Production of mAbs. Spleen cells were fused with Sp2.0 myeloma cells at a ratio of 5:1 following standard fusion protocols involving the addition of 75 mM polyethylene glycol (Marshall, 1990). Hybridoma supernatants were screened initially using a non-competitive ELISA with hyphal walls bound directly to the plate. Hybridomas were considered positive if their optical density was greater than 0.88 for *N. crassa* as the antigen and 0.78 if *Pax. involutus* was the antigen. These values are the mean plus four standard deviations when non-specific mAbs were tested in the ELISA for their reaction with the hyphal walls. The hybridomas with positive supernatants in the ELISA were then tested by IF. Hybridomas whose supernatants showed different fluorescence patterns were cloned. mAbs were typed by immunodiffusion tests against sheep anti-mouse antisera (ICN) raised against the antibody heavy-chain classes IgG₁, IgG_{2a}, IgG_{2b}, IgA and IgM.

ELISA. Because of initial difficulties in binding hyphal walls of *N. crassa* directly to ELISA plates, an extensive series of comparative experiments was completed (Marshall, 1990) to determine the most appropriate methodology (binding agents, drying times, etc.) and optimum assay conditions (antibody

concentrations, etc.) for an indirect non-competitive ELISA. The final protocol selected was determined initially with polyclonal sera (raised using standard protocols in New Zealand White rabbits using hyphal walls as immunogen). Strong and even binding of hyphal fragments to the ELISA plates was accomplished by initially coating wells with poly-L-lysine (10 $\mu g ml^{-1}$), then pipetting 100 μl hyphal wall suspension at 100 μg (freeze-dried weight) ml^{-1} in sterile distilled water into wells to give an antigen concentration of 10 μg per well. Material was then allowed to dry on to the plate at 37 °C overnight. BSA (250 μl 1%, w/v, in PBS: 8.0 g NaCl l^{-1} , 0.2 g KCl l^{-1} , 2.16 g Na₂HPO₄ · 7H₂O l^{-1} , 0.2 g KH₂PO₄ l^{-1} ; pH 7.0) was added to each well and the plate incubated for 1 h at 37 °C. The blocker was flicked from the wells and 100 μl of the first antibody was added and the plate incubated for 2 h at 37 °C. After incubation the antibody solution was flicked from the plate and the wells were washed six times for 5 min with 300 μl PBS containing 0.05% (v/v) Tween 20. Second antibody, either anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (Sigma) was diluted 1:500 in PBS and 100 μl added to each well. The plate was then incubated for 2 h at 37 °C and the same washing procedure followed as after the first antibody incubation. 2,2-Azinobis(3-ethylbenz-thiazolinesulphonic acid) was used as the substrate at a concentration of 0.8 mM in 0.1 M citrate buffer.

IF. Polyclonal antisera and hybridoma supernatants were screened using an indirect IF technique. Slides were coated with poly-L-lysine (100 $\mu g ml^{-1}$), by submersion of the slides in the poly-L-lysine solution for 15 min. Excess poly-L-lysine was removed by a wash in PBS and the slides air-dried. Ten microlitre aliquots of a suspension of test fungal material were placed onto the slides. For screening of hybridoma supernatants this comprised either 10 μl *N. crassa* germlings, or 10 μl *Pax. involutus* hyphal walls. The 10 μl aliquots were air-dried on the slides for 30–60 min at 25 °C. Ten microlitres of the first antibody was then placed onto each sample and the slides incubated at 25 °C for 1 h in moist conditions. The slides were then washed three times for 5 min with PBS. Excess PBS was drained from the slides and 10 μl of either anti-mouse IgG or anti-rabbit IgG FITC conjugate (Janssen) diluted 1:10 in PBS was placed on each sample before incubation at 25 °C for 1 h. The washing step (3 × PBS, 5 min) was repeated and the slides drained. A drop (10 μl) of Mowiol (Sigma) containing 10 $\mu g ml^{-1}$ *p*-phenylenediamine was added and samples were observed by epifluorescence using a Zeiss Axioskop microscope with a mercury lamp and a Zeiss standard dichroic filter set no. 10 for detection of transmitted light from FITC. Checks were made during all experiments that the fluorescence was not due to non-specific binding by comparing results with second antibody controls (i.e. preparations in which the first antibody but not the second was omitted) using fungal spores, hyphae and the undiluted culture medium as substrates.

Immunogold electron microscopy. Mini-cultures of *N. crassa* were grown in the wells of microtitre plates, fixed with 3.7% (w/v) formaldehyde containing 0.6% glutaraldehyde in Pipes-EDTA-magnesium (PEM) buffer for 1–2 h, and dehydrated and embedded in Lowicryl K4M (Chemische Werke Lowi) using the standard protocol involving infiltration during progressive lowering of the temperature prior to polymerization at –35 °C using UV light (Bendayan, 1984). Thin sections (150–190 μm) were cut using a Reichert ultratome and mounted on nickel grids for immunogold labelling. Subsequent steps were carried out by placing one drop (10 μl) of the appropriate solution onto each grid and incubating them in a bioassay plate at room temperature. Free aldehyde groups were neutralized by three 5 min washes in 20 mM glycine. The

Table 1. IF reactions of mAbs S4D1, S3b3 and Pax-1 with a taxonomically diverse range of fungi

+++ , Strong fluorescence; ++ , moderate fluorescence; + , weak fluorescence; — , no reaction. NT, combination was not tested.

Fungus	mAb		
	S4D1	S3B3	Pax-1
Ascomycota			
Sordariaceae			
<i>Neurospora crassa</i> IMI 142819	+++ (All surfaces)	+++ (Some conidial ends, septa in conidiophore chains)	+++ (Some conidial ends)
<i>Neurospora crassa</i> IMI 53240	+++ (All surfaces)	+++ (As above)	NT
<i>Neurospora sitophila</i>	+++ (All surfaces)	+++ (As above)	NT
<i>Neurospora tetrasperma</i>	+++ (All surfaces)	+++ (As above)	NT
<i>Sordaria fimicola</i>	++ (Asci, ascospores) + (Hyphae)	— —	NT
Chaetomiaceae			
<i>Chaetomium globosum</i>	—	NT	nt
Dipodascaceae			
<i>Geotrichum candidum</i>	—	—	—
Lasiosphaeriaceae			
<i>Podospora paucietia</i>	—	—	NT
Anamorphic fungi			
<i>Aspergillus nidulans</i>	+++ (Hyphae) ++/+ (Conidia)	—	—
<i>Colletotrichum gloeosporioides</i>	++ (Secondary germ tubes) — (Conidia, appressoria, primary germ tubes)	NT	+ (Germ tubes only)
<i>Penicillium chrysogenum</i>	+++ (Hyphae, conidiophores) — (Phialides, conidia)	— (Hyphae) + (Conidial ends)	+ (Conidial ends)
Basidiomycota			
Agaricaceae			
<i>Agaricus bisporus</i>	NT	—	+++
Amanitaceae			
<i>Amanita muscaria</i>	— (Hyphae) +++ (Basidiospores)	NT	NT
Paxillaceae			
<i>Paxillus involutus</i>	++ (Hyphae)	—	+++
Schizophyllaceae			
<i>Schizophyllum commune</i>	+++ (Skeletal hyphae) + (Generative hyphae)	—	+++
Zygomycota			
Mucoraceae			
<i>Mucor hiemalis</i>	—	—	—

grid was then washed once with PBS and incubated with the first antibody for 1 h. The mAb hybridoma supernatants were not diluted. The grid was then washed five times for 5 min with 1% BSA/PBS. Anti-mouse IgG whole-molecule 10 nm diameter gold conjugate (Janssen) was diluted 1:10 in 1% BSA/PBS and placed on the grid for 1 h before washing twice in 1% BSA/PBS for 5 min, followed by three 5 min washes in 0.1% BSA/PBS and two 5 min washes in PBS. The bound antibody was fixed to the sections by incubation in 2.5% (v/v) glutaraldehyde for 30 s. Sections were then stained using 5% (w/v) uranyl acetate in 1% (v/v) acetic acid and Reynold's lead citrate. Grids were observed using a Philips EM410

transmission electron microscope. Control preparations in which the first antibody (but not the second) was omitted were always run in parallel to check for non-specific binding.

RESULTS

Production of mAbs

After initial screening by ELISA of immunized mouse antisera and resulting hybridoma supernatants, hybridomas deemed positive by comparison to known polyclonal antisera were assayed by IF. Of 3360 super-

Figs 1–8. **Fig. 1.** IF micrograph of ungerminated conidia of *N. crassa* treated with mAb S4D1 showing even distribution of epitope over conidial surface. Bar, 15 μ m. **Fig. 2.** IF micrograph of germlings of *N. crassa* treated with mAb S4D1 showing even distribution of epitope over surface of germ tubes and conidia. Bar, 15 μ m. **Fig. 3.** PC micrograph of a mixture of germlings of *N. crassa* and arthroconidia of *G. candidum* treated with mAb S4D1. Bar, 15 μ m. **Fig. 4.** IF image corresponding to Fig. 3 showing selective binding of mAb to *N. crassa*. **Fig. 5.** PC micrograph of a conidiophore of *Pen. chrysogenum* treated with mAb S4D1. Bar, 15 μ m. **Fig. 6.** IF image corresponding to Fig. 5 showing localization of epitope on conidiophore but not on conidia or phialides. **Fig. 7.** PC micrograph of a germinating conidium, secondary germ tube (g) and appressorium (a) of *Col. gloeosporioides* treated with mAb S4D1. Bar, 15 μ m. **Fig. 8.** IF image corresponding to Fig. 7 showing even distribution of labelling over surface of secondary germ tubes only.

natants from hybridoma cell lines obtained from mice injected with *N. crassa*, 286 tested positive by ELISA and 102 of these recognized hyphal fragments of *N. crassa* as determined by IF. The patterns of fluorescence fell into four major groups: those which reacted with: (i) conidial ends and septa, (ii) hyphae and conidia, (iii)

hyphae only, and (iv) spores only. Hybridoma cell lines which fell into the first three categories were successfully cloned and were represented by mAbs S3B3, S4D1 and S1E5, respectively (all IgM). A cell line secreting antibody of the fourth category which recognized spores only was not successfully cloned.

Figs 9–14. **Fig. 9.** PC micrograph of spores and hyphae from a basidiome of *Amanita muscaria* treated with mAb S4D1. Bar, 15 μ m. **Fig. 10.** IF image corresponding to Fig. 9 showing localization of epitope on basidiospores but not on hyphae. **Fig. 11.** PC micrograph of hyphae from a mycelial culture of *Schiz. commune* treated with mAb S4D1. A thick-walled skeletal hypha (s) is visible. Bar, 15 μ m. **Fig. 12.** IF image corresponding to Fig. 11 showing localization of epitope on hyphal surface. **Fig. 13.** Electron micrograph of longitudinal section through a conidium of *N. crassa* treated with gold-labelled mAb S4D1 showing location of the epitope within both the outer (o) and inner (i) wall layers of the conidium. Bar, 0.5 μ m. **Fig. 14.** IF micrograph of ungerminated conidia of *N. crassa* treated with mAb S3B3 showing localized distribution of the epitope at some ends of the conidia. Bar, 15 μ m.

From 1440 hybridomas produced using hyphae of *Pax. involutus* as immunogen, 108 proved positive by ELISA, and 29 of these were also positive in the IF assay. There was only one pattern of reactivity as described below. A representative was cloned and the cell line designated Pax-1, and the antibody it produced, also an IgM, cross-reacted with all fungi against which it was tested (Table 1).

mAb S4D1

S4D1 recognized an epitope which was distributed over the entire surface of both the dormant conidia (Fig. 1) and hyphae (Figs 2–4); the same immunolabelling pattern was seen with the immunogen *N. crassa* IMI 142819, and another isolate of *N. crassa*, IMI 53240, and with other members of the genus (Table 1). S4D1

reacted to a more limited extent with *Sor. fimicola*, and showed no reaction with the hyphae of *Pod. paucieta*, nor with *Chaetomium globosum*, or arthroconidia of *Geotrichum candidum*. If *N. crassa* germlings and *G. candidum* arthroconidia were mixed together and screened for reaction with S4D1 by IF, the mAb clearly differentiated between the two species (Figs 3 and 4).

The reaction of S4D1 with genera outside the Ascomycota was also studied (Table 1). With *Pen. chrysogenum*, fluorescence was exhibited along the surface of hyphae and conidiophores, but not on the phialides or conidia (Figs 5 and 6). With *Aspergillus nidulans*, the fluorescence was seen on the conidial surface but it varied in intensity with different conidia (not illustrated). Bright fluorescence was seen on the hyphae, as with *Pen. chrysogenum*. In the case of *Col. gloeosporioides*, fluorescence was not seen on the surface of the conidium, appressorium or its subtending primary germ tube, but did occur on the surface of germ tubes ('secondary germ tubes') which were produced after an appressorium had developed (Figs 7 and 8). S4D1 showed no reaction with the hyphae from the fruiting body of *Amanita muscaria* but fluorescence was observed on the basidiospore surface (Figs 9 and 10). With other members of the Basidiomycota, S4D1 labelled an epitope on the hyphal surface, e.g. with *Schiz. commune* (Figs 11 and 12) fluorescence was seen particularly along the long unbranched skeletal hyphae, which have thick walls, along with some fluorescence on fragments of thin-walled generative hyphae. It is possible that some of this could be due to autofluorescence.

Thin sections of conidia from a 3-d-old colony of *N. crassa* had a distinct, thin outer wall layer and a thicker, lightly staining inner wall. S4D1 recognized an epitope present throughout the wall layers of the conidium (Fig. 13). The gold labelling was associated with the wall rather than the plasma membrane. The epitope occurred throughout the septal region and was also present between the conidia in chains. The hyphal wall was much thinner than the conidial wall and labelling occurred throughout, although it was not as heavily labelled as the conidial wall (results not illustrated).

Fractions I, II, III and IV from the hyphal walls of *N. crassa* were tested for their reaction with S4D1 by ELISA. S4D1 showed a negligible reaction with Fraction II (monosaccharides), but reacted with the other three fractions (Table 2). It did not react with purified chitin nor with its subunit *N*-acetylglucosamine. The reaction of S4D1 with Fraction IV (mainly chitin) was not, therefore, due to a reaction with chitin but to a reaction with another hyphal wall component that was also extracted in Fraction IV. Fraction III (glucans) reacted strongly with S4D1, suggesting that the antigen was a major part of this wall fraction. S4D1 also cross-reacted with Fraction I which contained glucan, galactosamine and peptides. The antigen may, therefore, be any one of these, and Fraction III may have contained one of these components as well as the glucan. S4D1 had a negligible reaction with galactosamine, a component of Fraction I

Table 2. A_{410} values for the ELISA reaction of mAb S4D1 with hyphal wall fractions from *N. crassa* and with hyphal wall components prepared from other fungi

Results are expressed as a mean of four replicates (and range of values). Figures quoted have been corrected by subtraction of readings obtained for a second antibody control.

Component	A_{410}
Whole <i>N. crassa</i> walls	1.10 (1.02–1.20)
Fraction I	0.83 (0.81–0.89)
Fraction II	0.12 (0.10–0.13)
Fraction III	1.31 (1.27–1.35)
Fraction IV	0.57 (0.55–0.59)
<i>A. nidulans</i> 1,3- α -glucan	0.88 (0.87–0.90)
<i>Schiz. commune</i> S-glucan (1,3- α)	0.70 (0.65–0.79)
<i>Schiz. commune</i> R-glucan (1,3- β , 1,6- β)	0.24 (0.21–0.26)
Chitin	0.00
<i>N</i> -Acetylglucosamine	0.00
Galactosamine	0.00

but reacted with a 1,3- α -glucan isolated from hyphal walls of *A. nidulans* and a 1,3- α -glucan (or S-glucan) isolated from hyphal walls of *Schiz. commune* (Table 2). In contrast, it reacted only very weakly with 1,3- β , 1,6- β -glucan (or R-glucan) from hyphal walls of *Schiz. commune* (Table 2).

mAb S3B3

S3B3 recognized an epitope present at the ends of some conidia (Figs 14, 16 and 18). The epitope, however, was not always present at both or even one end of all detached conidia (Fig. 14). In a conidial chain, the fluorescence occurred in the septal region (Figs 15–18). Conidial chains of *N. crassa* develop basipetally, and the position of a conidium in the chain may correlate with the presence or absence of the epitope. For example, in Figs 17 and 18 in the older portion of the conidiophore, the septal region showed fluorescence, whereas in the developing conidial chains there was no fluorescence until the septal crosswalls formed.

S3B3 cross-reacted in a similar fashion with the other *Neurospora* isolates but showed no reaction with *Sor. fimicola* or *Pod. paucieta* (Table 1). It did not react with the ends of the arthroconidia of *G. candidum*, which are also formed basipetally, or with conidia from *A. nidulans*, but reacted weakly with the ends of conidia from *Pen. chrysogenum*. No reaction was seen with any member of the Basidiomycota or Zygomycota tested.

Immunolabelling at the electron-microscopic level also showed the epitope recognized by S3B3 to be localized at the ends of conidia, as might be expected from the IF pattern shown by light microscopy, but the label was also seen more generally within the inner walls of conidia (Figs 19, 20), as well as within the hyphal walls

Figs 15–24. **Figs 15 and 17.** PC micrographs of developing conidial chains of *N. crassa* treated with mAb S3B3. Bar, 5 μ m. **Figs 16 and 18.** IF images corresponding to Figs 15 and 17 respectively showing localized distribution of epitope only between the ends of mature conidia. **Figs 19 and 20.** Electron micrographs of longitudinal sections through conidial wall of *N. crassa* treated with gold-labelled mAb S3B3 showing location of the epitope within the inner electron-lucent wall layer (i) and not in the outer wall (o). Bar, 0.5 μ m (Fig. 19), 0.1 μ m (Fig. 20). **Fig. 21.** PC micrograph of germlings and ungerminated conidia of *N. crassa* treated with mAb S1E5. Bar, 15 μ m. **Fig. 22.** IF image corresponding to Fig. 21 showing localized distribution of the epitope over the surface of germ tubes but not on the conidial surface. **Fig. 23.** PC micrograph of hyphae from a mycelial culture of *Pax. involutus* treated with mAb Pax-1. Bar, 15 μ m. **Fig. 24.** IF image corresponding to Fig. 23 showing localization of epitope over hyphal surface.

and in the septal region of hyphae. In sections of conidial chains, gold labelling was also seen in the septal region (not illustrated). Because the binding pattern of this

mAb resembled that of Calcofluor, which binds to chitin and other β -linked glucans, the antibody was also screened by ELISA against pure chitin and *N*-acetyl-

glucosamine (as described for S4D1) but was found not to bind to either.

mAb S1E5

S1E5 reacted with an epitope present on the surface of germ tubes of *N. crassa*, but which was not present on dormant conidia (Figs 21, 22). Labelling of conidia was occasionally observed but these were usually swollen conidia in the process of germination. The reaction with heterologous fungi was not tested. Although the fluorescence pattern exhibited by conidia showed that the epitope was not present at the surface of conidia, immunogold labelling of thin sections of conidia revealed that the epitope was present in the inner wall layers, but was not present at the conidial surface (results not shown).

Pax-1

Pax-1 reacted with hyphal walls of *Pax. involutus* ITE 16 (Figs 23, 24). It also reacted with hyphal walls from *Pax. involutus* ITE32, and hyphae and basidiospores from a fresh basidiome of *Pax. involutus*. In a population of hyphal fragments, some showed fluorescence along their whole length, some show fluorescence along only part of the fragment and others showed no fluorescence at all. The strongest fluorescence seen was often associated with the ends of the fragments and this suggested that Pax-1 reacted with an internal antigen. Pax-1 possibly reacted with an epitope distributed at the surface only at certain times during hyphal development. The pattern seen on older mycelial strands did not differ significantly from that on younger wall fragments.

Pax-1 reacted by IF with hyphae and spores of 22 species of Basidiomycota tested (including species of *Agaricus*, *Amanita*, *Boletus*, *Clitopilus*, *Collybia*, *Coprinus*, *Gymnopilus*, *Hebeloma*, *Laccaria*, *Lactarius*, *Mycena*, *Pluteus* and *Russula*); the pattern of fluorescence was similar to that for *Pax. involutus* (data not shown). Pax-1 did not react with *Mucor hiemalis* but cross-reacted, however, with members of the Ascomycota (Table 1). Pax-1 showed the same pattern of fluorescence on conidia of *N. crassa* as S3B3. The different patterns of reactivity against other antigens, however, suggest that these two mAbs are not identical.

DISCUSSION

The four types of IF reaction reported here indicate the potential of mAbs in the localization of particular wall components. mAb S4D1, for example, recognized an antigen present on the surface of both conidia and hyphae in *N. crassa*, and in the glucan fraction of hyphal walls of *N. crassa*, and the 1,3- α -glucan fractions from *A. nidulans* and *Schiz. commune*. The presence of 1,3- α -glucans has been reported from the hyphal walls of *N. crassa* (Bull, 1970; Cardimil & Pincheira, 1979), and our results suggest that this component is distributed

throughout the walls of both hyphae and conidia and not localized in a specific region. mAb S4D1 was also used to localize the epitope in other fungi. All fungi tested from the Ascomycota and Basidiomycota reacted with the antibody, whereas fungi from the Zygomycota did not. This provides further circumstantial evidence that 1,3- α -glucan is not present in the hyphal walls of Zygomycota (Bartnicki-Garcia, 1968). mAbs have previously been found which react to epitopes within particular carbohydrate components of the hyphal wall, but these have usually been to β -linked glucans, the most important group of fungal glucans (Ruiz-Herrera, 1991).

The surface distribution of the S4D1 epitope varies between fungal species, and also within different structures of a single species. The differential expression and location of epitopes in different structures of the same fungus at varying development stages has been observed previously with other fungi (e.g. Hardham *et al.*, 1985, 1994; Wycoff & Ayers, 1990; Ollert & Calderone, 1990; Xia *et al.*, 1993). A comparable result was observed during IF and immunogold labelling of mycelium of *Penicillium islandicum* with a mAb raised to soluble surface molecules (Cole *et al.*, 1991). The antibody bound to outer wall layers in actively growing young hyphae and to regions within the wall of older mycelium. It did not bind to phialides or conidia. Conversely, an antibody raised to phialoconidia of *Trichoderma harzianum* reacted with the immunogen but not with the mycelium or chlamydospores of the same fungus (Thornton & Dewey, 1996). mAb S4D1 bound to hyphae but not to appressoria of *Col. gloeosporioides*. These latter structures are heavily melanized (Jeffries *et al.*, 1990) which may mask the relevant epitope. mAbs raised against *Colletotrichum lindemuthianum* have previously been shown to exhibit differential binding patterns (Pain *et al.*, 1992, 1994b). One antibody (UB20) labelled conidia strongly, but germ tubes and appressoria weakly; another (UB22) reacted strongly with germ tubes, especially in regions away from the apices, as well as labelling appressoria and conidia less strongly; a third antibody (UB25) bound only to intracellular hyphae and did not recognize conidia, appressoria or germ tubes.

Although a large number of hybridomas were raised which produced mAbs that gave positive results in both ELISA and IF screens against hyphal walls, there was a relative lack of variation in the IF patterns obtained. This suggests immunodominance by some wall components or a uniformity in hyphal wall composition. Immunodominance has been reported in previous studies. In *Aspergillus* and *Penicillium* species, for example, some extracellular polysaccharides are immunodominant (Notermans *et al.*, 1987, 1988) and the use of crude mycelial extracts as immunogens has been shown to induce cross-reactive antibodies. Many of the cell lines we obtained gave IF patterns similar to mAb S3B3, whereas the other binding patterns were only demonstrable with a few cell lines. Thus, immunodominance was most strongly exhibited by the material which accumulates between conidia during sporogenesis. The

morphological development of conidiophores of *N. crassa* involves the septation of a preconidial stage such that individual conidia become delimited by crosswalls (Springer & Yanofsky, 1989). When the interconidial junction has reached the furrow stage, IF staining of this region using Calcofluor reveals two thick bands of material similar to those obtained when reacted with mAb S3B3. Calcofluor binds to chitin and other β -linked glucans whereas mAb S3B3 does not react to chitin or to N-acetylglucosamine, the chitin subunit, thus it is evident that these two fluorescence probes are reacting to different molecules which have a similar distribution.

In our work, the use of crude mycelial homogenates has revealed a degree of diversity of responses to unrefined immunogens. The individual reactions of mAbs S4D1, S3B3, S1E5 and Pax-1 to hyphae of *N. crassa* show differential surface localization of their respective epitopes. Previous tests using ELISA with homologous antigens have also shown that different mAbs raised against the same immunogens can react differently with different fungal structures (Xia *et al.*, 1992). Immunodominant antigens, however, can mask these more specific interactions and this emphasizes the need to screen a large number of cell lines to obtain a full spectrum of responses. By this means it is possible to get a range of mAbs that are excellent probes for small regions of hyphal wall morphogenesis and these should be very useful in determining the structural complexity of hyphal development.

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