Immunological recognition of fungi

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by

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No part of this thesis has been submitted in support of an application for any degree or qualification of The University of Kent or any other University or institute of learning.

Margaret Marshall.

Margaret Marshall

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To my parents

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Abstract

Polyclonal antisera were raised against the fungi *Neurospora crassa* and *Paxillus involutus*, respectively. These antisera were used in the development of an ELISA for subsequent use in monoclonal antibody (MAb) screens. Fungal cell walls were chosen as the most suitable immunogen for *in situ* ecological studies. Hyphal walls were isolated and bound to a poly-*l*-lysine coated ELISA plate. Both antisera showed cross-reactions with other species of fungi. These reactions were not diminished by pre-absorption of the antisera with cell walls from species of fungi which react with the antiserum.

The ELISA was then used in the production of specific MAbs raised against fungal cell wall antigens. Hybridoma supernatants were screened following fusions of spleen cells from immunised mice and Sp2.0 myeloma cells. A successful immunisation protocol was established using fungal cell walls, and a titre of tailbleed plasma used to determine whether a mouse would be used for fusion. Four MAb cell lines were established (S4D1, S3B3, S1E5 and Pax-1).

Of these MAbs, three were derived from mice immunised with cell walls of *N. crassa*, and the fourth from a mouse immunised with the cell walls of *P. involutus*. In immunofluorescence studies the three MAbs raised against *N. crassa* recognised epitopes which show different patterns of distribution at the cell surface of *N. crassa*. MAb S4D1 recognises an epitope which is present on the surface of both conidia and hyphae; MAb S3B3 an epitope seen only at the end of conidia or in the septal region of hyphae and conidial chains; and S1E5 an epitope present on the surface of hyphae, but not mature conidia. MAb Pax-1, raised against *P. involutus*, reacts with cell wall fragments of *P. involutus* and reacts with *N. crassa* conidia in a similar way to S3B3 in immunofluorescence studies, *i.e.* with septa and the ends of conidia. The MAbs S3B3 and Pax-1 are, however, differentiated by their reaction with *P. involutus*, Pax-1 reacts with *P. involutus*, but S3B3 does not.

S4D1 reacted with an epitope found in α-1,3 glucan fractions from different fungi. The surface distribution of this epitope varies across a taxonomic spectrum. It is found on the surface of both conidia and hyphae of *N. crassa* and *Aspergillus nidulans*, but only on the basidiospore surface of *Amanita muscaria*. Conversely it appears on the hyphae but not the conidia of *Penicillium chrysogenum*. Immunogold studies revealed that the epitope was present throughout the wall of *N. crassa*. In quantification studies, S4D1 showed a linear relationship between the reaction of the MAb with its epitope, recorded as absorbance in an ELISA, and cell wall biomass (freeze-dried weight).

S3B3 and Pax-1 also reacted with other fungi. Pax-1 cross-reacted with all fungi tested except for a member of the Zygomycotina. The nature of the epitopes of these two MAbs was not determined, nor was that of S1E5. Immunogold studies revealed that epitopes of these three MAbs were not present at the surface in all the morphological forms of *N. crassa*, but that they were present in inner layers of these walls. For example, the epitope of Pax-1 is found in a discrete layer next to the plasma membrane of *N. crassa* cells but not at the surface, except in the septal region. The epitope of Pax-1 is not chitin.

The MAbs produced did not show species-specificity. The specific reaction of the MAbs with their epitopes enabled the localisation of the epitopes at the cell surface of fungi and their distribution throughout the wall of *N. crassa* to be studied. The distribution of the epitopes within the walls of hyphae and conidia has been schematically represented.

Abbreviations

ABTS	2,2'-azinobis(3-ethylbenz-thiazolinesulphonic acid)
BSA	Bovine serum albumin
DMSO	Dimethyl sulphoxide
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
HRPO	Horseradish peroxidase
LSB	Laemmli sample buffer
MAb/s	Monoclonal antibody/ies
MEA	Malt extract agar
MMN	Modified Melin Norkrans medium
NCA/B	*Neurospora crassa* culture agar/broth
NCMM	*Neurospora crassa* minimal medium
PBS-7	Phosphate buffered saline, pH7
PEG	Polyethylene glycol
PSB	Phosphate sample buffer
PVP	Polyvinyl pyrrolidone
USB	Urea sample buffer, 10M
ddH_2O	Double distilled water
sdH_2O	Sterile distilled water
YEPD	Yeast extract peptone digest medium

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Chapter One

Introduction

The recognition and differentiation of one fungal species from another is fundamental to mycology. One of the earliest mycological publications to describe and differentiate fungi was *Nova Plantarum Genera* (Micheli, 1729), in which 900 species of fungi were illustrated. Nowadays the situation has become more complex with over 50,000 species named, but the taxonomic literature is also much more advanced, with a plethora of taxonomic keys in existence, and so it is relatively easy to identify most fungi in pure culture, as long as they have sexual or asexual structures present. Occasionally problems arise when spores are difficult to tell apart, *e.g.* spores of endomycorrhizal fungi, but these cases are unusual. When fungi do not sporulate, however, the problem of identification is compounded.

Wherever fungi are encountered in the natural environment, hyphae form the bulk of the biomass. Most hyphae lack distinguishing characteristics and are difficult to identify. It is possible to culture many fungi and induce sporulation to enable morphological identification, but frequently the mycologist is looking for a quick, reliable answer and it is here that other rapid methods of identification have been investigated. Speed and accuracy of identification are particularly important in diagnosis of disease, either plant or animal, and the quicker identification is confirmed, the sooner treatment can begin and the more likely it may succeed.

With the potential release of wild-type and genetically manipulated organisms into the environment quick, reliable means of identification take on new importance: not only for locating the organisms released and quantifying their survival, but also for following the

impact of the released organisms on the population of indigenous species. This statement is true, not only for fungi, the organisms considered in greatest depth in this thesis, but for other micro-organisms too. One of the greatest barriers to the release of micro-organisms is the lack of information available regarding their fate within the environment.

The information needed to follow micro-organisms in any environment includes detection, identification and quantification. All three of these can be investigated at different levels of specificity *e.g.* species, genus or family. Techniques used to uncover this information may also enable taxonomic discrimination, which is closely linked to identification, and experimental investigation of the micro-organism *in vitro*. The aim of this project was to develop immunological techniques to enable such information to be obtained.

It is not within the scope of this introduction to discuss general aspects of immunology; the role of antibodies and their helpers in natural immune systems. Immunological techniques, however, have been used in microbiology since the beginning of the twentieth century (Seeliger, 1960). They have been used in all of the four areas outlined above: detection, identification, quantification and investigation. In this introduction the contribution of immunological studies to these four areas has been considered, along with other methods which also have been used to detect, identify, quantify and investigate fungi. None of the techniques in isolation answer all the questions in a particular area of interest, but consideration of all of the information gleaned by the various methods enables a fuller picture to emerge whatever the question.

1.1. Immunology in Microbiology.

1.1.1. The antibodies.

Immunological techniques can enable micro-organisms to be detected, identified, quantified and investigated, biochemically or structurally, by utilising the specific

recognition of antigens by antibodies. In the natural immune system of mammals foreign antigens are recognised by a number of means, but the one which has been the most exploited experimentally, and is the basis of work discussed here, is the production of monospecific antibody by B lymphocytes. Each B lymphocyte has the potential to make monospecific antibody. The origin of this specificity is complex, but involves extensive rearrangement of the DNA sequences which code for antibody polypetide chains and also somatic mutation during clonal development (Tonegawa, 1983). This means that if a foreign antigen is introduced deliberately into an animal its immune system will respond and one of the responses will be specific antibody production by B lymphocytes. If the animal is bled following immunisation the antiserum prepared should recognise the introduced antigen. The antiserum, however, contains antibodies from lots of B lymphocytes and so may show cross-reactivity with a number of antigens. Such polyclonal antisera were the first immunological probes used in microbiology.

Polyclonal antisera have long been very useful to microbiologists in detection, identification, quantification and investigation, but they have a number of properties which can be disadvantageous. One, as mentioned above, is cross-reactivity. This may be removed by absorption of the serum, but frequently this lowers the titre (Gerik, Lommel & Huisman, 1987) and does not always remove all cross-reactivity (Dewey *et al.*, 1984; Chard, Gray & Frankland, 1983, 1985b). Cross-reactivity, however, can be useful if a low level of specificity is required *e.g.* detection of all fungal contaminants in tomato paste (Robertson, Patel & Sargeant, 1988). The biggest disadvantage polyclonal antisera have is their variability between different animals and also between different bleeds of the same animal. These differences between antisera have led to different results between laboratories using antisera raised against the same organism *e.g.* Mohan (1988) and Amouzou-Alladaye, Dunez & Clerjeau (1988) raised antisera to *Phytophthora fragariae*, the former did not record a

reaction with *Pythium middletonii* whereas the latter did. Therefore, standardisation of diagnostic tests and taxonomic classification using antisera is difficult.

In 1975 Köhler & Milstein presented their classic paper on monoclonal antibody (MAb) technology. In it they reported the successful fusion of B lymphocyte and myeloma cell lines to produce "cells secreting an antibody of predefined specificity". They had been able to combine the monospecific antibody properties of B lymphocytes with the infinite lifespan of the myeloma cells to produce cells, called hybridomas, secreting one type of antibody and having an infinite lifespan. A myeloma cell line is prepared from a tumorous B lymphocyte cell line and the first myeloma cell lines secreted antibody themselves. Now a number of myeloma cell lines are available which do not secrete antibody, thus ensuring that all antibody secreted by the hybridoma cell line originates from the B lymphocyte parent.

In MAb technology the animal is immunised as for polyclonal antibody production, but instead of the animal's antibodies being used directly, it is the cells which actually secrete the antibodies which are utilised. These cells, the B lymphocytes, are produced in the bone marrow but are concentrated in the spleen three to four days after immunisation and the spleen is taken as the source of these cells for a fusion.

When a fusion is performed not only do spleen cells fuse with myeloma cells, but also spleen cell with spleen cell and myeloma cell with myeloma cell. Unfused spleen and myeloma cells are also present. It is, therefore, necessary to selectively remove unwanted cells from the fusion mixture. The spleen cells have a finite lifespan in culture and do not survive; but the myeloma cells do survive and, if allowed, would outgrow the hybridomas. To overcome this problem myeloma cell lines used for fusion are selected for a self-destruct mechanism.

The myeloma cells are grown in the presence of one of the following drugs: 8-azaguanine, 6-thioguanine or bromodeoxyuridine. The myeloma cells are normally killed by

these drugs, but some mutated cells survive by switching off the production of the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT) in the case of 8-azaguanine and 6-thioguanine selected cells, and the enzyme thymidine kinase (TK) in the case of bromodeoxyuridine selected cells. HGPRT is part of purine salvage pathway, whereas TK is part of the pyrimidine salvage pathway. When the HGPRT or TK negative cells are grown in the presence of aminopterin, hypoxanthine and thymidine the cells will die due to combination of the following: first, aminopterin blocks the main biosynthetic pathways for purine and pyrimidine synthesis; and second, the HGPRT or TK negative cells are unable to utilise their nucleoside salvage pathways and use the hypoxanthine and thymidine supplied in the medium to make purines and pyrimidines. The sites of aminopterin blockage are shown in detail by Campbell (1984).

If the fusion mixture is grown in medium containing hypoxanthine, aminopterin and thymidine (HAT) the parent spleen cells will die as stated above, but because of the use of the HGPRT or TK negative myeloma cells all myeloma cells which have not fused with a spleen cell partner will also die. Only hybridoma cells will be able to grow in the HAT medium, because they possess the ability to grow infinitely from the myeloma cells and the genes to produce HGPRT or TK from the parent spleen cells.

Following fusion the successful production of a useful MAb cell line requires screening of the hybridomas for the desired antibody. This can be done by a number of methods which include ELISA, immunofluorescence and immunoblotting. The best method is the one in which the antibody is to be ultimately used as antibodies can perform differently in different screening tests *e.g.* certain MAbs produced against *Corynebacterium sepedonicum* reacted with the bacterial cells in immunofluorescence tests but failed to agglutinate the same cells in agglutination tests (de Boer & Wieczorek, 1984). Once an antibody is found which recognises the test antigen, the hybridoma cells which secreted it are cloned. Cloning is most

commonly carried out by the limiting dilution technique. In this technique aliquots of low dilutions of the hybridoma cells are placed in the wells of 96 well plastic microtitre plates accompanied by feeder cells. If the cell dilution per well is calculated as 1 cell/well then the number of cells in the wells will form a Poisson distribution about 1 according to Goding (1980). Some cells will therefore possess clones that originated from one cell and it is the supernatants from these wells that are subsequently tested for antibody activity to ensure that the antibody is from one clonal cell and hence "monoclonal". Three successive cloning operations are usually the minimum number carried out to ensure monoclonality.

The MAb secreted by the hybridoma reacts with only one epitope. An epitope is the specific site on an antigen to which the antibody binds by means of its hypervariable region. This specificity makes MAbs better probes than polyclonal antibodies for investigative studies. Levels of affinity between MAbs and their epitopes differ from one MAb to another. For example a MAb of high stringency can be selected using high stringency conditions in a selective screen. The cross-reactivity of a MAb with other antigens depends on the distribution of its epitope in other antigens. It is possible to select the specificity of the monoclonal antibody at the screening stage. For example, if a mixed antigen is used for immunisation, but an antibody is wanted to specifically recognise one component of the mixture, the screen is devised to select antibodies which show no cross-reactivity with the other components.

The other advantage MAbs have over polyclonal antibodies is their infinite lifespan and the supply of a known antibody which never differs. A summary of comparisons between polyclonal antisera and MAbs is shown in Table 1.1.

1.1.2. The antigens.

Potentially an antigen can be any part of a micro-organism or the whole. With polyclonal antisera the more specific the antigen the more specific the resulting antiserum

Polyclonal	versus	Monoclonal
relatively easy		difficult
cheap		expensive
quick (1-2 months)		slow (6-12 months)
broad range of specificity		high specificity
finite life		indefinite life
binds to many epitopes		binds to one epitope

Table 1.1. A summary of the different properties of polyclonal antisera and MAbs.

will be, because less non-specific antibodies will "contaminate" the antiserum. One of the reasons for the notorious cross-reactivity of fungal antisera has been the complex nature of the antigens used for injection. This contrasts with the relative success of polyclonal antibodies specific for plant viruses (Clark, 1981), where the immunogen is a less complex organism with fewer antigenic determinants.

As will be discussed in Sections 1.2.1.1 and 1.2.2.1 it has made relatively little difference to the specificity of fungal antisera whether cell wall or cytoplasmic components have been used as the antigens. Some workers have reported that antisera raised against cell wall antigens show less cross-reactivity (El-Nashaar, Moore & George, 1986; Kough, Malajzcuk & Linderman, 1983; Fitzell, Fahy & Evans, 1980; Halsall, 1976; Malajzcuk, McComb & Parker, 1975; Holland & Choo, 1970): whereas others (Gerik *et al.*, 1987; Aldwell, Hall & Smith, 1983, 1985) found antisera against cytoplasmic fractions showed less cross-reactions with other fungi. In medical mycology exoantigens have proved useful for raising diagnostic antisera (Kaufman & Standard, 1988), but variability between antisera has been reported (Kaufman & Standard, 1988). The antisera raised to fungi which have the least reported cross-reactivities have been those raised against defined antigens *e.g.* cerato-ulmin (Svircev *et al.*, 1988); lignin peroxidase (Daniel, Nilsson & Petersson, 1989); β-glucanase (Dickerson & Pollard, 1982) and laccase (Wood, 1979).

Monoclonal antibody technology enables a population of B lymphocytes to be screened for a desired antibody, providing successful fusion of each particular B lymphocyte occurs. This enables a complex antigen to be used for immunisation and the desired antibody selected by careful screening. It should be noted, however, that in practice it is always better to purify the antigen to give a higher probability of successful production of the wanted antibody. Where this is not possible, such as in the case of producing species specific MAbs to fungi, careful screening or use of techniques to enrich a desired B lymphocyte population

(George & Converse, 1988; King & Morrow, 1988) is essential.

The type of antigen used for immunisation for either polyclonal or MAbs is also determined by the technique in which the antibody is to be used. For example, cell wall antisera have been found to be better for detecting fungi using immunofluorescence techniques (Malajzcuk *et al.*, 1975; Kough & Linderman, 1986; Fitzell, Evans & Fahy, 1980a). Where antibodies have been used in ELISAs to detect fungi, soluble antigens have proved easier to bind to ELISA plates than particulate antigens (Banowetz, Trione & Krygier, 1984) and, therefore, antibodies have been raised against soluble antigens (Wright, Morton & Sworobuk, 1987; Dewey, MacDonald & Phillips, 1989a; Dewey, Munday & Brasier, 1989b).

1.2. Antibody uses: Detection and Identification.

There are relatively few medically important fungi compared to the numbers of medically important bacteria and viruses. Bacteria and viruses are also much more difficult to identify quickly and consequently more use has been made of antibodies to identify these organisms than to identify fungi. The first antibody detection tests for any of these organisms used polyclonal antibodies, but MAbs are now used more frequently. For example, MAbs are used to detect varicella-zoster virus (Rawlinson *et al.*, 1989), cytomegalovirus (Lucas *et al.*, 1989), both clinical and environmental isolates of *Legionella* spp. (Brindle, Stannett & Tobin, 1987) and *Neisseria gonorrhoeae* (Carlson *et al.*, 1987; Tam & Sandstrom, 1988; Young & Moyes, 1989).

Similarly in non-medical microbiology antibodies have also been used more frequently to recognise bacteria and viruses, especially viruses. Clark (1981) and Lommel, McCain & Morris (1982) have reviewed the uses of antisera to recognise plant viruses, and Halk & de Boer (1985) and Martin (1987) the use of MAbs in the detection of plant viruses. By 1985

plant viruses from 20 families could be identified using MAbs. In the same review (Halk & de Boer, 1985) only six examples of MAbs to plant bacteria and two to plant fungi were cited. Schaad (1979) reviewed the use of antisera to recognise plant bacteria, but since then antibody recognition of bacteria has advanced considerably and Macario & Conway-Macario (1985-6, 1988) have reviewed the uses of MAbs in all types of bacteriology. Reference to these reviews provides a useful background for the antibody recognition of micro-organisms other than fungi, and therefore, except for particular examples, the uses of antibodies reviewed here refer to the recognition of fungi.

Detection can occur at a number of different taxonomic levels *e.g.* fungus from bacterium, fungal genus from genus, fungal strain from strain; the differentiation depending on the level of antibody specificity.

1.2.1. Detection of medically important fungi.

1.2.1.1. Using polyclonal antisera.

In medical mycology detection of a fungus by immunological methods has often not been by use of reference antisera to detect fungal antigens, but rather use of reference antigens to detect circulating antibody in a patient's serum. Although the test procedures are the same the interpretation of the results is not necessarily the same. A negative result from antigen identification with reference sera is a true negative, but a negative from antifungal antibody identification may be due to several factors. These include incorrect test antigen and lack of antibody in the infected patient, which may have two causes: no immune response or insufficient immune response at the test date.

It is the absence of a normal host immune response in many mycotic patients which has prompted the increased use of serological tests that recognise fungal antigen. In the past 10-15 years there has been an increase in the incidence of mycoses, mainly due to the increased use of immnosuppressive drugs and the emergence of Acquired Immune

Deficiency Syndrome (AIDS) (Degregorio *et al.*, 1982; Horn *et al.*, 1985; Chandler, 1985; Campbell & White, 1989). Patients either on these drugs or with AIDS are unable to produce antibodies in response to fungal infection and so serological tests that recognise fungal antigens have been developed.

Some of the more successful diagnostic tests that have been accepted as reliable are exoantigen immunoidentification tests. Samples from a patient are cultured and exoantigens extracted from the subsequent pure cultures, usually by merthiolate treatment. One of the first fungi to be accurately identified by exoantigen tests was *Histoplasma capsulatum. H. capsulatum* possesses two unique exoantigens, H and M. Using reference antisera raised against these two antigens, and with reference antigens as controls, H and M exoantigens can be identified by reverse agar gel double diffusion or counter-immunoelectrophoresis (Standard & Kaufman, 1976). Similarly, *Blastomyces dermatitidis* can be identified by its A exoantigen (Kaufman & Standard, 1978; Kaufman, Standard & Padkye, 1983); *Coccidioides immitis* by its HS (heat stable) and HL and F (heat labile) exoantigens (Kaufman & Standard, 1978; Huppert, Sun & Rice, 1978); and *Paracoccidioides brasiliensis* by its protein antigen E and antigens 2 and 3 (Standard & Kaufman, 1980). Commercial exoantigen reagents are available for identification of *H. capsulatum*, *C. immitis* and *B. dermatitidis* (Immuno-Mycologics, Inc., Norman, Oklahoma and Nolan Scott Biological Laboratories, Inc., Tucker, Georgia). False positive reactions have not been encountered, but false negatives may occur (Kaufman & Standard, 1987). These may be attributable to technical error, absence of specific homologous antibody in the reference antiserum, or inadequate antigen concentration.

Exoantigen tests are relatively quick compared with conventional morphologic tests but they still require a culturing step rather than direct testing of a sample. Direct testing of samples excludes this step and speeds identification, however, the sampling technique

should not be traumatic for a patient. For example, transbronchial specimens can be obtained by bronchoscopy for direct identification of a fungus by immunofluorescence or conventional staining, but bronchoscopy is often a hazardous procedure in a critically ill patient. Therefore, direct identification of antigen is best performed on easily available body samples *e.g.* blood, urine or bronchoalveolar lavage fluid.

One of the major causes of morbidity and mortality in immunocompromised patients is candidiasis and *Candida* antigens have been detected in body fluids by several serological techniques, including enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and latex agglutination (LA). Antigens originating from both the cytoplasm and cell wall of *Candida* spp. have been detected and commercial tests kits are available which detect either of these type of antigens. The CAND-TEC latex agglutination test (Ramco Laboratories, Houston, Texas) is based on the work of Gentry *et al.* (1983) and detects a circulating heat-labile glycoprotein; whilst the LA Candida Antigen Detection System (Immuno-Mycologics, Inc., Norman, Oklahoma) detects circulating *Candida* mannan.

Different workers have reported different correlations between the antigens detected and the occurrence of systemic candidiasis. For example, several workers have compared the detection of cytoplasmic and mannan antigens using antisera specific to either of these antigens in retrospective studies of sera from patients with known medical histories. In some cases the two commercial kits (CAND-TEC and LA Candida) have been compared (Fung, Donta & Tilton, 1986) and in others one of the kits has been compared with "in-house" antisera (Kahn & Jones, 1986 and Bailey *et al.*, 1985). Some workers have found that specific detection of circulating cytoplasmic *Candida* antigens correlated with systemic candidiasis, and that it was possible to differentiate between colonisation and deep-seated infection by the different titres given by the circulating cytoplasmic antigens in the respective cases (Araj *et al.*, 1982, Gentry *et al.*, 1983, Fung *et al.*, 1986 and Price & Gentry,

1986). These workers all reported low or negative titres for the detection of mannan antigens in the same sera. Other workers (de Repentigny *et al.*, 1985, Bailey *et al.*, 1985 and Kahn & Jones, 1986) found that detection of mannan did correlate with deep-seated *Candida* infections. Kahn & Jones (1986) claimed also that serological mannan antigen detection had greater diagnostic sensitivity than cytoplasmic antigen detection.

Matthews & Burnie (1988) used an affinity purified rabbit antiserum that recognised a circulating 47Kd protein antigen found in patients' sera to diagnose systemic candidiasis. This 47Kd protein had been found to be immunodominant during earlier studies (Matthews, Burnie & Tabaqchali, 1987). Matthews & Burnie (1988) found that the antiserum raised against the 47Kd protein was effective at detecting *C. albicans* but not other *Candida* species. There were also false negatives that occurred when testing sera from neutropenic patients with *Candida* infections and false positives from patients with autoantibodies. False positives were also reported with the CAND-TEC system when the rheumatoid factor was present (Gentry *et al.*, 1983 and Price & Gentry, 1986). The level of reaction, however, was lower than the normal level of reaction for diagnosis of candidiasis, although Price & Gentry (1986) reported that the agglutination increased as the rheumatoid factor titres increased.

The conclusion reached by de Repentigny *et al.* (1985) that diagnosis of *Candida* infection should involve two methods, in their case blood cultures and enzyme-linked assays for mannan, appears to apply to all of the studies. It is inadvisable to rely on one technique as the sole means of diagnosing candidiasis.

Latex agglutination using a polyclonal antiserum is also used to diagnose cryptococcosis. The capsular polysaccharide antigens of *Cryptococcus neoformans* can be detected in body fluids by this technique. There are four serotypes of the capsular polysaccharide and a polyclonal antiserum enables detection of all four serotypes, although there may be bias towards one particular serotype (Eckert & Kozel, 1986).

The detection of antibodies that recognise *Aspergillus* antigens as a means of diagnosing *Aspergillus* infections has been reported by a number of workers and was reviewed by de Repentigny & Reiss (1984). Diagnosis of *Aspergillus* infections by serological detection of circulating *Aspergillus* antigens has become more prevalent because of the reasons outlined previously. Diagnosis of *Aspergillus* infections has relied on the detection of circulating galactomannans, whether the antisera has been used in ELISA, RIA or latex agglutination tests (de Repentigny & Reiss, 1984).

1.2.1.2. Using MAbs.

For use as a routine means of detecting the presence of fungi which cause mycoses MAbs have the particular advantage of enabling standardisation because of their properties outlined in Table 1.1. Difficulties arise, however, not only in producing a MAb to a species-specific epitope, but also in selecting an epitope which will be indicative of the presence of infection. Also, the difficulty of correlating the presence of an antigen with invasive rather than non-invasive disease, and the detection of the disease in time to start a successful disease control programme, do not recede because of the use of a MAb rather than a polyclonal antiserum.

This was found to be the case in work carried out by Reiss *et al.* (1986). They used MAbs raised against *Candida tropicalis* mannans, which recognise mannans from *C. albicans* serotype A and *C. tropicalis*, to detect circulating mannans during infection using both ELISA and immunofluorescence. Although sensitivity was in the low ng ml^{-1} range, 70%, at best, of the sera from patients with disease tested positive for mannans. In comparison, however, with detection of the candidal metabolites, arabinitol and mannose, using gas-liquid chromatography, the detection of mannans using the MAbs was found to be more specific and sensitive. The figure of 70%, however, is no improvement compared with some of results found using polyclonal antisera. For example, Ness *et al.* (1988) reported a

68% correlation between invasive candidiasis and detection of circulating antigen using the CAND-TEC kit.

De Repentigny (1986) postulated that the rapid formation and clearance of immune complexes may explain the relative low level of detection of antigen compared with the level of disease. Maida & Buckley (1986), however, used a MAb which recognised the immunodominant 48Kd protein frequently found in patient sera to show that this is not so. They found that circulating immune complexes did not play a significant role in the difficulties associated with detecting circulating *C. albicans* antigens, when they tested sera from 12 neutropenic patients. The MAb used in the survey of Maida & Buckley (1986) was one of three derived from mice immunised with *C. albicans* (Strockbine, Largen & Buckley, 1984). The 48Kd protein of Maida & Buckley (1986) may well correspond to the immunodominant 47Kd protein used to raise a polyclonal antiserum (Matthews & Burnie, 1988), as described in 1.2.1.1.

Chardès *et al.* (1986) produced 239 hybridomas secreting anti-*Candida* antibodies from 4870 hydridomas tested. They studied seven in some detail and found that two of these seven recognised *C. albicans* and *C. tropicalis*, but not other *Candida* spp.; whereas the other five recognised *C. albicans* serotype A, and not serotype B, but cross-reacted with all other *Candida* spp. tested. The use of these MAbs to identify circulating antigens in patients has not yet been reported.

Another MAb raised against *C. albicans* which has not yet been used to identify an infection is that of Hopwood *et al.* (1986). This antibody recognises glycoproteins from *C. albicans* wild type strains, but not those from a mannan deficient mutant of *C.albicans* or from a wild type strain of *S. cerevisiae* when tested using immunofluorescence. Some of the epitope was present in the vesicles of *S. cerevisiae* blastoconidia.

Eckert & Kozel (1986) raised four MAbs which recognise capsular polysaccharides of the four serotypes of *Cryptococcus neoformans*. Two of the MAbs were derived from mice immunised with serotype A capsular polysaccharide. One of these MAbs reacted with polysaccharides of all four serotypes (A, B, C and D) and the other with polysaccharides of serotypes A, B and D. The other two MAbs were derived from a mouse immunised with serotype A polysaccharide and one of these MAbs also reacted with polysaccharide from serotypes A, B and D. The other MAb derived from the same mouse reacted with seroptype A and D polysaccharides.

Kurup (1989) produced MAbs which bind to specific antigens of *Aspergillus fumigatus* associated with allergic bronchopulmonary aspergillosis and Phillips & Wiener (1987) have diagnosed invasive aspergillosis using MAbs in immunocytochemical staining of tissue removed by bronchoscopy.

MAbs which recognise *Coccidioides immitis* antigens rather than antigens from other pathogenic fungi (*e.g. C. albicans, H. capsulatum, Cryptococcus neoformans*) have been raised by Kraeger *et al.* (1986). These MAbs recognise different forms of *C. immitis*. The use of these MAbs in assays to detect *C. immitis* antigens has not yet appeared.

These examples illustrate that MAbs can be successfully raised against medically important fungi. The MAbs, however, cannot yet be used in routine practice to replace other methods of detecting mycoses, although they are useful additional means of testing for infection.

1.2.2. Detection and identification of non-medical fungi.

Immunological identification of non-medical fungi has concentrated on species which can not be identified easily and quickly by morphology. Fungi falling into this category include soil and seed borne plant pathogens, endo- and ecto- mycorrhizae, plant endophytic fungi, food contaminants and litter decomposers.

As will be discussed in later sections antibodies can be used in other studies of fungi besides detection and identification, but the main rational behind the use of antibodies so far has been to enable quick and easy detection. For example, the aims of antibody detection of fungi have included the quantification of fungal contamination in food; the surveillance of seed plants; and the detection of fungi such as endo- and ecto-mycorrhizae in experiments designed to test the efficacy of inoculation of sterile plots with mycorrhizal fungi and their subsequent infection of plants and spread through the soil.

1.2.2.1. Using polyclonal antibodies.

Cross-reactivity can hinder the use of polyclonal antibodies, but it can also prove useful. For example, Robertson *et al.* (1988) used a mixture of antigens from five different fungi to raise a pan-fungal antiserum which would recognise any fungal contaminant of comminuted tomato products such as juices and pastes. There are legislative limits on the amount of decayed fruit that can be incorporated in these products. At present assessment of the amount of decayed fruit is by the Howard Mould Count (Howard, 1911), which relies on a correlation between the proportion of decayed fruit in the product and the number of hyphae counted by microscopy. The pan-fungal antiserum was used to stain hyphae in an immunofluorescence technique. This enabled the hyphae to be seen more clearly against the background thus enabling more accurate counting and a reduction in the time taken to perform the count.

The example of Robertson *et al.* (1988) illustrates the need to consider the aim for immunological identification. For their purposes a polyclonal antiserum had the necessary properties; for other workers, whose aim has been more specific recognition tools, polyclonal antisera have not readily been the specific probes they need to be for *in situ* studies.

For example Johnson *et al.* (1982) developed a detection system for the endophyte *Epichloe typhina* in tall fescue using an antiserum in a sandwich ELISA. Initially they tested

the antiserum against 14 other soil fungi and found it only reacted with three of these (*Fusarium, Rhizoctonia* and *Claviceps*), and then at only one tenth the absorbance of *E. typhina*. Siegel *et al.* (1984a, b) used the ELISA to investigate the incidence and dissemination of *E. typhina* in experimental plots and fields, and to evaluate control methods in field plots. However, Siegel *et al.* (1988) have since reported that they no longer use the assay routinely because of reactions with other endophytes, which were not originally screened for cross-reactivity.

The example of Siegel's group illustrates the most common problem with serological identification *in situ*, namely cross-reactivity with other fungi which inhabit a similar ecological niche. El-Nashaar *et al.* (1986) found this with antisera raised against *Gaeumannomyces graminis* var. *tritici*. They found that the antiserum raised against a cell wall fraction cross-reacted with seven species of soil and rhizosphere fungi, namely *Rhizopus* sp., *Trichoderma* sp., *Fusarium graminearum, Fusarium roseum, Phoma* sp., *Colletotrichum* sp., *Rhizoctonia* sp. and *Gliocladium* sp.. This cross-reactivity of the cell wall antiserum was less than that of the cytoplasmic antiserum, five more species reacting with the cytoplasmic antiserum. This corresponded with the findings of Holland & Choo (1970) and Choo & Holland (1970) that cell wall antisera were more specific for *Ophiobolus (Gaeumannomyces) graminis* than antisera raised to soluble and whole cell fractions. However, because of the cross-reaction with other soil fungi, El-Nashaar *et al.* (1986) were only able to use their ELISA system to study *G. graminis* var. *tritici* in trial pots of wheat seedlings.

Similarly, Mohan (1988) was able to detect *Phytophthora fragariae* in strawberry plants using an antiserum raised to *P. fragariae* soluble proteins. *P. fragariae* causes the notifiable disease, red core, and fungal antigens could be detected by ELISA 6-8 days after inoculation, and before macroscopic symptoms appeared, making this technique very

attractive as an early and rapid means of detection. However, the antiserum also reacted with *Phytophthora cactorum*, leathery rot of strawberries, and so could not be a specific test for *P. fragariae in situ*. Amouzou-Alladaye *et al.* (1988) also raised an antiserum to soluble proteins of *P. fragariae*. Their antiserum showed similar cross-reactivity to that of Mohan (1988), *i.e.* cross-reaction with *P. cactorum*, but it also cross- reacted with *Pythium middletonii*.

Other antisera raised against *Phytophthora* spp. have also cross- reacted within the genus and to varying extents with other members of the Oomycetes, but in general they have not cross-reacted with genera outside the Oomycetes: making them some of the more specific antisera raised (Burrell *et al.*, 1966; Morton & Dukes, 1967; Merz, Burrell & Gallegly, 1969; Malajzcuk, McComb & Parker, 1975; Halsall, 1976; MacDonald & Duniway, 1979). Unfortunately, closely related *Phytophthora* spp. often inhabit similar niches, so thwarting specific serological identification, as the examples of Mohan (1988) and Amouzou-Alladaye *et al.* (1988) show.

The previous examples illustrate how the cross-reactions of antisera with other fungi in the habitat of the studied fungus invalidate any attempt to identify specifically the fungus in question. Nevertheless, not all studies have had such problems. Gleason, Ghabrial & Ferriss (1987) were able to use an antiserum to detect soybean seed decay by ELISA. Soybean seed decay is caused by members of the *Diaporthe/Phomopsis* complex. Gleason *et al.* (1987) found that their antiserum could distinguish between members of this complex and seven other seed-borne fungi, although the antiserum was unable to differentiate between members of the complex. Likewise, antisera raised against *Phoma exigua* var. *foveata* and *P. e.* var. *exigua*, which both cause gangrene in potatoes, were unable to discriminate between the two varieties; but they did distinguish the two gangrene fungi from other potato pathogens such as *Phytophthora infestans* and *Fusarium* spp. (Aguelon & Dunez, 1984). Therefore, these

antisera could be used in an ELISA as a specific test for gangrene-causing fungi. Cross-reaction of the antisera with *Phoma tracheiphila* did not interfere with the test and give false positives, because *P. tracheiphila* does not occur in potatoes. It does, however, occur in lemons causing mal secco disease and Nachmias *et al.* (1979) were able to detect its presence in infected lemons by ELISA. The reactivity of the antiserum with *P. exigua*, similarly, not interfering with the assay because *P. exigua* is not present on lemons.

Several workers have also found that, although their antisera raised have shown cross-reactivity outside closely related fungi, they have been able to use the antisera in *in situ* studies because the fungi with which they cross-react are not usually present in the environment of the test fungus. An example of this type is the antiserum raised to *Colletotrichum* sp. by Barker & Pitt (1988). They raised an antiserum to a cell wall fraction from an isolate of a *Colletotrichum* sp., which causes leaf curl in anemones. The antiserum cross-reacted strongly with all other leaf curl isolates tested and with three other closely related species (*Colletotrichum acutatum, C. gloeosporioides* and *Glomerella cingulata*): as well as weakly with *Sordaria fimicola, Botrytis cinerea, Phycomyces blakesleeanus, Rhizopus sexualis* and *Penicillium notatum.* However, these cross-reactions led to no false positives in the diagnosis of leaf curl infection in anemones by ELISA because none of the cross-reacting fungi were isolated from diseased corms. Nor should the cross-reactions lead to false positives in other hands, as the cross-reacting fungi are not usually isolated from corms with leaf curl.

Another example of this type was that of Gerik *et al.* (1987), who raised an antiserum to *Verticillium dahliae*. They were able to specifically detect *V. dahliae* in cotton root tissue. Unger & Wolf (1988) have reported the successful use of an antiserum to specifically recognise *Pseudocercosporella herpotrichoides*, which causes invasive eyespot of winter-sown cereals. It should be noted, however, that Dewey (1988) reported that she had been

unable to dilute out a strong cross-reaction with *Rhizoctonia solani* given by an antiserum raised against *P. herpotrichoides* and that Unger & Wolf (1988) did not test their antiserum against *R. solani.*

Although antisera have shown cross-reactivity with closely related fungi and with fungi in the surrounding habitat, it has been possible to use them in controlled experiments. For example Kough & Linderman (1986) used a cell wall antiserum which did not cross-react with species outside the genus *Glomus* to detect *Glomus* spp. in VA mycorrhizal infected soils. The antiserum was able to indicate the distribution of *Glomus* spp., but was not able to discriminate between the initial antigenic species, *Glomus versiforme*, and other *Glomus* spp.; thus making this a genus, rather than a species, specific study. In a similar study, Aldwell & Hall (1986) were able to exploit antigenic differences between *Glomus mosseae* and *Acaulospora laevis* to follow the spread of *G. mosseae* through soil infested with *A. laevis.* Aldwell *et al.* (1983) had compared the antigenic relationship between four species of VA mycorrhizae using antisera raised to the four species in an ELISA. Their results suggested that *G. mosseae* and *A. laevis* are not closely related and antisera raised to both species will differentiate between the two in an ELISA. However, Aldwell *et al.* (1983) also suggested that *Gigaspora margarita* and *A. laevis* are very closely related antigenically and antisera could not be used to differentiate these two species in the soil.

Schmidt *et al.* (1974) studied the ectomycorrhizal association between red pine and *Pisolithus tinctorius* by immunofluorescence using an antiserum prepared against a *P. tinctorius* mycelial homogenate. Although the antiserum reacted against other fungi this study (Schmidt *et al.*, 1974) showed the potential of antibodies for investigation of ectomycorrhizal associations as well as endomycorrhizal associations. In a similar study Fitzell, Evans & Fahy (1980) used an antiserum raised against *Verticillium dahliae* cell walls to show that colonisation of plant roots by *V. dahliae* occurred directly by germ tubes

germinated from microsclerotia.

Chard, Gray & Frankland (1985b) found the antiserum they raised against *Mycena galopus* showed cross-reactions with other *Mycena* spp. as well as with several species from the Asco- and Deuteromycotina. Absorption of the antiserum with cross-reacting fungi reduced the level of fluorescence with most cross-reacting species to an acceptable level, although the fluorescence of some fungi remained bright, *e.g. Collybia* spp. and other species of *Mycena*. They concluded that the antiserum did have potential use in laboratory investigations, *e.g.* competition between *M. galopus* and *Marasmius androsaceus*, when species were differentiated by the antiserum.

1.2.2.2. Using MAbs.

The relative lack of specificity of polyclonal antisera plus the other advantages of MAbs as outlined in Table 1.1 has led several workers to investigate MAbs as detection and identification probes.

Banowetz *et al.* (1984) tried to produce MAbs which would differentiate between two *Tilletia* species, *Tilletia controversa* which causes dwarf bunt disease of wheat and *Tilletia caries* which causes common bunt. They wanted to develop an assay to distinguish between the two species in grain shipments. Certain countries, such as China, ban any shipments with *Tilletia* spores present because they do not have indigenous *T. controversa* (dwarf bunt) infections and wish to keep it that way. Current methods of differentiation between the two species take too long for routine examination of grain shipments. Fixed teliospores or water or potassium iodide extracts of the teliospores were used as immunogens. Two sets of mice also received either cyclophosphamide treatment or co-immunisation with a MAb which reacted with immunodominant antigens of both *T. controversa* and *T. caries* teliospores. All antibodies secreted from the hybridomas reacted with both *T. controversa* and *T. caries*, none of the immunisation protocols producing species-specific antibodies. Quantitative

differences were found, with homologous teliospores binding quantitatively more antibody. Quantitative differences are, however, of no value to differentiate between two species if both are present. All of the antibodies reacted with polysaccharides. The antibodies also cross-reacted with the sporidial stage of *Ustilago scitaminea*, the sugar cane smut fungus.

Hardham *et al.* (1985) stated that "the investigation of *Phytophthora cinnamomi in situ* would be greatly helped by definitive probes" and they described a panel of MAbs raised against *P. cinnamomi* which showed discrimination at various taxonomic levels including strain differentiation. Hardham's group, however, have not used their monoclonal antibodies *in situ*, chosing instead to use them to investigate the organism further as will be discussed in Section 1.7. Callow, Estrada-Garcia & Green (1987) have also raised MAbs to an Oomycete fungus. They raised six MAbs which show specific recognition of *Pythium aphanidermatum* and two MAbs which react with other Oomycetes tested.

The most specific MAb to date for detection of a fungus is that of Wright *et al.* (1987). This MAb was raised against spores of the VAM fungus, *Glomus occultum*. It recognises a soluble protein (Wright & Morton, 1989) and is specific for *G. occultum*. The detection of spores using this antibody in an ELISA is sensitive to one *G. occultum* spore and in the presence of other VAM spores the signal is only strong when *G. occultum* spores are present. The protein antigen is also present in the hyphae and Wright & Morton (1989) devised an immunoblotting technique to detect *G. occultum* in roots. They squashed infected roots on to a nitrocellulose filter and then followed a modified immunoblotting procedure to show specifically the presence of *G. occultum* in roots, even when other VAM fungi were also present. This technique is particularly useful since *Gl. occultum* is one of the VAM fungi which are not stained by conventional techniques (Morton, 1985). The immunoblotting technique neatly overcomes the problem of discovering the extent of colonisation of a root with a MAb to an internal rather than an external antigen.

Dewey *et al.* (1989b) produced MAbs to the Dutch elm fungus *Ophiostoma ulmi*. 33 MAbs were produced and six specifically recognised *O. ulmi*, but not other fungi. Two of the MAbs showed discrimination between insoluble mycelial antigens of aggressive and non-aggressive isolates, but did not discriminate between soluble mycelial antigens of the different isolates. The pattern of cross-reactivity of the species-specific MAbs with elm tissue was also varied: one of the species-specific MAbs, 18FH1, reacted more strongly with heathly host tissue than with infected host tissue, but the other species-specific MAbs showed little or no cross-reaction.

Dewey *et al.* (1989a) raised a MAb to *Humicola lanuginosa*, a fungus which causes discolouration of rice, for use in detection kits. Although this MAb showed some cross-reactivity with *C. sepedonium*, *Penicillium diversum* var. *aureum* and *Penicillium variabile*, it was suitable for use in detection kits as these fungi do not occur in rice. The MAb also showed no cross-reaction with rice grains which some of the other MAbs raised against *H. lanuginosa* did. Dewey has also raised a species-specific MAb to *Penicillium islandicum* (Dewey, pers. comm. 1989). Both this MAb and the MAb to *H. lanuginosa* were raised against surface washings of the two fungi. The components of these surface washings were analysed by MacDonald, Dunstan & Dewey (1989) and were found to contain low molecular weight glycoproteins. Some of these surface components appear very specific, *e.g.* for *H. lanuginosa* and *P. islandicum*, and are obvious targets for future work. Dewey has found, however, that this technique does not work for *Pseudocercosporella herpotrichoides*, since very little material is removed from a culture by surface washing (Dewey, pers. comm. 1989).

Combination of a panel of MAbs can overcome some of the problems of detection caused by cross-reactivity. For example, Pepin & Prager (1988) raised MAbs against *Phytophthora fragariae* and found that most of the MAbs cross-reacted with more than one

race, but using a combination of MAbs with different cross-reactivity with different races they were able to differentiate each race.

The arrival of MAbs, with their uniformity and infinite supply, has led to attempts to exploit MAbs in the market place for diagnosis of plant diseases. Bio-Reba was the first company to supply MAbs in kit form for the diagnosis of plant diseases, plant viruses rather than fungi being the pathogens involved. The certification of disease free plants, particularly virus free material, has led to several openings for such kits. The market, however, is small, with the potential turn-over estimated to be £5million in 1990.

The market for fungal diagnostic kits is but a small part of this. However, Agri-Diagnostics Associates (Cinnaminson, New Jersey, U.S.A.) now sell detection kits which can be used on site and the test, based on a double antibody sandwich ELISA, takes only 30mins to complete. The three "Reveal" tests for turf disease detection each test for one of three turf grass diseases common on U.S. golf greens: *Pythium* blight caused by a number of *Pythium* spp., but especially *P. aphanidermatum*; brown patch caused by *Rhizoctonia solani* and dollar spot caused by *Sclerotinia homoeocarpa*. None of the MAbs use in these kits are species-specific, but the MAbs used have been selected so that they diagnose for these diseases whether or not other fungi are present. For example, the *Pythium* test kit MAb was raised against *P. aphanidermatum* (Miller *et al.*, 1986) but cross-reacts with 10 other species of *Pythium*. These cross-reactions do not interfere with diagnosis of *Pythium* blight of turf grass since these other species may be involved as well as the predominate causal agent, *P. aphanidermatum*. On site surveys of *Pythium* blight test reading with the potential danger of disease and recommends fungicide treatment above a certain threshold level.

Agri-Diagnostics also supply conventional 96 well ELISA tests kits for experimental field surveys and are presently co-operating with Dr. John Menge (University of California, Riverside) on the potential use of their *Phytophthora* ELISA kit for the diagnosis of

Phytophthora root diseases in Californian fruit groves (Miller, pers. comm. 1989). There is a maximum statutory level for *Phytophthora* infection of fruit tree seedlings above which seedlings are rejected from sale. At present infection is assessed by plate counts and is very laborious, and a rapid ELISA test for assessment of infection would be preferable.

Several MAbs have been raised to mycotoxins to enable detection of these toxins in food stuffs.

1.3 Other methods of detection and identification of fungi.

1.3.1. Microscopic identification.

The classic method of fungal identification is microscopic examination of the morphology of the fungus from pure cultures. In most cases, specimens are examined directly by bright field or phase contrast optics, but various types of reagents have also been used to aid microscopic examination. For example, dyes such as lactophenol blue, methylene blue, periodic acid Schiff and Mayer's mucicarmine for *Cryptococcus*, enable fungi to be distinguished from the surrounding substrate, making morphological identification easier.

Similarly, fluorescent brighteners can enable better visualisation of fungi and there are examples of their application both in medical and plant pathology. Wachsmuth (1988) showed that Uvitex 2B and Calcofluor White M2R were able to selectively stain *Candida albicans* and *Blastomyces dermatitidis* in mouse lung and kidney sections but both fluorochromes also stained elastic fibres, so counter staining with Evans blue was needed to enhance contrast between fungal and background fluorescence. In plant pathology Calcofluor has also been used to observe fungal parasites in leaves of tomatoes (Rohringer *et al.*, 1977).

Similarly, Mills *et al.* (1982) showed the fluorescent dye acridine orange differentially stained fungal structures in plant tissue enabling easier examination of the fungus. Counter-

staining with malachite or methyl green was used to quench excessive background fluorescence.

Söderström (1977) detected fungi in soil samples using fluorescein diacetate. This fluorogen is actively metabolised by fungal esterases to give fluorescent fluorescein so that fungal structures containing functional esterases are stained. Fluorescein diacetate is also metabolised by other micro-organisms and there have been differing reports as to the efficiency of bacterial staining by fluorescein diacetate (Ziegler, Ziegler & Witzenhausen, 1975 and Weaver & Zibilske, 1975). Söderström (1977) reported that staining of bacteria did occur in his soil samples.

Fluorochrome-conjugated lectins can also be used to detect fungi. Lectins recognise specific sugar residues which are found in fungal cell wall polysaccharides, *e.g.* wheat-germ agglutinin (WGA) recognises n-acetyl-glucosamine residues, which are present in fungal chitin. Using FITC conjugated WGA Meyberg (1988) specifically detected fungi in lichens, mycorrhizal roots and diseased plants.

Karyanannopoulou, Weiss & Damjanov (1988) found that FITC conjugated Concanavalin A (ConA), WGA and pokeweed mitogen (PWM) reacted with fungal pathogens commonly found in nosocomial infections. They showed that succinylated ConA and PWM gave the least background fluorescence and were hence best for showing the fungal infection.

Most of these reagents differentiate between fungi and other organisms by recognition of specific fungal components, thus enabling detection of fungi in different substrates. However, a major disadvantage of these reagents is cross-reactivity. The cross-reactivity across the fungi, or even outside this group, occurs because, although the reaction of the reagents with their targets is specific, the distribution of the targets is not. This cross-reactivity can be either an advantage or a disadvantage depending on the detection

specificity needed.

1.3.2. Molecular identification.

Distinct and reproducible differences have been found between the protein patterns of related species and strains by 1 and 2-D gel electrophoresis; and have been used to identify fungi. An early example of fungal identification using PAGE was Chang, Srb & Stewart (1962), who were able to recognise differential proteins patterns between *Neurospora crassa, N. sitophila, N. intemedida* and a wild-type *Neurospora* from the Philippines. Other early examples of differentiation by gel electrophoresis include identification of *Phytophthora spp.* by Clare & Zentmeyer (1966) and three *Penicillium* species by Bent (1967).

Although Glynn & Reid (1969) and Sorenson, Larsh & Hamps (1971) expressed doubts as to the taxonomic value of such studies there has been continued use of PAGE to analyse fungal species and strains. Most recent PAGE work has involved the study of closely related plant pathogenic species were morphological identification is often difficult. This technique is relatively slow, especially if 2-D gels are run, and has been used more in taxonomic studies, but has also been used as a means of routine identification. For example, Bielenin *et al.* (1988) have separated six species of *Phytophthora* associated with deciduous fruit crops. Taxonomic studies of fungi using gel electrophoresis include a comparison of polypeptides from the wheat bunt fungi, *Tilletia laevis*, *Tilletia tritici* and *Tilletia controversa*, from which Kawchuk, Kim and Nielsen (1988) proposed that these three species be reclassified as one species, *Tilletia tritici*, with three varieties, *laevis*, *tritici*, and *controversa.* Other examples include separation of 26 isolates of *Phytophthora megasperma* f. sp. *medicaginis* into two groups which correlated with cardinal temperatures for growth, pathogenicity and oogonium size (Faris, Sabo & Cloutier, 1985); confirmation of traditional cultural criteria based species grouping of three species of *Sclerotinia* (Tariq, Gutteridge & Jeffries, 1985);

and differentiation of four physiologic races of *Puccinia graminis* f. sp. *tritici* (Howes, Kim & Rohringer, 1982).

Gel electrophoresis has been used in conjunction with enzyme staining to differentiate species of vesicular-arbuscular mycorrhizal fungi. Differences in the isozyme pattern revealed by enzyme staining can show inter- and intra- specific variation if a combination of enzymes are used. Sen & Hepper (1986) used this method to separate six species of *Glomus* by esterase (E.C. no. 3.1.1.1.) staining and Hepper *et al.* (1988) used other enzymes in conjunction with esterase to compare different geographical isolates of *Glomus clarum, Glomus monosporum* and *Glomus mosseae*. In the latter case they found that it was not possible to separate some closely related species by this method *e.g. G. monosporum/G. mosseae* and *G. clarum/Glomus manihotis*. It may be, however, that these two pairings represent examples that are probably co-specific (Walker, pers. comm., 1988).

Although identification by means of PAGE can differentiate many fungi from their close relatives it does not lend itself readily to detection of fungi in environmental samples. The multiplication of the number of bands or spots and the probable overlapping of host and fungus polypeptides make the method impracticable. For example, Hepper, Sen & Maskall (1986) found that although they could identify the presence of *Glomus caledonium* and *G. mosseae* in the leek (*Allium porrum* L.) cultivar Musselburgh by the use of any of the enzyme stains they used they could not identify *G. mosseae* in leek cv. Early Market by its characteristic PEP activity because the leek cultivar had an additional band of PEP activity with similar mobility to the diagnostic fungal bands. They also found that infection needed to be above a threshold level of 15% before it could be detected. In a subsequent study from this work Rosendahl *et al.* (1989) used this technique to study the infection of leek roots (*Allium porrum* cv. Musselburgh) by three *Glomus* spp., *G. mosseae, G. caledonium* and *Glomus* sp. type E3. They showed that not only could they detect the vesicular-arbuscular

mycorrhizal fungi on the basis of enzyme activity, but that they could use the enzyme activity to quantify each fungus.

At the Foreign Disease-Weed Science Research Unit (Frederick, Maryland, U.S.A.) starch gel electrophoresis in conjunction with enzyme staining is used to distinguish the two wheat bunt species, *Tilletia indica* and *Tilletia barclayana* (Bonde, Peterson & Matsumoto, 1989) on a routine basis.

1.3.3. Genetic identification.

Advances in molecular biology enable genetic methods to be considered as means of detection and identification. Targeting DNA as the means of recognition should enable a micro-organism to be identified whatever its phenotypic state since, DNA is present at all stages of a life cycle, unlike other targets which may vary throughout a life cycle.

In the case of fungi the genetic target may be genomic, ribosomal or mitochondrial DNA. Different workers have used different types of DNA, although for ease of handling most have chosen mitochondrial or ribosomal DNA. It is also important to note that the a type of DNA which enables differentiation of some fungi, may not enable differentiation of others. For example, Kistler *et al.* (1987) were able to differentiate strains of *Cochliobolus heterostrophus* by comparison of ribosomal DNA, but found that rDNA of form species of *Fusarium oxysporum* were too conserved to enable differentiation. However, they were able to differentiate between the *F. oxysporum* form species if they used mitochondrial DNA.

Comparison of fungal DNAs has been carried out most commonly by RFLP analysis. This involves cutting the chosen DNA with restriction endonucleases and then separating the resulting DNA fragments by agarose gel electrophoresis. If genomic DNA is used in this technique the result is a smear of DNA fragments running the whole length of the gel, from which only repetitive DNA fragments can be distinguished because they produce definite bands. These repetitive DNA fragment bands can be compared, usually, however, DNA

probes are used in Southern blot hybridisation and the resulting patterns analysed. Coddington *et al.* (1987) and Manicom *et al.* (1987) were able to differentiate species, form species, races and isolates of *Fusarium* by analysis of genomic DNA using random probes of genomic DNA.

More commonly RFLP and Southern blot hybridisation analysis of fungi has been carried out using ribosomal or mitochondrial DNA and most examples of fungal differentiation by this technique have been differentiation of yeasts. For example, strains and species of *Candida* have been differentiated using ribosomal DNA (Magee, D'Souza & Magee, 1987) and *Histoplasma capsulatum* isolates have been differentiated using both ribosomal and mitochondrial DNAs (Vincent *et al.*, 1986). Recently, identification of some plant pathogenic fungi has been undertaken. Förster *et al.* (1989) analysed RFLPs of the mitochondrial DNA of *Phytophthora megasperma* isolates. Their analysis showed *P.megasperma* f. p. *glycinea* isolates were very different to those of *P. megasperma* f. p. *medicaginis*, but that isolates from the same sub-species showed limited variation. Similarly, Hulbert *et al.* (1988) have analysed the lettuce downy mildew fungus, *Bremia lactucae*, using 55 genomic and cDNA probes. 61 RFLP loci were identified among three hetereothallic isolates of *B. lactucae*. Henson (1989) cloned a 4.3kb mitochondrial DNA fragment from *Gaeumannomyces graminis* which hybridised with all three varieties of *G. graminis* but showed little homology with DNA from other fungi. Henson (1989) reported that the probe was useful for identification of *G. graminis* strains as different strains exhibited characteristic hybridisation patterns with the probe.

Other electrophoretic techniques are now also being for genetic identification. Resolution of chromosome sized DNA molecules into an electrophoretic karotype using orthogonal field alternation gel electrophoresis (OFAGE) has been used to compare clinical isolates of *Candida* (Merz, Connelly & Hieter, 1988) and to differentiate serotypes of

Cryptococcus neoformans (Polachek & Lebens, 1989). Pulsed field electrophoresis has been used to produce electrophoretic karyotypes for *Neurospora crassa* (Orbach *et al.*, 1988), *Schizosaccharomyces pombe* (Smith *et al.*, 1987) and *C. albicans* (Snell & Wilkins, 1986), and clamped homogeneous electric field electrophresis (CHEF) has also been used to produce an electrophoretic karyotypes for *C. albicans* (Magee *et al.*, 1988). The advantage of the three latter techniques over RFLP analysis if that they enable fungal genomic DNA to be more easily studied and thus enable comparison between the genomic DNA of different species to be made more readily.

1.4. Antibody uses: Quantification.

The specific reaction of antibodies with their epitopes lends itself to quantification of the micro-organism present by measurement of the antibody-antigen reaction. The ELISA technique, whether sandwich or direct binding, particularly lends itself quantification, but adaptation of microscopic methods by addition of an immunofluorescence step can be used.

Many of the polyclonal antisera and MAbs raised to detect and identify fungi have also been used to quantify fungal biomass. Using ELISA techniques Johnson *et al.* (1982); El-Nashaar *et al.* (1986); Gleason *et al.* (1987); Amouzou-Alladaye *et al.* (1988) and Mohan (1988) have all quantified fungal biomass using polyclonal antisera. As described in 1.3.1 all of these antisera show cross-reactivity with other fungi in the environment of interest and so, although, the relationship between fungal biomass and ELISA absorbance was linear over a certain range in all of these examples, quantification *in situ* is not meaningful unless only one reactive fungus is present. These enables use in trials but not in the field.

Where antisera show cross-reactivity with other fungi but the cross-reacting fungi are not present in the habitat of interest the antisera can be used for quantification of the test fungus. Examples of such antisera include those of Barker & Pitt (1988) and Gerik *et al.*,

(1987). The level of specificity required also influences whether or not the antiserum can be used for quantification. For example, Fallon & Newell (1989) measured total fungal biomass in standing-dead leaves of the saltmarsh cordgrass, *Spartina alterniflora*, using an antiserum raised to the fungus, *Phaeosphaeria typharum*. The antiserum cross-reacted with other fungal species common to the saltmarsh but not with non-fungal material. They estimated that the fungal biomass could account for more than 10% of the carbon in standing-dead leaves of *S. alterniflora*.

Similar results to those observed with polyclonal antisera have been obtained for quantification of fungi using MAbs, *i.e.* where the MAbs are not species-specific their usefulness in quantification depends on the presence or absence of cross-reacting material. For example, the quantitative differences between the level of reaction of MAbs raised against *Tilletia* spp. could not be used to quantify the level of *T. caries* or *T. controversa* in grain shipments since both fungi are present (Banowetz *et al.*, 1984).

Although the test kits from Agri-Diagnostics do not possess species-specific MAbs they have been used to quantify the fungal biomass specifically associated with the disease to be diagnosed. The co-ordination of the ELISA reading has been related to fungal biomass and risk of disease as described in 1.2.2.2.

If a MAb is species-specific the problems of cross-reactions with other fungi *in situ* are avoided. Wright *et al.* (1987) were able to use a MAb which was specific to *Glomus occultum* to quantify spores of the fungus in amongst spores from other VA mycorrhizal species. The MAb was used in an ELISA to prepare a calibration curve of spore number against absorbance at 405nm and could detect 1-20 spores, above 20 the reading exceeded the maximum reading of the plate reader. Using the MAb in an ELISA Wright *et al.* (1987) were able to detect one *G. occultum* spore amongst 40 spores from other VAM species.

Antibodies have also been used in conjunction with microscopic techniques to quantify fungi. Malajczuk, Bowen & Greenhalgh (1978) compared a combined fluorescent antibody and soil sieving technique with a modified sieving method to count chlamydospores of *Phytophthora cinnamomi* in soil. In the modified sieving technique chlamydospores were washed on to a selective medium and the germinating chlamydospores counted after incubation at 24°C for 20h. They counted five times as many spores using the combined technique than by the sieving method. Malajzcuk *et al.* (1978) postulated that the difference in counts could be due to two reasons: the counting of dead spores by the fluorescent antibody technique as the antibody did not differentiate between dead and living material and secondly the lower counts using the sieving technique could be because the chlamydospores did not germinate on the medium used.

Frankland *et al.* (1981) used an antiserum raised against *Mycena galopus* to estimate mycelial biomass in leaf litter. Using the antiserum in combination with both the agar-film technique (see 1.5.1) and the membrane filter technique (see 1.5.2), they found that the fluorescence of the hyphae in the agar film technique was variable. They, therefore, chose the membrane filter technique with antibody staining to use in experiments to compare fungal biomass estimates using fluorescent antibody staining with a conventional technique, *i.e* the agar-film technique without antibody staining. They found that the length of fluorescent hyphae in *Quercus* litter at the base of *M. galopus* fruit bodies was $3267mg^{-1}$ of oven-dried litter compared with a hyphal length of $2863mg^{-1}$ of oven dried litter using the agar-film technique.

Mendgen (1986) reviewed serological estimation of fungal biomass and some earlier examples are referred to in this review. Two points that Mendgen (1986) mentioned in his review are particularly poignant for serological, and other, quantification of what might be referred to as "indicator" fungal molecules. They are: the non-uniformity of "indicator"

content in all stages of the fungus; for example, varying chitin content between spore and hyphal walls; and allied to this the differential expression of fungal components in different stages of the life-cycle, *e.g.* changes in protein patterns (Huang & Staples, 1982; Kim, Howes & Rohringer, 1982) and changes in surface carbohydrates (Mendgen, Lange & Bretschneider, 1985).

1.5. Other methods of fungal quantification.

These techniques have been used to quantify fungi on their own, but some have also been used in conjunction with antibodies as described in 1.4. and further joint applications may enable more accurate fungal biomass estimations to be made. Matcham, Jordan & Wood (1984) reviewed estimation of fungal growth on solid substrates. Some further methods of quantification, not considered here, were reviewed by these workers.

1.5.1. Agar-film technique.

This is the most commonly used means of fungal quantification in soil. It was first described by Jones & Mollinson (1948) and involves the homogenisation of a soil sample diluted in water for an appropriate length of time The homogenate is further diluted, suspended in molten agar and sub-samples pipetted on to a haemocytometer slide. The samples are usually stained, e.g. with phenol aniline blue, and finally mounted *e.g.* in Euparal, for microscopic examination. The length of hyphae is counted and converted to a fungal biomass estimate by taking into account various sources of error (for a conversion formula see Frankland, Lindley & Swift, 1978).

Frankland (1974) reported that the method of microscopic examination can have a significant effect on the number of hyphae counted. She found observation by phase contrast microscopy enabled 'ghost' hyphae to be seen and counted which were invisible when using bright-field as the method of observation: up to 42% of hyphae in leaf litter samples were

only visible using phase-contrast microscopy. Similarly, Bääth & Söderström (1980) found that the magnification factor could be an important source of error when estimating hyphal lengths in soil samples. They also found significantly higher values for hyphal length using the agar-film technique as compared with the membrane-filter technique.

1.5.2. Membrane-filter technique.

First described by Hansen, Thingstad & Goksøyr (1974) the membrane-filter technique is similar to the agar-film technique but instead of suspending the soil sample hyphae in agar they are trapped on a membrane filter. The filter is cleared in immersion oil and covered with a cover-slip. The method is much faster than the agar-film technique and for this reason has become used increasingly, although as already mentioned in Section 1.5.1. the hyphal length values measured have been found to be less than in the agar-film technique (Bääth & Söderström, 1980). West (1988), however, developed a membrane-filter technique using the fluorescent brightener, Calcofluor, which was superior to the agar-film technique in both speed and increased estimation of hyphal length.

1.5.3. Chitin assay.

With a few exceptions, notably the Oomycetes and Hemiascomycetidae, most fungi have chitin, in varying amounts, as a component of their cell walls and an assay for chitin might, therefore, bear relation to the amount of fungal biomass present. However, a chitin assay is only able to measure total fungal biomass, as it is not able to differentiate between different species. Therefore, a chitin assay can be useful if a measure of total fungal biomass is needed in a mixed population or in experiments where only the test fungus is present, but it cannot be used to measure the biomass of one species in a mixed population. For example, the assay has been used to estimate total fungal contamination in tomato products (Jarvis, 1977; Bishop, Duncan & Evancho, 1982) and also as an experimental estimate of the amount of infection present in tomato plants infected with *Fusarium oxysporum* f. sp. *lycopersici*

(Ride & Drysdale, 1972).

Frankland *et al.* (1978) compared the chitin assay with the agar-film technique as a means of estimating fungal biomass in leaf litter and found a correlation between the two. Similarly, Bethlenfalvay & Ames (1987) compared the assay with the membrane technique in order to quantify the extra-radical mycelium of vesicular-arbuscular mycorrhizae. They found a significant correlation ($P<0.01$) between the fungal biomass estimated by the two methods.

Frankland *et al.*(1978) concluded that although they obtained satisfactory results with both methods the sources of variation in the chitin assay were more likely to be significant in mixed cultures and therefore chitin assays are most useful in monocultures. Bethlenfalvay & Ames (1987) also referred to the assumptions needed to be made about chitin content, *e.g.* constant chitin content in all stages of the life cycle. The chitin assay can therefore be useful in giving an estimate af total fungal biomass, but sources of error need to be considered especially in mixed cultures.

1.5.4. Ergosterol assay.

Ergosterol is a fungal specific sterol and extraction and estimation of ergosterol has been used to estimate fungal biomass; although, as for the chitin assay, it measures total fungal biomass and does not discriminate between species. It has been used to estimate the fungal invasion in grains (Seitz *et al.*, 1977; Sietz *et al.*, 1979); to measure the fungal infection of spruce needles to indicate the contribution of fungi to a decrease in spruce growth (Osswald, Holl & Elstner, 1986); and to estimate the ectomycorrhizal infection of Scots pine (Salmanowicz & Nylund, 1988). Zill, Engelhardt & Wallnofer (1988) used ergosterol determination as the measure of fungal growth when correlating formation of the mycotoxin, zearalenone, with growth of *Fusarium graminearum* strains. Good correlation between chitin, laccase and ergosterol assays has been shown for *Agaricus bisporus*

(Matcham, Jordan & Wood, 1985) and they suggested that ergosterol content gives a more sensitive index for fungal growth. However, Nout *et al.* (1987) showed that ergosterol content was influenced by substrate composition, as well as aeration and growth phase. This again emphasises the problems of estimating fungal biomass by indicator fungal components as the levels of the indicators may not always be proportional to biomass in every phase or stage of the life cycle. West, Grant & Sparling (1987), however, showed that ergosterol could be linearly correlated with fungal surface area in a common function for two types of soil tested. They found there was 0.16g ergosterol cm^{-2} fungal surface area.

1.5.5. Iodonitrotetrazolium (INT) assay.

Sylvia (1988) used the tetrazolium salt INT to measure the activity of external hyphae of vesicular-arbuscular mycorrhizal fungi. Hyphae attached to roots or extracted from soil were collected by the membrane filter technique and then incubated on the filter with INT and NADH in 0.2M Tris buffer for 8h. Fungal dehydrogenases reduce the violet INT to its red reduced salt, and so the proportion of red stained hyphae was taken to be "active metabolising" hyphae by Sylvia (1988). He found most hyphae attached to roots were "active" while the proportion of "active" hyphae extracted from soil ranged from 0 to 32%. But, as with the chitin assay, the INT assay estimates fungal biomass for all fungi and to estimate fungal biomass for a particular fungus, either it needs to be the only fungus present or background hyphae must be eliminated by some means. Abbott, Robson & De Boer (1984) and Abbott & Robson (1985) subtracted the length of hyphae in control treatments from the lengths in experimental treatments, where vesicular-arbuscular mycorrhizal fungi had been used as the inoculants.

When alternative methods for fungal biomass have been compared the majority of workers have found correlations between the estimates. Sylvia (1988), however found differing results from Bethlenfalvay & Ames (1987) in the estimate of fungal colonisation of

the soil around a root. Sylvia (1988) found the production of active external hyphae lagged behind root colonisation by several weeks, whereas Bethlenfalvay & Ames (1987) found that the total quantity of external hyphae peaked after 10wk, while internal fungal biomass increased up to 19wk. The differences between these two findings emphasise the need to consider both total biomass present and the amount of that biomass which is metabolically active.

1.5.6. Fluorescein diacetate estimation.

Söderström (1977) used fluorescein diacetate to label fungi specifically in soil samples (see Section 1.3.1). As fluorescein diacetate is only converted to its fluorescent derivative by actively metabolising hyphae, Söderström (1978) used it to estimate active fungal biomass by using fluorescein diacetate in the agar-film technique rather than aniline blue. He found good correlation between relative staining efficiency, growth rate and respiration. It should be noted, however, that actively metabolising fungal biomass estimates do not necessarily correlate with viable fungal biomass. Söderström (1977) reported fully developed conidia of *Penicillium chrysogenum* did not fluoresce and so would not be included in active fungal biomass estimates, even though many of them would be viable.

All of these quantification methods can be used to measure fungal biomass, but none offers the potential for specific recognition of one particular fungus. MAbs offer this potential.

1.6. Antibody uses: taxonomic classification.

Data from the study of fungi using immunological probes can be used to aid taxonomical classification of fungi. The taxonomy of bacteria and viruses makes large use of immunological data. Some immunological studies of fungi have concentrated primarily on these taxonomic relationships and some of these are considered below, illustrating another

use for antibodies in mycology.

1.6.1. Using polyclonal antisera.

Serological identification has been used also as an aid to taxonomy; the antigenic relationships being compared with conventional taxonomic designations. For example, Aldwell *et al.* (1985) investigated, using antisera in ELISAs, the immunological relationships of 14 members of the Endogonaceae. They found that the immunological groups backed conventional taxonomic classification. Similarly, Ianelli *et al.* (1982) concurred with the proposed differential classification of *Fusarium moniliforme* and *Fusarium oxysporum* f. sp. *dianthi* and *F. o.* f. sp. *lycopersici*. In their study Ianelli *et al.* (1982) were able to remove cross-reactions by absorption to make the antisera specific to the *formae speciales* against which they were raised. Using antisera raised against three *Sclerotinia* spp., Scott (1981) found that *Sclerotinia sclerotiorum* and *Sclerotinia minor* share more common antigens with each other than either of them do with *Sclerotinia trifoliorum*.

Other examples of serology as an aid to taxonomy include a number of studies of Oomycete species. Morton & Dukes (1967) showed *Pythium aphanidermatum* was serologically different from *Phytophthora parasitica* var. *nicotianae* and *Phytophthora parasitica*. Merz *et al.* (1969) found that *Phytophthora cinnamomi* was serologically distinct from five other species of *Phytophthora*, although two isolates of *Phytophthora palmivora* belonged to different antigenic groups. Similarly, Halsall (1976) was able to discriminate between *P. cinnamomi* and *Phytophthora cambivora*, and between those and *Phytophthora crytogea* and *Phytophthora dreschleri*. There was no serological differentiation between *P. cambivora* and *P. dreschleri*, confirming their close taxonomic relationship.

Antigenic relationships between fungi have also been studied using antisera raised to extra-cellular antigens. Notermans & Soentoro (1986) studied the immunological

relationship of extra-cellular polysaccharide antigens produced by different fungal genera. Using these extra-cellular polysaccharides as immunogens they found the antisera raised were predominately genus specific, except for those raised against *Penicillium* spp. which reacted with *Aspergillus spp.*; and those raised against *Mucor racemosus* which reacted with *Rhizopus* spp.. Their results reflected accepted taxonomic relationships, *e.g.* the close relationship of the genera, *Penicillium* and *Aspergillus*. Similar work by Polonelli *et al.* (1988) showed that three species of *Rhizopus* are closely related. In this case, however they were able to obtain a species-specific reference antiserum for *Rhizopus chinensis* by cross absorption with heterologous antigens from other *Rhizopus* spp..

1.6.2. Using MAbs.

The specificity of MAbs for one epitope enables their use in taxonomic studies. Even when a MAb is not specific for one species quantitative differences may occur in the level of reaction between different genera, species, form species or isolates. These can be exploited taxonomically as in the following examples.

Ianelli *et al.* (1983) extended their study of the taxonomic classification of the genus *Fusarium* using MAbs instead of polyclonal antisera (Ianelli *et al.*, 1982). Using a panel of 11 MAbs raised against *Fusarium oxysporum* f. sp. *lycopersici* spores they were able to differentiate between different *Fusarium* species, *e.g. F. oxysporum* could be distinguished from *Fusarium moniliforme* and *Fusarium xylarioides* by differential binding to different MAbs. Four form species, *lycopersici, dianthi, pisi* and *melonis* could also be distinguished by their differential binding patterns to different MAbs in the panel.

Ferguson, Wycoff & Ayers (1988) assessed the use of a panel of MAbs for taxonomic differentiation of phytopathogenic fungi. They used 43 MAbs raised against *Phytophthora megasperma* f. sp. *glycinea* in ELISAs to test the level of reaction of 14 fungi with the MAbs. They then used cluster analysis to assess the data generated. They found clusters

which corresponded very closely to classical taxonomic designations. In agreement with the findings of Ianelli *et al.* (1983) for *Fusarium* species they also found that differences between *Phytophthora* species were quantitative rather than qualitative.

The MAbs raised by Hardham, Suzaki & Perkin (1985, 1986) to cysts and zoospores of *Phytophthora cinnamomi* showed different levels of taxonomic specificity from genus to isolate specific MAbs. Two MAbs were specific to zoospores of the *P. cinnamomi* isolate 6BR and four others showed species-specificity for both zoospores and cysts of *P. cinnamomi*. These MAbs were therefore specific taxonomic probes for *P. cinnamomi* zoospores and cysts and could be used on their own to identify *P. cinnamomi* 6BR and other *P. cinnamomi* isolates. Similarly, Callow, Estrada-Garcia & Green (1987) raised six MAbs which enable differentiation of the Oomycete, *Pythium aphanidermatum*, from other *Pythium* spp., as well as from other members of the Oomycetes.

Fox & Hahne (1988) have reported MAbs which enable the differentiation of different isolates of *Armillaria mellea* on the basis of quantitative differences found in ELISAs tests, in a similar manner to the taxonomic cluster analysis of *Phytophthora* spp. (Ferguson *et al.*, 1988).

Morace, Amalfitano & Polonelli (1986) used MAbs which recognise soluble antigens from *Microsporum canis* and *Trichophyton rubrum* to show for the first time that different serotypes exist within these species.

1.7 Antibody uses: Investigation.

Antibodies, both polyclonal and monoclonal, show different levels of taxonomic recognition. Similarly, they can be used to investigate an organism at different levels of recognition. For example, they can be used to differentiate one organism from another; one morphological form of an organism from another; and single components of the organism

(enzyme, carbohydrate *etc.*).

Antibodies can be used to investigate a fungus at the organism level. Particular use has been made of antibodies to follow the infection process of plants during infection by pathogenic fungi. Using immunofluorescent, immunoperoxidase and immunogold methods the infection process can be followed more easily by the differentiation of fungal tissue from plant. Some examples of this have already been discussed in 1.2.2.1 (Schimdt *et al.*, 1974; Fitzell *et al.*, 1980a); and in 1.2.2.2 (Wright & Morton, 1989). Other examples of the use of antibodies to enable the investigation of infection of plants by fungi include Cuypers & Hahlbrook (1988), who used a polyclonal antibody raised against surface material of *Phytophthora megasperma* f. sp. *glycinea* to follow the compatible and non-compatible interaction of potato leaves with *Phytophthora infestans* and *P. m.* f. sp. *glycinea*, respectively; and Mueller, Tessier & Englander (1986) who used polyclonal antisera to show that *Rhododendron* roots contain several different endophytes.

Many fungi show a wide range of morphological forms during their life-cycles and antibodies can be used to investigate whether different molecules are present in these different morphological forms. This is particularly interesting with regard to cell walls. For example, Hunsley & Kay (1976) raised antisera against three *N. crassa* wall fractions, as well as whole cell walls. Using an immunofluorescence technique, they found that the antiserum raised against Fraction I, the glucan-peptide-galactosamine complex, showed fluorescence on hyphae in the apical and subapical region, but the fluorescence decreased markedly behind the subapical region. The antiserum raised against Fraction III, the glucan fraction, showed fluorescence along the length of the hyphae but only faint fluorescence at the apex. The antiserum raised against Fraction IV only exhibited bright fluorescence at hyphal fractures. Hunsley & Kay (1976) also showed that wall antigens other than those of the four major wall fractions defined by Mahadevan & Tatum (1965) existed. Fraction II

largely consists of monosaccharide residues and is not antigenic.

Similarly, Jones & Gooday (1978) demonstrated that antisera raised against zygophore surface antigens of *Mucor mucedo*, (+) and (-) mating types, were specific for the zygophore surface; whereas antisera raised against vegetative surface antigens reacted with all cell surfaces of both mating types. The zygophore specific antisera reacted with both mating types regardless of the type used for immunisation. These antisera also reacted with the zygophore surface of *Mucor hiemalis*, (+) and (-), but not with the surface of *Phycomyces blakesleeanus* zygophores. The cross-reactivity of the antisera with the heterologous mating strain could, however, be removed by absorption of the antisera with heterologous zygophore material.

Several workers have used antisera to investigate the antigenic composition of medically important fungi. For example, Cole *et al.* (1983) have studied the important antigens of *Coccidioides immitis* and showed that the different conidial wall fractions, *i.e* outer conidial wall fractions I and II, and the inner conidial wall fractions, contain different components and show differing degrees of immunogenicity when used to immunise burros. Similarly, Hearn, Proctor & MacKenzie (1980) compared the wall antigens of various *Aspergillus* spp.. They found many of the antigens were common to all of the species tested. Many aspects of medical mycology, especially the antigenic nature of the fungi, have been the topic of a book recently produced by Reiss (1986).

Following the production of MAbs which recognise fungi, some workers have now used these in investigative studies. The reaction between a MAb and its epitope is very specific and should enable collection of data concerning that one epitope only, rather than several as is the case with a polyclonal antiserum.

The MAbs raised by Hardham *et al.* (1985, 1986) exhibited several patterns of fluorescence with the surfaces of *Phytophthora* spp. and *Pythium* spp. zoospores and cysts.

The patterns can be summarised as follows:

Fluorescence pattern	Fungus	Specificity
Whole surface of zoospores, including flagella, whole surface of cysts.	*Phytophthora cinnamomi* 6BR	Isolate
Groove region of zoospore	*Phytophthora cinnamomi*	Species
Whole surface of cysts, but not zoospores	*Phytophthora cinnamomi*	Species
Whole surface of zoospores, but not to cysts	*Phytophthora* spp.	Genus
Whole surface of zoospores, whole surface of cysts	*Phytophthora* spp. *Pythium* spp.	Family

Hardham & Suzaki (1986) used one of the MAbs, Zf-1, which bound to the surface of both flagella, to study the encystment of zoospores. They found that binding of the MAb to the surface of the flagella induced encystment of the zoospores. Encystment was also induced by the lectin, ConA, which binds to the entire surface of the zoospores including the flagella. Hardham & Suzaki (1986) postulated that the surface components that bind ConA and MAb Zf-1 are involved in the critical step of triggering encystment at the surface of a potential host during infection.

Gubler & Hardham (1988) used another two of the MAbs, Cpa-2, which binds to *P. cinnamomi* cysts only, and Lpv-1, which binds to large peripheral vesicles, to follow the secretion of adhesive material during encystment of *P. cinnamomi* zoospores. Immunogold electron micrographs with these two MAbs showed that two types of peripheral vesicle occur. Gubler & Hardham (1986) concluded that the adhesion of *P. cinnamomi* cysts to plant

roots involves the secretion of two types of adhesives from two types of cyst vesicle. They also showed that these adhesives were high molecular weight glycoproteins which bound the two MAbs on Western blots.

The MAbs raised against *P. aphanidermatum* by Callow *et al.* (1987) also show differential patterns of fluorescence on zoospores and cysts. They found that MAbs which recognise the cyst wall as well as the entire zoospore surface reacted with all Oomycete fungi tested; whereas MAbs which only recognised the zoospore surface were species-specific (White, pers. comm., 1989). The patterns shown on the zoospore surface were similar to those observed by Hardham *et al.* (1986). One of the MAbs which reacts with the flagella was found to react specifically with the mastigonemes when immunogold electron microscopy was undertaken.

The variation of fungal surface antigens revealed by immunofluorescence using MAbs is also seen in medically important fungi. For example, Kraeger *et al.* (1986) produced a panel of seven IgM MAbs which recognise different surface antigens in the different forms of *Coccidioides immitis*. The patterns seen fell into the following categories:

Fluorescence pattern	Morphological form
Small, random patches	Spherule
Whole surface	Spherule
Septum	Hypha
Tip only	Hypha

The MAb which recognises a *C. albicans* glycoprotein (Hopwood *et al.*, 1986) shows a differential staining pattern on *C. albicans* yeast and mycelial phase cells. The epitope is present on the yeast phase cells, and on germ tubes at the start of their development; however, as the germ tube develops so the fluorescence decreases along the germ tube

length. This may be due to either loss of the antigen or, the development of a further wall layer over the antigen which prevents antigen-antibody binding.

Specific individual fungal components have also been followed using antibodies. For example, Daniel *et al.* (1989) used a polyclonal antiserum to investigate the localisation of lignin peroxidase during the degradation of wood by the white rot fungus, *Phanerochaete chrysosporium*, using immunogold labelling. They showed that the lignin peroxidase was located around the cell wall of the fungus, and was associated with membraneous vesicular structures in the cytoplasm. The enzyme was also located in the degrading wood fibre elements.

Dickerson & Pollard (1982) followed the localisation of β-glucanase during infection of rye plants by *Claviceps purpurea* using a polyclonal antiserum. The fluorescence labelling pattern showed that the enzyme was not present until 10 days after the onset of infection, and was present predominantly at hyphal tips and the sites of sclerotial development.

Benhamou *et al.* (1985a) raised two MAbs which recognised a phytotoxic glycopeptide produced by *Ophiostoma ulmi*. These two MAbs showed no cross-reactivity with closely related compounds. The toxin was localised in experimentally infected young elm seedlings (Benhamou *et al.*, 1985b) and was present in pit membranes, the innermost wall layer of paratracheal parenchyma cells, intercellular spaces and middle lamella. The immunogold labelling increased in intensity as the disease progressed. Ouellette & Benhamou (1986) discussed the potential for MAbs in the study of fungal components in a review paper based on their own findings during their study of the *O. ulmi* toxin.

MAbs can be used to further investigate a fungal component in the same way they have been used to study many biological molecules. For example, Mischak *et al.* (1989) have raised MAbs which recognise two functionally different domains in cellobiohydrolases found in *Trichoderma reesei*. Similarly, Lemmon, Lemmon & Jones (1989) have raised

eight MAbs to clathrins prepared from *S. cerevisiae* cells which will enable further characterisation of yeast clathrins.

Other workers have studied fungal tubulins using both MAbs raised against fungal tubulins and MAbs raised against other tubulins which react with fungal tubulins. Barton & Gull (1988) used anti-tubulin MAbs raised against both *S. cerevisiae* and *Trypanosoma brucei* tubulins to follow the microtubule cytoskeleton of *C. albicans* during the life-cycle. Similarly, Runeberg, Raudaskowski & Virtanen (1988) followed the cytoskeletal elements of *Schizophyllum commune* hyphae using two MAbs raised against yeast tubulin and a polyclonal antiserum raised against the actin filaments of sperm cells.

1.8. The Fungal Cell Wall.

Bartnicki-Garcia (1968) in his classic paper on fungal cell walls stated: "..., with few exceptions, the wall, more than any other cellular part, defines a fungus and distinguishes it from other living creatures". The aim of this project was to make immunological probes which would specifically recognise the wall of one fungal species, and which could be used to follow the fungus in the environment using immunofluorescence. It is, therefore, useful to consider the components that have been found in fungal cell walls, which may be recognised by immunological probes.

1.8.1. Cell wall composition.

Many papers discuss the chemical composition of a wide range of fungal cell walls, and all agree with the close correlation between the major chemical wall components and taxonomic grouping put forward by Bartnicki-Garcia (1968), see Table 1.2.

From 80 to 90% of the fungal wall is made of the major polysaccharide components. These include cellulose, glucan, mannan and chitin. Walls also contain lipids and proteins; as well as other minor polysaccharides. It is not necessary to consider all of the data

Chemical category	Taxonomic group	Distinctive features
Cellulose-glycogen	Acrasiales	pseudoplasmodia
Cellulose-glucan	Oomycetes	biflagellate zoospores
Cellulose-chitin	Hyphochytridiomycetes	zoospores with anterior flagella
Chitosan-chitin	Zygomycotina	zygospores
Chitin-glucan	Chytridiomycetes	zoospores with posterior flagella
	Ascomycotina	septate hyphae, ascospores
	Basidiomycotina	septate hyphae, basidiospores
	Deuteromycotina	septate hyphae
Mannan-glucan	Saccharomycetaceae	yeast cells, ascospores
	Cryptococcaceae	yeast cells
Mannan-chitin	Sporobolomycetaceae	yeasts (caroteniod pigment) ballistospores
	Rhodotorulaceae	yeasts (carotenoid pigment)
Polygalactosamine-galactan	Trichomycetes	arthropod parasites

Table 1.2. Cell wall taxonomy of fungi. (After Bartnicki-Garcia, 1968 and Webster, 1980)

published on fungal cell wall composition in this introduction and it would to a large extent only repeat the review of cell wall composition made by Wessels & Sietsma (1981). The occurrence of cell wall polysaccharides in different fungal species has been listed in detail by Barreto-Berger & Gorin (1983) and these two papers, along with that of Bartnicki-Garcia (1968) give an excellent introduction to fungal cell wall composition.

N. crassa was used in this project because of the relatively large amount of data available on its cell wall composition. It was thought easier to study wall antigens of a fungus, the wall of which has been partially characterised. It is, therefore, useful to consider some of this work, briefly, in this introduction.

Glucose and glucosamine are the main basic components of the *N. crassa* cell wall and account for approximately 60% of the wall. Mahadevan & Tatum (1965) found the hyphal wall contained, at least, four major complexes: galactosamine-peptide-glucan; two glucose polymers, one of which was a β-1,3 glucan; and chitin. Cardimil & Pincheira (1979) showed the *N. crassa* wall contains a glucan with 1,4 and 1,3 linkages with, perhaps, an α-D configuration. It may well be that this 1,4, 1,3 linked glucan was the second glucose polymer reported by Mahadevan & Tatum (1965). Wall peptides have proved difficult to extract, however, a high molecular weight glycopeptide was released from hyphal walls by treatment with ammonium hydroxide (Wrathall & Tatum, 1973). From varying analyses they proposed that the glycopeptide is a highly ordered structure of peptides linked together by a branched carbohydrate.

The conidial walls also contain glucan, chitin, protein and lipid. β-1,3 glucan forms a major part of the glucan (Mahadevan & Mahadkar, 1970). The conidia also possess a surface rodlet layer which is composed mostly (91%) of protein (Beever, Redgewell & Dempsey, 1979). Other spore rodlet layers have also been shown to consist mainly of protein *e.g.* in *Syncephalastrum racemosum* (Hobot & Gull, 1981a) and *Aspergillus niger* (Cole *et al.*,

1979).

The levels of the four major structural wall fractions in hyphal walls are different in colonial mutants compared with the wild type. For example, the colonial mutant B233 had altered levels of Fractions I, III and IV, whereas the colonial mutant B28 only showed an increased level of Fraction III (Mahadevan & Tatum, 1965). Chiba, Nakajima & Matsuda (1988) found an alteration in the β-glucan fraction (Fraction III) of a morphological mutant compared to its wild type. The mutant glucan had 2.5 times as many branches in its structure than the wild-type glucan and far fewer 1,3 linked glucose residues. The wall also had more carbohydrate and less proteins than the wild type walls and the mutant lacked the ability to form spores.

The percentages of the amino sugars, galactosamine and glucosamine, vary between conidia and hyphal wall fractions (Schmit, Edson & Brody, 1975). Up to 10% of the hyphal cell wall is galactosamine, whereas this sugar is only present in trace amounts in the conidial wall. Glucosamine forms a major part of both types of wall, due to the presence of its n-acetylated polymer chitin. The glucosamine level in fully developed hyphae and conidia is approximately the same, but the level of glucosamine increases markedly (2.5 times) in young germlings.

These examples illustrate the fundamental relationship between cell wall composition and morphological form which exists in fungi.

Paxillus involutus was the other fungus used for immunisations of mice during this project. No detailed analysis of the *P. involutus* hyphal cell wall has been published. *P. involutus* is a member of the family Agaricales, an order of the Basidiomycotina. *Schizophyllum commune* is the representative of this order about which most is known of the nature of the cell wall. The cell wall of this fungus has been the subject of a detailed study over several years by Wessels and his co-workers at the University of Groningen (Wessels,

1965, 1988; Wessels & Sietsma, 1981; Wessels *et al.*, 1972; Sietsma & Wessels, 1981, 1988; Siestma, Sonnenberg & Wessels, 1985; Sonnenberg, Sietsma & Wessels, 1985). Much of their earlier work was reviewed by Wessels & Sietsma (1981). The main components of the *S. commune* wall are glucans with only a relatively small amount of chitin being present. The water-insoluble glucans can be separated on the basis of their solubility in alkali. The alkali-insoluble glucan (termed R-glucan) contains both β-1,3 and β-1,6 linkages; whereas the alkali-soluble glucan (S-glucan) contains α-1,3 linkages (Wessels *et al.*, 1972). *S. commune* produces a mucilage at its surface which is also a glucan. This mucilage glucan has a β-1,3 linkages with single glucose residues attached to the main chain by β-1,6 linkages.

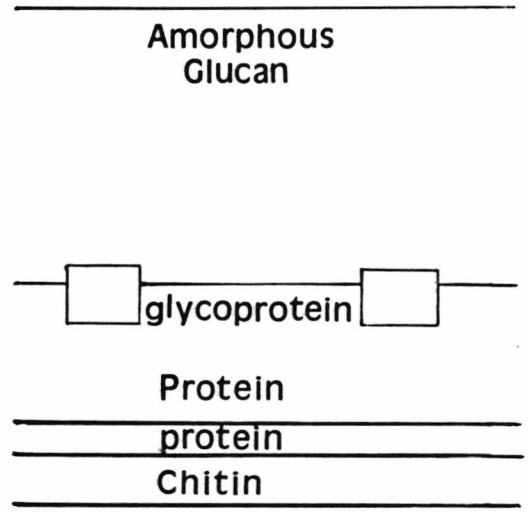
1.8.2. Cell wall ultrastructure.

The chemical composition shows which "building blocks" are present in a fungal cell wall, but it does not give any information regarding the arrangement of these "building blocks" in the wall. Various techniques have been used to discover the structural arrangement of cell walls, including the use of antibodies (Hunsley & Kay, 1976; Jones & Gooday, 1978) as discussed in 1.7.

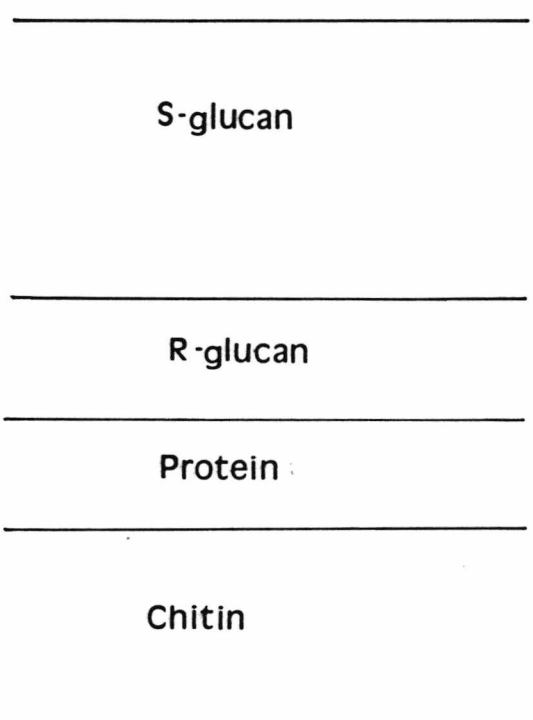
One of the classical papers on fungal cell wall ultrastructure is that of Hunsley & Burnett (1970) who postulated wall structures for the hyphae of *N. crassa* and *S. commune*. The structures proposed for these cell walls following electron microscopy of enzyme dissected walls are illustrated in Fig. 1.1.

The layered pattern proposed for *N. crassa* cell walls by Hunsley & Burnett (1970) was substantiated by the findings of Hunsley & Kay (1976) in their study of wall structure using polyclonal antisera (see section 1.7). The outermost layer of *N. crassa* hyphae and conidia was shown to be different ultrastructurally by Dempsey & Beever (1979). They showed conclusively that the outermost layer of the conidia consists of a regularly arranged rodlet

Fig. 1.1. Fungal cell walls



Neurospora crassa hyphal wall



Schizophyllum commune
hyphal wall

(after Hunsley & Burnett, 1970)

layer, composed mainly of protein, whereas no rodlet layer is seen on the hyphal surface. This differential presence of rodlets on spores but not hyphae was also shown in *Syncephalastrum racemosum* by Hobot & Gull (1981b).

The layered wall model proposed by Hunsley & Burnett (1970) was challenged by Wessels (1988), although he and fellow workers (Wessels *et al.*, 1972) had previously agreed with the layer model proposed for *S. commune* by Hunsley & Burnett (1970). This is discussed further in Chapter 9.

Some workers have used autoradiographic techniques to investigate where wall precursors are deposited. For example, Sonnenberg *et al.* (1985) showed that the R-glucan layer of *S. commune* contains more β1,6 links in older hyphae and Hunsley & Gooday (1974) showed that chitin is a major component of the *N. crassa* septum.

Schmit & Brody (1976) made a major review of the life-cycle of *N. crassa* and further fundamental information can be accessed through that paper. *P. involutus* has been the subject of considerable interest in the study of mycorrhizae, but fundamental knowledge about the fungus itself has not been published.

1.9.Concluding remarks.

"Immunological recognition of fungi" is one of those all embracing labels which can be interpreted differently by different people. Many take this statement to include only the specific recognition of one species of fungus from another, but this is only one aspect of the information immunological probes can reveal about a fungus. The initial aim of this project was, indeed, to distinguish one species of fungus from another; in this case the aim was the specific recognition of the ectomycorrhizal fungus *P. involutus*, but the development of such a probe revealed information in other areas. These have been introduced in the previous pages and, throughout this thesis, the use of immunological probes in all areas of "immunological recognition of fungi" will be described and discussed.

Chapter Two

Materials and Methods

2.1 Fungal Cultures.

2.1.1. Standard cultural procedures.

The fungi cultured in this project are listed in Table 2.1. The fungi were maintained on the media listed in Table 2.1 at 25°C in the dark. The exceptions to this were the *Neurospora* spp. which were grown at 30°C under a fluorescent light to induce the maximum production of conidia. The constituents of the culture media used are described in detail in Appendix I.

The fungi were maintained on media in Petri dishes, with stock cultures stored at 4°C on media slopes in Universal bottles. Large quantities of conidia from *Neurospora* spp. were obtained from cultures grown on NCMM slopes in 250 and 500ml medical flat bottles.

2.1.2. Germination of *N. crassa* conidia in batch culture.

Germinating conidia were grown in the following way and used in immunofluorescence studies of MAb epitope distribution along *N. crassa* germ tubes. *N. crassa* (CMI 142819) conidia were harvested from a 20-30 day old slope culture by washing the surface of the culture with sterile 0.1% 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. 250ml Erlenmeyer flasks containing 25ml of *Neurospora* germination medium or NCB were inoculated with sufficient of the conidial suspension to give a concentration of 1 x 10^6conidia ml^{-1}. Duplicate flasks were prepared for each time point (up to 12h). The

Species	Source	Number	Culture medium*	Temp. °C
Aspergillus nidulans	CMI	16040	MEA	25
Colletotrichum gloeosporioides	IK/UKC	24	MEA	25
Geotrichum candidum	UKC		MEA	25
Mucor hiemalis (+)	CMI	21216	MEA	25
Neurospora crassa	CMI	142819	NCMM/NCA	30
Neurospora crassa	CMI	53240	NCMM/NCA	30
Neurospora sitophila	CMI	63920	NCMM/NCA	30
Neurospora tetrasperma	CMI	34454	NCMM/NCA	30
Paxillus involutus	ITE	16	MMN	25
Paxillus involutus	ITE	32	MMN	25
Paxillus involutus	ITE	45	MMN	25
Penicillium chrysogenum	CMI	37767	MEA	25
Pisolithus tinctorius	ITE	FCOO6	MNN	25
Podospora paucieta (+)	CBS	29356	MEA	25
Saccharomyces cerevisiae	UKC		YEPD	30
Schizophyllum commune	UKC	17	MEA	25
Sordaria fimicola	CMI	105391	MEA	25
Thelephora terrestris	ITE	C	MMN	25

Table 2.1. Details of fungal cultures.

Cultures obtained from collections at the following institutions:

CBS	Centraalbureau voor Schimmelcultures
CMI	Commonwealth Mycological Institute
ITE	Institute of Terrestrial Ecology
UKC	University of Kent at Canterbury
IK/UKC	Irina Koomen at UKC.

* Culture media: these are listed in Appendix I

cultures were incubated at 30°C and agitated at 200 rev min^{-1}. At hourly time points duplicate flasks were removed from the shaker and 25ml of freshly prepared formaldehyde solution (7.4%w/v) was added, giving a final formaldehyde concentration of 3.7% (w/v).

2.1.3. Mini cultures of *N. crassa*.

In order to prepare samples of intact *N. crassa* conidiophores for immunofluorescence studies of epitope distribution using MAbs, mini cultures were grown. 100µl of NCMM was aliquoted into the wells of a 96 well microculture plate. Each well was seeded with approximately 100 *N. crassa* conidia. The plate was then incubated at 30°C, in the light, for three days. The fungus grew to form a thin mycelial mat on the surface of the medium. After three days the medium was removed by aspiration and the culture fixed by addition of 3.7 (w/v) freshly prepared formaldehyde.

2.1.4. Large scale culture of *N. crassa*.

To prepare sufficient mycelium of *N. crassa* for isolation of cell walls, large scale cultures were made. A 10l carboy containing 9l of NCMM mineral salts was autoclaved for 40min at 121°C, 15p.s.i.. 200g of sucrose in 750ml of dH_2O in a one litre side arm flask was sterilised for 20min at 121°C, 15p.s.i.. An empty one litre side arm flask was also sterilised for 20min at 121°C, 15p.s.i., and was used to transfer inoculum into the carboy. Both side arm flasks were fitted with appropriate steri-connectors to enable addition to the 10l carboy via steri-connectors.

Following sterilisation the sucrose was added to the 10l carboy and the final medium was allowed to equilibrate to 30°C. The inoculum of *N. crassa* conidia was obtained by preparing a conidial suspension of *N. crassa* as described in Section 2.1.2. The number of conidia per ml in the suspension was counted and the volume of the suspension to be added to the 10l carboy adjusted so that the final concentration of conidia in the carboy would be 1 x 10^5 conidia per ml. The correct volume of inoculum suspension was then placed in the

empty side-arm flask and added to the carboy via steri-connectors.

The carboy was incubated at 30°C on a magnetic stirrer and was connected to an air supply via an air inlet pipe. The air passed through a filter before being dispersed throughout the culture by a glass sparger. An air outlet pipe with filter allowed release of gas from the culture. The medium was agitated by means of a large stirrer bar. The culture was grown for 3 days under these conditions.

2.1.5. Culture of *Paxillus involutus, Thelephora terrestris* and *Pisolithus tinctorius* for cell wall preparation.

Two litre Erlenmeyer flasks containing 200ml of MMN were inoculated with one of the following species, *P. involutus, T. terrestris* or *P. tinctorius* using a plug from a Petri-dish culture. The flasks were then incubated at 25°C, in the dark, statically. The mycelium was collected when the mycelial mat covered the surface of the medium, usually after 1-2 months.

2.2. Basidiomycete fruiting body collection.

Fruiting bodies of 22 different members of the Basidiomycotina were collected from woodland sites on the UKC campus, in September and October 1988. The identifications given to these species is listed in Table 2.2. *Agaricus bisporus* was obtained from a local mushroom farm. In all, 14 genera were represented in this collection. All belonged to the order Agaricales.

Species	Family
Agaricus bisporus	Agaricaceae
Amanita citrina	Amantitaceae
Amanita muscaria	Amanitaceae
Boletus chrysenteron	Boletaceae
Boletus piperatus	Boletaceae
Clitopilus prunulus	Entolomataceae
Collybia fusipes	Tricholomataceae
Coprinus atramentarius	Coprinaceae
Gymnopilus junonius	Cortinariaceae
Gymnopilus penetrans	Cortinariaceae
Hebeloma sp.	Cortinariaceae
Laccaria amethystina	Tricholomataceae
Laccaria laccata	Tricholomataceae
Lacrymaria velutina	Coprinaceae
Lactarius sp. (1)	Russulaceae
Lactarius sp. (2)	Russulaceae
Lactarius sp. (3)	Russulaceae
Mycena galopus	Tricholomataceae
Mycena sp.	Tricholomataceae
Paxillus involutus	Paxillaceae
Pluteus cervinus	Pluteaceae
Russula atropurpurea	Russulaceae
Russula ochroleuca	Russulaceae

Table 2.2. Details of identification of fungal fruit bodies collected on the UKC campus.

All of the species collected were members of the Agaricales, an order of the Basidiomycotina.

2.3. Fungal cell wall preparations and fractionations.

2.3.1. Preparation of isolated cell walls.

Cell walls of *N. crassa, P. involutus, T. terrestris* and *P. tinctorius* were isolated for use in immunisation, ELISA and immunofluorescence protocols.

The mycelium obtained was removed from its culture medium by filtering onto four layers of muslin. It was then split into aliquots which were approximately 25g (wet weight) in mass. The aliquots not used immediately were stored at -80°C. Aliquots for immediate use were subjected to the following breakage and cleaning procedure:

Step 1 French pressure cell, 3x, 14,087 tonnes m^{-2}

Step 2 Spin, 4500r.p.m. in a MSE Centaur 1 bench top centrifuge (2250*g*), 5 min.

Step 3 Aspirate supernatant and resuspend pellet in sdH_2O. Sonicate at 16 amp microns amplitude peak to peak, 5 min in total, in 10s bursts with 10s intervals.

Step 4 Spin as in Step 2 and repeat Steps 2 and 3 until cytoplasmic contamination is removed.

The presence of cytoplasmic contamination was checked using phase contrast microscopy and Step 4 was repeated until no cytoplasmic contamination remained. The isolated cell walls were then lyophilised for storage.

2.3.2. Crude cell wall preparations from the fruiting bodies of members of the Basidiomycotina.

1mg (wet weight) of tissue was excised from a fruit body and was homogenised in an equal volume of sdH_2O, using a hand-held homogeniser in a test tube. The homogenised suspension was spun for 5 min at 4500r.p.m. (2250*g*) and the supernatant containing the

cytoplasm removed by aspiration. The cell walls and spores were resuspended in 1ml sdH_2O and sonicated for 1 min at 16 amp microns amplitude peak to peak. The suspension was then used in immunofluorescence studies.

2.3.3. *N. crassa* cell wall fractionation.

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 of galactosamine-peptide-glucan, monosaccharide sugars, glucan and chitin, respectively (see Section 1.8.1). The fractions obtained were lyophilised for storage.

2.4. Immunisations.

2.4.1. Immunisation of New Zealand White rabbits.

Two NZ White rabbits were injected with 30mg (freeze-dried weight) cell walls of *N. crassa* CMI 142819 and *P. involutus* 16, respectively. This was based on the 30mg injections of cell wall fractions Hunsley & Kay (1976) used to raise antisera in rabbits against cell wall fractions of *N. crassa*. The cell walls were suspended in a 1ml volume of sdH_2O and mixed with an equal volume of Freund's incomplete adjuvant. The rabbits were given subcutaneous injections at several sites on the back. Initially a course of six injections was given at 14 day intervals. The rabbits were bled 10 days after the sixth injection and the titre of the antisera established using ELISA. The rabbits were then given booster injections of 30mg of cell walls 10 days prior to any further bleed. Five booster injections were given over six months.

2.4.2. Immunisation of Balb/c mice.

Six week old Balb/c mice were injected intraperitoneally with cell wall fragments of either *N. crassa* or *P. involutus*. Cell walls was suspended in sdH_2O prior to injection. Development of an appropriate protocol is reported and discussed in Section 4.2.

2.5. Antiserum preparation.

2.5.1. Rabbit antiserum.

Blood was collected from the ear vein of the rabbits 10 days after an injection. The ear vein was constricted with finger and thumb above a point where a needle (25G) was inserted along the vein in the direction of the blood flow. The drops of blood from this wound were collected into a boiling tube. The blood was allowed to clot for 1h at room temperature. The clot was then detached from the sides of the tube using a wooden spatula and the tube was placed at 4°C overnight to allow the clot to retract. The serum was removed from the clot by Pasteur pipette and aliquoted into 1ml volumes which were stored in liquid nitrogen. A large quantity of serum was obtained from each rabbit by a final cardiac puncture.

2.5.2. Mouse plasma.

Mice were bled from the tail 10 days after an injection by cutting a small piece off the end of the tail and collecting the blood using a drawn Pasteur pipette. The blood was put into 0.5ml Eppendorf tubes which contained 100µl of saline (see Appendix III). The red cells were removed by centrifugation at 4500r.p.m. (2250*g*). The plasma was removed from the red cell pellet and either used immediately or stored at -20°C.

2.6. Production of hybridoma cell lines.

2.6.1. Culture medium and conditions.

Myeloma and hybridoma cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM); the components of which are listed in Appendix II. The medium was prepared from a 1x stock powder (Gibco, 074-2100) by dissolution in double distilled water. Sodium bicarbonate ($3.7g\ l^{-1}$) and sodium pyruvate ($0.11g\ l^{-1}$) were added to the DMEM and the pH adjusted to 7.3. This medium was filter sterilised through a 0.2µm bell filter (Acrocap) at a pressure of 10p.s.i.. The sterility of the medium was checked by incubation overnight of a

50ml test volume, at 37°C and 5.2% CO_2.

For growth of cells this medium was supplemented by sterile addition of the following:

Foetal calf serum	10%
Hypoxanthine	0.1M
Thymidine	0.016mM
Glutamine	2mM
Modified Eagle Medium Non-Essential Amino Acids (NEAA)	1x
2-mercaptoethanol	0.05mM

Hypoxanthine, thymidine and glutamine were stored as 100x stock solutions. 2-mercaptoethanol was stored as a 500x stock solution and the non-essential amino acids were obtained as a 100x stock solution from Gibco (Cat. no. 043-1140, see Appendix II). The supplemented DMEM is termed "SDMEM". For fusions this media (SDMEM) was supplemented with 0.4μM aminopterin. If necessary the media was supplemented with penicillin (50 units Penicillin) and streptomycin (50μg ml^{-1}). The supplemented medium was stored at 4°C and was warmed to 37°C prior to use.

The cell lines were incubated in a CO_2 incubator (Forma Scientific 3029) at 5.2% CO_2 and 37°C. All cell manipulations were carried out in a laminar flow clean air cabinet.

2.6.2. Cell lines.

Sp2.0 and X63/Ag-8.653 myeloma cell lines were grown in SDMEM in the conditions outlined in 2.6.1. The cells were maintained at concentrations between 5 x 10^5 and 5 x 10^6 cells per ml. The cells were subcultured when a concentration of 5 x 10^6 cells per ml was reached.

The myeloma cell lines were checked for reversion of the HGPRT mutation by addition of 8-azaguanine (6.7μM) to SDMEM. The cells were passaged through 8-azaguanine for one

subculture and then passaged three times in SDMEM without 8-azaguanine before use in fusions or before being frozen as stock cell lines.

Stocks of both the myeloma and hybridoma cell lines were stored in liquid nitrogen. The culture medium was removed from cells by centrifugation at 1500r.p.m. (250*g*) and subsequent aspiration of the culture medium. The cells were resuspended in 1ml of freezing medium (90% foetal calf serum and 10% DMSO) in 1.8ml cryotubes. The cells were placed at -80°C overnight before transfer to liquid nitrogen. The slower freezing rate in a freezer is thought to prevent excess damage caused by the formation of ice crystals.

Cell lines were revived from frozen stocks by removal from the liquid nitrogen and immediate placement in a 37°C water bath. On thawing the cells were added to 10ml of SDMEM and centrifuged at 1500r.p.m. (250*g*). The cells were aspirated, resuspended in 10ml of SDMEM and incubated at 37°C, 5.2% CO_2

2.6.3. Fusion of myeloma cells and spleen cells.

One week prior to fusion the myeloma cell lines were revived from frozen storage and grown for 7 days to obtain 1-5 x 10^7 cells in total. The cells were carefully monitored over this period to ensure that over 95% of the cells were viable at the time of fusion. The viability of the cells was observed by addition of 50µl of cell suspension to 50µl of trypan blue (0.5%w/v). Dead cells take up the stain, whereas live cells do not and so the total number of cells and the percentage of cells that are viable can be assessed.

On the day of fusion the myeloma cells were counted to check the viability was 95%, or above, and to ensure that at least 5 x 10^7 cells were available for fusion. A mouse with a tailbleed titre in excess of 1/200 (see Sections 4.2 and 4.5), which had been given a booster injection four days beforehand, was sacrificed by dislocation of the neck and dipped in 70% alcohol. The spleen was removed in a sterile cabinet using sterile forceps and scissors, and placed in 25ml Universal tube containing 10ml of DMEM. The sterile cabinet used for

isolation of the spleen was situated outside the tissue culture room. Following removal from the mouse the spleen was transferred into the main tissue culture suite and all procedures carried out in a laminar flow cabinet.

The spleen was washed three times in DMEM and then placed in a Nitex bag in a dish containing 10ml DMEM. The spleen was pierced with forceps and the cells expressed from the spleen with the plunger from a 1ml disposable syringe. The Nitex bag retains any large cell debris. The spleen cells were transferred to a 50ml centrifuge tube and the number of lymphocytes counted. Following this count the appropriate number of myeloma cells was removed from the cultures to give a ratio of 5 spleen cells to 1 myeloma cell. This was approximately 1×10^8 spleen cells and 2×10^7 myeloma cells.

Both sets of cells were spun at 1500r.p.m. (250*g*) for 6 min. The supernatants were aspirated and both sets of cells were resuspended in 10ml DMEM. Two 10µl samples of each suspension were placed separately into four wells of a 96 well microculture plate. The spleen cells were then added to the myeloma cells. The volume of this mixture was made up to 50ml by addition of DMEM and the mixture was spun at 1500r.p.m. (250*g*) for 8 min. As the cells were spun, 100ml of HAT medium was prepared. This was done by addition of aminopterin (0.4µM) to SDMEM. 200µl of the HAT medium was placed in each of the four wells seeded with the parental cells.

When the mixture of cells had been spun together to form one pellet, the DMEM was removed and the pellet incubated for 5 min in a water bath at 37°C. 1ml of PEG mixture was then added to the pellet, slowly, over a 2 min period. The pellet and PEG was stirred continuously during this time with the tip of the pipette used to add the PEG. The PEG mixture contained 75mM polyethylene glycol (M.W. 1500) and 5% DMSO. This was pre-heated to 37°C prior to use. 2ml of DMEM was then added slowly to the fusion mixture over a period of 3 min. The mixture was stirred continuously. Next, 7ml of SDMEM was added

slowly to the mixture over a 3 min period. The fusion mixture was then expanded to 100ml in volume by addition of 90ml of the HAT medium (*i.e.* SDMEM with aminopterin). The fusion mixture was then dispensed in 100µl aliquots into 476 wells of five microculture plates using an 8-channel Titertek pipette.

The fusion plates were incubated at 37°C and 5.2% CO_2. The plates were checked for contamination on all days following fusion, but were not fed until small clumps of hybridoma cells could be seen (after 5-9 days). The first feed was simply addition of 100µl of HAT medium. Subsequent feeds required aspiration of 100µl of culture supernatant before addition of 100µl of new HAT medium.

The hybridoma cell lines were screened for antibody production when the majority of hybridoma colonies could be seen by eye (12 to 20 days after fusion). 100µl of supernatant was removed from each well using an 8-channel Titertek pipette and placed in the well of an ELISA plate containing the appropriate fungal cell wall (*N. crassa* or *P. involutus*). The ELISA was carried out as described in Section 3.3.4. The cells in wells secreting desired antibody were removed from the microculture plate wells by gently scraping the bottom of the well with a Pasteur pipette. The cells and medium was transferred to the well of a 24 well microculture plate containing 1ml of HAT medium and 5 x 10^5 spleen cells from an unimmunised mouse. The cells were grown in this well for two days when a 10µl sample of supernatant was removed for screening by immunofluorescence (see Sections 2.8). If the cell line secreted a desired antibody, as judged by the immunofluorescence pattern (see Section 4.3), it was cloned when sufficient cells for cloning were available.

2.6.4. Limiting dilution cloning.

Cells to be cloned were resuspended using a Pasteur pipette and placed into a 25ml Universal. A 50µl sample of the cells was counted using a haemocytometer (Neubauer) and trypan blue as in Section 2.6.2. The cells were then diluted in 25ml SDMEM to

concentrations of 50, 25 and 5 cells per ml, respectively. Spleen feeder cells were added to give a concentration of 5 x 10^5 feeder cells per ml. 200µl aliquots of each cell suspension were then pipetted into all wells of three 96 well microculture plates using an 8-channel Titertek pipette. The cloning plates, therefore, contained 10, 5 and 1 cell per well, respectively.

The plates were incubated until small clones could be seen using an inverted microscope. At this time, before the cells were fed, the wells containing single clones were marked. The clones were fed when necessary by addition of SDMEM. When the clones were visible by eye, supernatants from the single clone wells were screened.

Cell lines from single clone wells which secreted a desired antibody were expanded in 24 well plates, as in Section 2.6.3, and re-cloned by the same limiting dilution technique. Cell lines were frozen after first, second, third and, if necessary, fourth clones.

2.7. ELISA.

The development of a successful ELISA for screening hybridoma supernatants for specific antibodies against fungal cell walls is described in detail in Chapter 3, Section 3.3. A detailed overview of the protocol is also given in Section 3.3.4.

2.8. Immunofluorescence.

Polyclonal antisera and hybridoma supernatants were screened using an indirect immunofluorescence technique. Slides with a single frost at one end were coated with poly-*l*-lysine (100µg 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-7. The slides were allowed to air dry. 10µl aliquots of an appropriate fungal suspension were placed onto the slides. For screening of hybridoma supernatants this comprised either of 10µl of *N. crassa* germlings, prepared as

described in Section 2.1.2, or 10µl of *P. involutus* cell walls. Three aliquots were placed on each slide to reduce the number of slides processed in hybridoma supernatant screens. In subsequent investigations of the MAbs three aliquots were placed on each slide to allow duplicate tests with a second antibody control on each slide.

The 10µl aliquots were allowed to air dry onto the slides for 30-60 min at 25°C. 10µl of the first antibody was then placed onto each sample and the slides incubated at 25°C for 1h in moist conditions. The moist conditions were obtained by placing the slides in 25cm^2 bioassay plates on damp kitchen towel.

Following first antibody incubation the slides were washed three times with PBS-7, 5 min each wash. Excess PBS-7 was drained from the slides and 10µl of either anti-mouse IgG or anti-rabbit IgG FITC conjugate diluted 1 in 10 in PBS-7 was placed on each sample. The slides were then incubated at 25°C for 1h. The washing step (3x PBS-7, 5 min) was repeated and the slides drained to remove excess PBS-7. 10µl of Mowiol containing 10 ml^{-1} *p*-phenylene-diamine was placed on each sample and the samples were covered with a 50mm coverslip for observation.

Samples were observed using a Zeiss Axioskop microscope with a mercury lamp and a dichroic filter set containing appropriate filters for detection of transmitted light from FITC. FITC is excited at 488nm and the transmitted light emitted at 530nm. The patterns of fluorescence seen were recording using an Olympus camera and Ilford XP1 400 film.

For observation of conidial chain fluorescence seen with the MAb S3B3 (see Section 6.3.) mini cultures of *N. crassa* were grown as described in Section 2.1.3. Prior to immunofluorescence staining formaldehyde was removed by aspiration and the colony washed in PBS-7. All of the immunofluorescence steps described above were then carried out, but instead of the procedure taking place on a slide it was performed in the wells of the microculture plate. The volumes used were scaled up to 200µl and all solutions were

removed by aspiration between steps. The colony was carefully placed onto a slide for observation. Mowiol was not used, but *p*-phenylene-diamine was added to PBS-7 to try to prevent excess fading of the FITC.

2.9 Antibody typing.

Antibody from the final MAb clones was tested to establish the class of immunoglobulin to which the antibodies belonged. The antibody from 1ml of hybridoma supernatant was precipitated from the supernatant by addition of 50% ammonium sulphate on ice. The supernatant was removed and the precipitate resuspended in 10µl of PBS-7. This solution was placed into the middle well of an immunodiffusion gel.

The immunodiffusion gel was 1% agarose with 0.1% sodium azide. The molten agarose was pipetted onto a acid-washed glass slide, so that an even, thin layer (no more than 2mm thick) was formed. 10µl wells were cut in the gel using a hole punch. This cuts six outer wells surrounding one inner well. The antibody sample was placed into the inner well and 10µl aliquots of sheep anti-mouse antisera (ICN) raised against the antibody heavy chain classes IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgA and IgM were placed in the six outer wells. The gel was placed at 25°C for 24h and then stained using Coomassie blue for observation of the precipitin lines of reaction between the test antibody and the control antibody of the same class. The gel was stained for 30 min and then de-stained in a solution of 5% methanol and 8% acetic acid in distilled water.

2.10. Immunogold electron microscopy.

Mini cultures of *N. crassa* were grown in the wells of 96 well microculture plates as described in Section 2.1.3; but were fixed with 3.7% (w/v) formaldehyde containing 0.6% glutaraldehyde in PEM buffer (see Appendix III). The dehydration and embedding steps are

summarised in Fig. 2.1. Following polymerisation of the Lowicryl blocks containing *N. crassa*, thin sections (150-90μm) were cut using a Reichert ultratome. The thin sections were placed on EM nickel grids. They were then ready for immunogold labelling.

All of the subsequent steps were carried out by placing one drop of the appropriate solution onto the grid using a Pasteur pipette. The grids were placed on parafilm, which was placed on moist kitchen towel. The grids were incubated in a bioassay plate at room temperature for each step.

Firstly, any free aldehyde groups were neutralised by three 5 min washes in 20mM glycine. The grid was then washed once with PBS-7, and the first antibody was placed on grid for 1h. The MAb hybridoma supernatants were not diluted. The grid was then washed five times with 1% BSA/PBS-7, 5 min each wash. Anti-mouse IgG whole molecule gold conjugate (Janssen) was diluted 1 in 10 in 1% BSA/PBS-7 and placed on the grid for 1h. The grid was then washed twice in 1%BSA/PBS-7 for 5 min, followed by three 5 min washes in 0.1%BSA/PBS-7 and two 5 min washes in PBS-7. The bound antibody was fixed to the sections by incubation in 2.5% glutaraldehyde for 30s. The sections were then negatively stained using 5% uranyl acetate in 1% acetic acid and lead citrate (Reynolds, 1963).

The grids were observed using a Philips EM410 transmission electron microscope and photographs taken using Ilford EM film.

2.11. 1-D Polyacrylamide gel electrophoresis.

1-D PAGE was carried out using the method and buffers of Laemmli (1970).

2.11.1. Sample preparation.

25 mg (freeze-dried weight) of cell walls of *N. crassa, P. involutus* and *Pisolithus tinctorius* were each suspended in 1ml of the appropriate extraction solution. The extraction solutions used were Laemmli sample buffer, LSB, (Laemmli, 1970) at three times normal

concentration; 10M urea sample buffer (USB); 1M NaOH and 0.5% SDS. The cell walls were boiled in the extraction solutions for 5 min and the cell wall debris then removed from the extraction solution by a spin at 13,000r.p.m. (11,650*g*). The extraction solutions containing 1M NaOH and 0.5% SDS, respectively, were further treated by precipitation on ice in TCA and resuspension of the pellet in phosphate sample buffer, PSB. Extraction solution components are given in Appendix III.

2.11.2. PAGE gels.

Two glass plates (14 x 8 or 14 x 16cm) were clipped together with 2mm spacers in between them and three edges sealed with 1% agarose. The main gel mix (see Appendix III) containing the appropriate percentage of acrylamide (10, 12.5 or 15) was poured between the plates and allowed to polymerise, under an overlay of iso-butanol. On polymerisation the butanol was removed and the top of the gel washed with water. The stacking gel was mixed and poured onto the top of the main gel and a lane spacer inserted. When the stacking gel was set, the lane spacer was carefully removed to leave a series of wells across the gel. The gel was placed in a gel tank and covered with running buffer. The samples were placed into the wells using a Hamilton syringe and the gels run at a current of 10 to 15mA until the dye front was 1.5 to 2cm above the bottom of the gel.

The gels were then either silver stained using the method of Wray *et al.* (1981) or the proteins transferred onto a nitrocellulose filter for immunoblotting (see Section 2.12).

2.12. Immunoblotting.

2.12.1. Western blotting.

This was carried out using the electroblot transfer technique of Towbin, Staehelin & Gordon (1979) to transfer proteins from a polyacrylamide gel to a nitrocellulose filter. The nitrocellulose filter was then immunoblotted in the following manner.

The nitrocellulose paper was removed from the electroblot transfer system and placed in ddH_2O. The nitrocellulose was then stained for 5 min with Ponceau red (0.2% w/v in 3% TCA) to check the proteins had been transferred and to enable the filter to be cut if necessary. The excess stain was removed by ddH_2O to enable visualisation of the bands and the stain was removed before blotting by a wash in TBS-Tween (see Appendix III). The first antibody was diluted in TBS-Tween and the filter incubated with the first antibody at room temperature for 1-2 h. After incubation, unbound first antibody was removed by three 5 min washes in TBS-Tween, one 5 min wash in HS-Tween (see Appendix III), and two 5 min washes in TBS-Tween. The nitrocellulose filter was then incubated for 1 -2h with peroxidase conjugated second antibody, diluted 1 in 500 in HS-Tween. The peroxidase conjugated second antibody was either anti-mouse or anti-rabbit IgG whole molecule antibody raised in rabbit or sheep, respectively. After incubation, the unbound conjugate antibody was removed by three, 5 min, washes in TBS-Tween, a 10 min wash in HS-Tween, three, 5 min, washes in TBS-Tween and a final 5 min wash in TBS only. 4-chloro-1-naphthol was added as substrate and was converted from colourless to purple in the presence of peroxidase and H_2O_2. When the colour reaction had developed the nitrocellulose paper was washed free of substrate using ddH_2O and the result recorded by photography using Ilford FP4 film.

2.12.2. Slot blotting.

A nitrocellulose filter was placed in a slot blot apparatus (BRL). The slot blotter was attached to a water vacuum pump and 100µl of cell wall material extracted in LSB was placed in each slot. The cell wall extract was attached to the filter by suction. The filter was removed from the blotter and cut into appropriate pieces to enable the blotting steps described in 2.12.1 to be carried out. The results were recorded by photography.

Chapter Three

Immunological recognition of fungi using polyclonal antisera.

3.1. Introduction.

No MAbs were available as positive controls to test the efficacy of protocols for screening hybridoma supernatants for specific reactions to the fungi used in this project. It was therefore decided to raise polyclonal antisera to two fungi used in the project, *N. crassa* and *P. involutus*, to enable testing of protocols and also to study the antibodies produced in the immune response to cell walls of these fungi. The proviso must be added that the immune response to an antigen can vary from animal to animal within the same species, and from species to species, but nevertheless useful information can be gained.

Some workers have used polyclonal antibodies to identify antigens of interest and have subsequently used MAbs to further investigate such antigens, *e.g.* Sundstrom & Kenny (1984) characterised antigens specific to the surface of germ tubes of *C. albicans* using antisera from rabbits and humans. They followed this by study of surface mannoproteins of the same fungus using MAbs and shown specific localisation of different mannoproteins on different parts of fungal surface (Sundstrom *et al.*, 1988).

The monospecific nature of MAbs ensures that only one epitope is studied at a time, but useful information as to the range of antigenic components of a fungus can still be gained by using polyclonal antibodies.

3.2. Production of polyclonal antisera.

Antisera to *N. crassa* and *P. involutus* cell walls were raised in New Zealand White rabbits. Blood was obtained by bleeding from the ear and serum prepared using standard protocols.

3.3 Development of an indirect non-competitive ELISA.

The choice of cell walls as the fungal antigen meant that standard ELISA protocols for soluble antigens were not suitable, since they do not facilitate binding of wall antigens to the ELISA plate throughout the whole of the assay. The development of an indirect non-competitive ELISA with cell walls bound directly to the plate was undertaken. This type of ELISA was considered useful for determining the end point titration of polyclonal antisera and their cross-reactivity with other fungal cell wall, as well as being a simple and relatively fast method for screening hybridoma supernatants. The different parameters necessary for consistent repetition of the ELISA were determined. *N. crassa* cell walls were used initially to establish the parameters.

3.3.1. Determination of optimum binding conditions.

Five types of flat-bottomed plate were used and are listed in Table 3.1.

Name	Make	Type	No. wells
Immuno-II	Nunc	Rigid	96
Falcon	Becton-Dickinson	Rigid	96
Dynatech	Dynatech	Rigid	96
Titertek	Flow	Floppy	96
Falcon	Becton-Dickinson	Floppy	96

Table 3.1. ELISA plates used in determination of optimum binding conditions.

The ELISA plates were all initially coated with poly-*l*-lysine ($10\mu gml^{-1}$) by placing 100µl of poly-*l*-lysine into each well and incubating the plates for 2h at room temperature. The poly-*l*-lysine solution was flicked from the wells and *N. crassa* cell wall suspensions added to the appropriate wells. The *N. crassa* cell wall fragments were suspended in either sdH_2O or PBS-7 at $100\mu gml^{-1}$. Homogenous suspension of the fragments was achieved by sonication using an amplitude of 16 amp microns peak to peak for 1min in 5s intervals with 10s between each interval. The suspension was checked by phase contrast microscopy to ensure that fragments were not clumped together. A two-fold dilution series of the cell wall suspension was prepared and 100µl of appropriate suspension added to the appropriate well. Plates were then placed in the following conditions listed in Table 3.2.

	Dilutant	Temperature °C	Time h	Other
1	sdH_2O	25	o/n	-
2	sdH_2O	37	o/n	-
3	PBS-7	25	o/n	-
4	PBS-7	37	o/n	-
5	sdH_2O	25	2	vacuum oven
6	sdH_2O	37	2	vacuum oven
7	sdH_2O	60	2	vacuum oven

Table 3.2. Conditions used to determine optimum binding of cell wall fragments to ELISA plates.

Following the treatments any remaining dilutant was flicked from the plates and the plates observed, at a low magnification (x10), using an inverted microscope (Olympus IM). The degree of binding was assessed visually. The results obtained are shown in Table 3.3.

The most successful binding was achieved with cell wall fragments suspended in sdH_2O and left to dry on to the plate at 37°C overnight. A similar treatment with the cell walls suspended in PBS-7 also bound the cell wall fragments to the plate but the presence of contaminating crystals from the PBS-7 was considered undesirable. Suspension of cell wall fragments in sdH_2O followed by drying at 37°C overnight was chosen as the standard method for binding cell fragments to ELISA plates.

3.3.2. Determination of optimum antigen concentration.

The three types of plate used, Nunc, Falcon rigid and Falcon floppy, were initially coated with poly-*l*-lysine ($10\mu g ml^{-1}$) as described in 3.3.1. A $100\mu g\ ml^{-1}$ suspension of *N. crassa* cell walls in sdH_2O was prepared as in 3.3.1. A two-fold dilution series was made and 100µl of each dilution was pipetted into the appropriate wells. The plates were placed at 37°C overnight. 100µl 1%BSA/1%PVP in PBS-7 was put in each well and the plates again incubated overnight at 37°C. The ELISA plates will non-specifically adsorb antibodies by hydrophobic interactions. Once the antigen has been bound to the plate it is therefore necessary to block any other sites by use of a blocking agent. Common blocking agents include BSA, non-specific serum, dried milk solutions, such as Marvel, and bovine γ-globulin. The 1%BSA/1%PVP solution was flicked out from the wells and 100µl of anti-*N. crassa* rabbit polyclonal antiserum diluted in DMEM was added to each well. A two-fold dilution of the polyclonal antiserum was made and the dilutions put into appropriate wells to enable a chequerboard ELISA to be performed. Decreasing antigen concentrations were placed in the rows down the plate and decreasing antibody concentrations were placed in rows across the plate. Following incubation of the polyclonal antibody at 37°C for 2h the

Plate name	Treatment						
	25°C sdH_2O o/n	37°C sdH_2O o/n	25°C PBS-7 o/n	37°C PBS-7 o/n	25°C sdH_2O 2h	37°C sdH_2O 2h	60°C sdH_2O 2h
Nunc	-	+	-	+*	-	-	+/c
Falcon (rigid)	-	+	-	+*	-	-	+/c
Falcon (floppy)	-	+	-	+*	-	-	+/c
Dynatech	-	+	-	+*	-	-	+/c

Table 3.3. Binding of cell wall fragments to ELISA plates following different binding treatments.

Notes:

- no binding of fragments
+ even binding of fragment to plate
+* binding of fragments but presence of crystals from PBS-7
+/c binding of fragments but in uneven clumps.

antibody was flicked out and the wells were washed 3 times with PBS-7 for 5mins to remove excess antibody. 100µl anti-rabbit IgG HRPO conjugate, diluted 1/500 in second antibody diluent, was added to each well and the plate incubated for 2h at 37°C. The second antibody was flicked out and a similar washing regime employed as that used after the first antibody incubation (*i.e.* 3x PBS-7 for 5mins). ABTS was added as substrate and after 30mins the absorbance value for each well was read at 410nm using a Dynatech plate reader. The results of this chequerboard ELISA are shown in Table 3.4.

This screen was intended to find the optimum antigen concentration of cell wall antigen to use subsequently in the ELISA protocol. No decrease in absorbance was found as the antibody concentration decreased (Table 3.4) on all three types of plate (Nunc, Falcon rigid and Falcon floppy) used (Table 3.4a; b; c), *e.g.* with an antigen concentration of 10µg/well no decrease in absorbance was observed. The absorbance values did decrease with a decrease in antigen concentration at lower concentrations of antibody, *e.g.* at an antibody titre of 1/1024 the absorbance decreased as the antigen concentration decreased on all three types of plate. Antibody dose-response curves generally have a larger range than antigen dose-response curves and often exceed more than four $\log_{10}$ dilutions (Tijssen, 1985). This helps to explain the lack of a dose-response when antibody was diluted in a two-fold series as opposed to the observed dose-response when antigen was diluted.

The ELISA protocol used in this screen produced very high levels of background absorbance even when no antigen was present. At lower antibody concentrations this background absorbance was higher on the Falcon plates than on the Nunc plate (0.46 and 1.36 on the Falcon rigid and floppy respectively compared to 0.07 on the Nunc plate at an antibody titre of 1/1024). The level of background shown by wells incubated with second antibody only was also lower on the Nunc plate (compare the final column in Tables 3.4a, b and c)

Table 3.4. Three chequerboard ELISAs to determine the optimum *N. crassa* cell wall antigen concentration

Notes:

Pre8 pre-immune antiserum from the rabbit subsequently injected with *N. crassa* cell walls, diluted to 1/8

2nda/b the cell walls were not incubated with an antibody in the first antibody step, but were incubated with the HRPO conjugate antibody in the second step

Cell wall	Reciprocal antibody titre											
µg dry wt	2	4	8	16	32	64	128	256	512	1024	Pre8	2nda/b
10	>2	>2	>2	>2	>2	>2	>2	>2	>2	>2	>2	0.29
5	>2	>2	>2	>2	>2	>2	>2	>2	>2	1.56	>2	0.26
2.5	>2	>2	>2	>2	>2	>2	>2	>2	0.72	1.31	>2	0.15
1.25	>2	>2	>2	>2	>2	>2	>2	>2	0.36	0.37	1.03	0.14
0.63	>2	>2	>2	>2	>2	>2	>2	1.54	1.20	0.56	>2	0.07
0.32	>2	>2	>2	>2	>2	>2	0.39	1.44	0.65	0.22	1.70	0.06
0.15	1.60	1.73	>2	>2	>2	>2	1.52	0.26	0.43	0.13	0.32	0.09
0.0	0.99	1.26	1.12	1.32	1.21	0.94	0.77	0.39	0.10	0.07	0.74	0.06

Table 3.4a. Chequerboard ELISA to determine optimum antigen concentration on a Nunc Immuno-II plate.

Cell wall	Reciprocal antibody titre											
µg dry wt	2	4	8	16	32	64	128	256	512	1024	Pre8	2nda/b
10	>2	>2	>2	>2	>2	>2	>2	>2	>2	>2	>2	0.37
5	>2	>2	>2	>2	>2	>2	>2	>2	>2	>2	>2	0.24
2.5	>2	>2	>2	>2	>2	>2	>2	>2	>2	>2	>2	0.31
1.25	>2	>2	>2	>2	>2	>2	>2	>2	>2	>2	>2	0.29
0.63	>2	>2	>2	>2	>2	>2	>2	>2	1.31	0.89	>2	0.26
0.32	>2	>2	>2	>2	>2	>2	>2	1.69	1.00	0.61	>2	0.21
0.15	>2	>2	>2	>2	>2	>2	>2	1.53	0.96	0.77	1.03	0.19
0.0	1.75	>2	>2	>2	>2	1.22	1.05	0.71	0.51	0.46	0.68	0.15

Table 3.4b. Chequerboard ELISA to determine optimum antigen concentration on a Falcon rigid plate.

Cell wall	Reciprocal antibody titre											
µg dry wt	2	4	8	16	32	64	128	256	512	1024	Pre8	2nda/b
10	>2	>2	>2	>2	>2	>2	>2	>2	>2	1.18	>2	0.97
5	>2	>2	>2	>2	>2	>2	>2	>2	>2	>2	>2	0.27
2.5	>2	>2	>2	>2	>2	>2	>2	>2	>2	.2	>2	0.36
1.25	>2	>2	>2	>2	>2	>2	>2	>2	1.74	1.14	>2	0.28
0.63	>2	>2	>2	>2	>2	>2	>2	>2	1.36	1.12	>2	0.28
0.32	>2	>2	>2	>2	>2	>2	>2	1.26	1.11	0.74	1.25	0.17
0.15	>2	1.59	>2	>2	>2	>2	>2	1.00	1.11	0.61	1.60	0.17
0.0	>2	>2	>2	>2	>2	1.01	1.07	0.80	0.94	1.36	0.24	0.14

Table 3.4c. Chequerboard ELISA to determine optimum antigen concentration on a Falcon floppy plate.

The absorbance levels recorded when pre-immune serum diluted 1/8 was used as the first antibody shows that non-specific binding of antibody occurred. This binding occurred to both wells with cell walls and wells without, *e.g.* on the Nunc plate with 10μg of cell wall present in the well the absorbance was above 2 and even with no antigen present the absorbance was 0.74. An ELISA using pre-immune serum as the first antibody should have been carried out alongside the anti-*N. crassa* serum to establish the level of binding at comparative titres, but was not done due to a shortage of pre-immune serum. Tijssen (1985) stressed that serial dilutions with negative sera should be carried out since the assumption that non-specific binding is constant regardless of the dilution factor violates the law of mass-action.

Conventionally, using a chequerboard ELISA, the antigen concentration is chosen by assessing the end-point titre of the antiserum at the highest antigen concentration and then seeing what is the lowest antigen concentration which still gives a positive result at this end-point titre. This is the antigen concentration then chosen for subsequent use. It was not possible to do this from these results as an end point titre could not be established. But a decision was made to use 10μg/well as the appropriate concentration of cell wall antigen for subsequent experimentation. This concentration gave a strong positive absorbance value even at an immune antibody dilution of 1/1024, using this titre as if it were the end-point titre. Although the pre-immune serum gave a positive at this antigen concentration this was at a titre of 1/8 and so cannot strictly be compared with the test antiserum at a concentration of 1/1024 as the non-specific binding is at different levels at the different antibody concentrations. Some of the other antigen concentrations, 5 and 2.5μg/well, also gave high absorbance readings at 1/1024, but in the case of the Nunc plate these were not as high as when 10μg/well was used as the antigen concentration.

Microscopic examination of the different antigen concentration wells at the start and end of the experiment showed that at 10μg/well the cell walls fragments covered the whole of the well in an even monolayer. Below this concentration the fragments did not cover the whole well and as a result of this large holes occurred in the cell wall fragment monolayer. This observation corroborated the decision to use 10μg/well cell wall as the antigen concentration since it was thought that the uneven distribution of the cell wall fragments and the areas of the well not covered by antigen at lower antigen concentrations might interfere with the assay; for example in the blocking of non-specific binding sites and in the exaggerated effect any leaching of antigen from the wells might have on the final absorbance.

It was also decided to use the Nunc Immuno-II plate for all subsequent ELISA as this plate showed a lower level of background absorbance than the other two plates tested.

3.3.3 Determination of optimum blocking and washing procedures.

The high levels of background absorbance seen in the results from 3.3.2 are most commonly caused by an ineffective blocking step and/or inadequate washing procedures following antibody incubations. Both of these lead to excess non-specific antibody, both first and second, interfering with the final substrate step to give a higher than expected absorbance values. Determination of the blocking and washing procedures which would give little background interference was undertaken.

Antigen-free ELISA plates were blocked overnight using 1%BSA/PVP in PBS-7 or 2% Marvel in PBS-7. Two plates were blocked using 1%BSA/PVP, one was placed at 25°C and the other at 37°C. Only one plate was blocked with 2% Marvel and was put at 25°C. 100μl of blocker was added to each well. Following the overnight incubation the blocker was flicked out and 100μl PBS-7 added to each well and the plates incubated at 37° for 2h. The plates were then washed 3 times with PBS-7, 5mins for each wash. 100μl of the appropriate

dilution of second antibody was added to each well and the plates incubated at 37°C for 2h. The second antibody (goat anti-rabbit IgG conjugated to HPRO) was flicked from the wells and the washing step with PBS-7 repeated. In this case *o*-phenylenediamine was used as substrate. When *o*-phenylenediamine is added to wells with HRPO present it is oxidised from a colourless to an orange compound. This reaction can be stopped by adding 4M sulphuric acid and the absorbance measured at 490nm. Stopping the reaction after a set incubation time is very useful for ensuring that the absorbance values measured are directly comparable. The absorbance values were read at 490nm using a plate reader (Dynatech). The results are summarised in Table 3.5.

1%BSA/PVP overnight at 25 or 37°C effectively blocked non-specific binding sites of anti-rabbit IgG at 1/500 (Table 3.5). 2% Marvel was also an effective blocking agent, but at 1/1000 a "rogue" well with an absorbance reading >2 produced a large standard error (σ = 0.48). The blocking of anti-mouse IgG non-specific sites was not as effective with either 1%BSA/PVP or 2% Marvel at 25°C overnight ($\bar{x}$ = 0.30 and 0.28 respectively). In the case of the 2% Marvel further "rogue" wells occurred at 1/500 and 1/1000 causing high means with large standard errors *e.g.* at 1/500 $\bar{x}$ = 0.52 and σ = 0.60. The reason for the appearance of "rogue" wells is not clear, but their occurrence disqualified Marvel as the first choice of blocking agent.

These results suggest that when rabbit polyclonal antibody is being tested a second antibody concentration of 1/500 would be suitable to give a low background when used in conjunction with 1%BSA/PVP as a blocker and with three PBS-7 washes of 5mins following antibody incubation. The results for anti-mouse IgG suggest, even at 1/1000, the background is relatively high and if non-specific binding of first antibody were added to non-specific binding of second antibody the resulting absorbance would be unacceptably high. The chequerboard ELISA in Section 3.3.1 showed high non-specific binding of pre-

Antibody	Blocker		
	1%BSA/PVP 25°C	1%BSA/PVP 37°C	2%Marvel 25°C
Anti-rabbit IgG			
1/300	0.08±0.07	-	0.10±0.07
1/500	0.07±0.02	0.04±0.01	0.08±0.03
1/1000	0.06±0.02	-	0.20±0.48
Anti-mouse IgG			
1/300	0.30±0.08	-	0.28±0.17
1/500	0.30±0.06	-	0.52±0.60
1/1000	0.21±0.12	-	0.53±0.58

Table 3.5. Absorbance at 490nm for different blocking treatments and three washes of 0.05%Tween/PBS-7 in an ELISA (±S.D.).

Antibody	Blocker	
	1%BSA/PVP	1%BSA
Anti-rabbit IgG 1/500	0.06±0.02	0.05±0.01
Anti-mouse IgG 1/500	0.16±0.05	0.12±0.03
Substrate only	0.05±0.03	0.05±0.02

Table 3.6. Absorbance at 490nm for different blocking treatments and using six washes of 0.05%Tween/PBS-7 (±S.D.)

immune serum (1/8) both to antigen filled wells and wells with no antigen (see Table 3.4) and it was decided to investigate whether the blocking time could be shortened to enable the assay to be performed on one day, and also to determine if more extensive washing could overcome the high background problems.

The same protocol as already described in this section was followed except that 1%BSA/PVP in PBS-7 and 1%BSA only in PBS-7 were used as the blockers and the blocking time was reduced from overnight to 1h at 37°C. Following the second antibody incubations (2h, 37°C), six washes of 5min with PBS-7 were carried out. 0.05% Tween 20 was added to the PBS-7 since this aids removal of non-specifically bound antibody (Campbell, 1984).

The results of this protocol (Table 3.6) show that there was little difference between 1%BSA/PVP and 1%BSA only as blockers of non-specific binding sites. Incubation of these blockers for 1h at 37°C in conjunction with six washes with 0.05% Tween 20/PBS-7 gave similar results to overnight blocking with 1%BSA/PVP at both 25 and 37°C for anti-rabbit IgG when only three washes were carried out and lower background absorbances with anti-mouse IgG.

3.3.4. Test of full ELISA protocol.

All of the individual parameters were combined to test their effectiveness in ELISAs for determining the end-point titre of the two polyclonal antibodies and for screening hybridoma supernatants. To be useful in screening hybridoma supernatants for presence of antibodies specific to the fungal cell wall in question it is necessary for the ELISA protocol devised to be able to discriminate between supernatants containing specific antibodies and those with non-specific antibodies. No positive hybridoma supernatants were available to test for specificity using this protocol, but several MAbs raised against other antigens were available to test to see the levels of non-specific binding that occurred, enabling a

Fig. 3.1. Overview of the ELISA protocol.

Step 1 Poly-*l*-lysine coating of wells

2h, R.T.

Step 2 Antigen binding

10µg (freeze-dried weight) cell walls per well in 100 sdH_2O

o/n, 37°C

Step 3 Blocking

1% BSA in PBS-7, 1h, 37°C

Step 4 First antibody incubation

Polyclonal antibody or hybridoma supernatant

100µl, 2h, 37°C

Step 5 Washing

6x 0.05% Tween 20/PBS-7, 5min each wash by hand, or use of plate washer

Step 6 Second antibody incubation

100µl second antibody at 1/500 in second antibody diluent

Step 7 Washing as in Step 5

Step 8 Substrate addition

100µl *o*-phenylenediamine, or ABTS, 30min

Step 9 Plate read at 410nm or 490nm

positive/negative threshold to be established.

The wells were initially coated with poly-*l*-lysine (10μg ml^{-1}). 100μl of cell wall suspension at 100μg (freeze-dry weight) ml^{-1} in sdH_20 was pipetted into appropriate wells to give an antigen concentration of 10μg/well. This was allowed to dry on to the plate at 37°C overnight. 250μl of 1% BSA in PBS-7 was added to each well and the plate incubated for 1h at 37°C. The blocker was flicked from the wells and 100μl of the appropriate first antibody or control put into the appropriate well. The first antibody was added and the plate incubated for 2h at 37°C. After incubation the antibody solution was flicked from the plate and the wells were washed six times with 300μl of 0.05% Tween 20/PBS-7. Each wash was of 5min duration. Second antibody, either anti-rabbit or anti-mouse IgG conjugated to HRPO was diluted 1/500 in second antibody diluent and 100μl added to each well. The plate was then incubated for 2h at 37°C and the same washing procedure followed as after the first antibody incubation.

100μl *o*-phenylenediamine was added to each well as substrate when rabbit polyclonal antiserum had been used as first antibody. When the first antibody had been a MAb 100μl ABTS was added as substrate. It was found that the oxidised green product of ABTS was easier to observe visually to give a quick assessment of positives and negatives than the orange colour of the oxidized product of *o*-phenylenediamine and so ABTS was chosen for use in hybridoma screens. An overview of the final ELISA protocol is shown in Fig. 3.1.

The results presented in Tables 3.7 and 3.8 show that the ELISA protocol developed can be used to screen polyclonal antisera. The results in the fourth column *i.e.* the mean of the absorbances following incubation of the three polyclonal antisera in wells without antigen present ($\bar{x}$/no antigen) indicate that, at high concentrations, the antisera show a high level of non-specific binding. This non-specific binding did not occur at lower antisera concentrations where the level of specific binding was still very high *e.g.* 1/512 and so does

Antibody titre	Antiserum			
	anti-Nc	anti-Pi	Pre	$\bar{x}$/no antigen
1/2	>2	>2	>2	0.83±0.18
1/4	>2	>2	>2	0.63±0.16
1/8	>2	>2	>2	0.53±0.02
1/16	>2	>2	>2	0.32±0.08
1/32	>2	>2	>2	0.25±0.06
1/64	>2	>2	>2	0.18±0.05
1/128	>2	>2	1.40	0.14±0.03
1/256	>2	>2	0.88	0.11±0.02
1/512	>2	>2	0.52	0.09±0.02
1/1024	1.67	1.58	0.38	0.08±0.02

Table 3.7. The absorbances at 490nm from an ELISA to determine the effectiveness of the ELISA parameters using polyclonal antisera as the test antibodies and *N. crassa* as the test antigen.

Notes:

anti-Nc antiserum raised against *N. crassa*

anti-Pi antiserum raised against *P. involutus*

Pre pre-immune antiserum from the anti-*N. crassa* rabbit

All of the above values are the mean of duplicates.

$\bar{x}$/no antigen the mean of the six replicates from above three polyclonal antisera when tested in wells without antigen.

Antibody titre	Antiserum			
	anti-Pi	anti-Nc	Pre	$\bar{x}$/no antigen
1/2	>2	>2	>2	0.97±0.35
1/4	>2	>2	>2	0.71±0.10
1/8	>2	>2	>2	0.45±0.13
1/16	>2	>2	1.79	0.33±0.10
1/32	>2	>2	1.73	0.23±0.06
1/64	>2	>2	1.54	0.21±0.04
1/128	>2	>2	1.16	0.14±0.04
1/256	>2	>2	0.89	0.15±0.10
1/512	>2	>2	0.65	0.09±0.04
1/1024	>2	>2	0.36	0.08±0.02

Table 3.8 To show the effectiveness of the ELISA protocol using polyclonal antisera as the test antibodies and *P. involutus* as the test antigen.

Notes: as for Table 3.7.

not interfere with the assay at working concentrations of the antisera. The level of binding of the pre-immune antisera to both *N. crassa* and *P. involutus* cell walls is relatively high. For example at an antibody titre of 1/128 absorbances of 1.4 and 1.16 were seen when *N. crassa* and *P. involutus* cell wall fragments were reacted with pre-immune antisera; at this antibody titre the level of non-specific binding to the well was only 0.14 and so did not significantly contribute to the reaction of the pre-immune antisera at this titre. At an antibody titre of 1/1024 the reaction of cell wall fragments and pre-immune antisera was low, but was still positive; if a positive/negative threshold value of the mean background absorbance with no antigen present plus four standard deviations of this mean was used. This threshold level was chosen as it has been found to exclude 99% of all negative antisera (Tijssen, 1985).

The hybridoma medium itself, SDMEM, did not produce a high background interference and little non-specific binding occurred with two MAbs which were raised against the steroid, zeranol (Table 3.9). The level of background absorbance caused by non-specific binding of the bull sperm MAbs 1 to 6 was slightly higher than that caused by the two zeranol MAbs, but was still acceptable. The mean of the background absorbances seen with the bull sperm MAbs was increased when the absorbance seen with BS7 was included, but not to unacceptable levels. The standard deviation did increase considerably, from 0.06 excluding absorbances seen with BS7 to 0.16 including the BS7 absorbances when *N. crassa* was the antigen.

The use of the ELISA to screen hybridoma supernatants requires a negative/positive threshold to be established. A threshold value of the mean plus four standard deviations of the mean was chosen as described previously for the polyclonal antisera. The mean of the nine non-specific MAbs plus four standard deviations of the mean gave threshold values of 0.88 for *N. crassa* and 0.51 for *P. involutus*. The validity of these values to ensure correct discrimination between positive and negative hybridoma supernatants was confirmed by

First antibody	Cell wall antigen		
	N. crassa	*P. involutus*	No antigen
SDMEM	0.15±0.01	0.13±0.02	0.09±0.01
9 Mabs	0.28±0.15	0.23±0.07	0.12±0.03
Z Mabs	0.19±0.03	0.18±0.04	0.10±0.01
BS1-6	0.25±0.06	0.22±0.05	0.11±0.01
BS1-7	0.31±0.16	0.25±0.07	0.12±0.02
BS7	0.66±0.03	0.39±0.01	0.18±0.03

Table 3.9. The absorbance at 410nm to show the level of non-specific binding of hybridoma supernatants to fungal cell walls in an ELISA.

Notes:

SDMEM hybridoma medium

9 Mabs all Mabs tested

Z Mabs zeranol Mabs, F4 and F6.

BS1-7 bull sperm Mabs 1-7

their rejection of BS7 as a positive hybridoma supernatant. Immunofluorescence observations of this MAb when incubated with the cell walls of the two fungi showed no fluorescence, thus confirming the ELISA result.

These tests of the ELISA protocol using both polyclonal and monoclonal antibodies show that the ELISA protocol developed does enable differentiation between antibodies which show specific binding to the fungal cell walls and those which do not.

3.4. Characterisation of the polyclonal antisera.

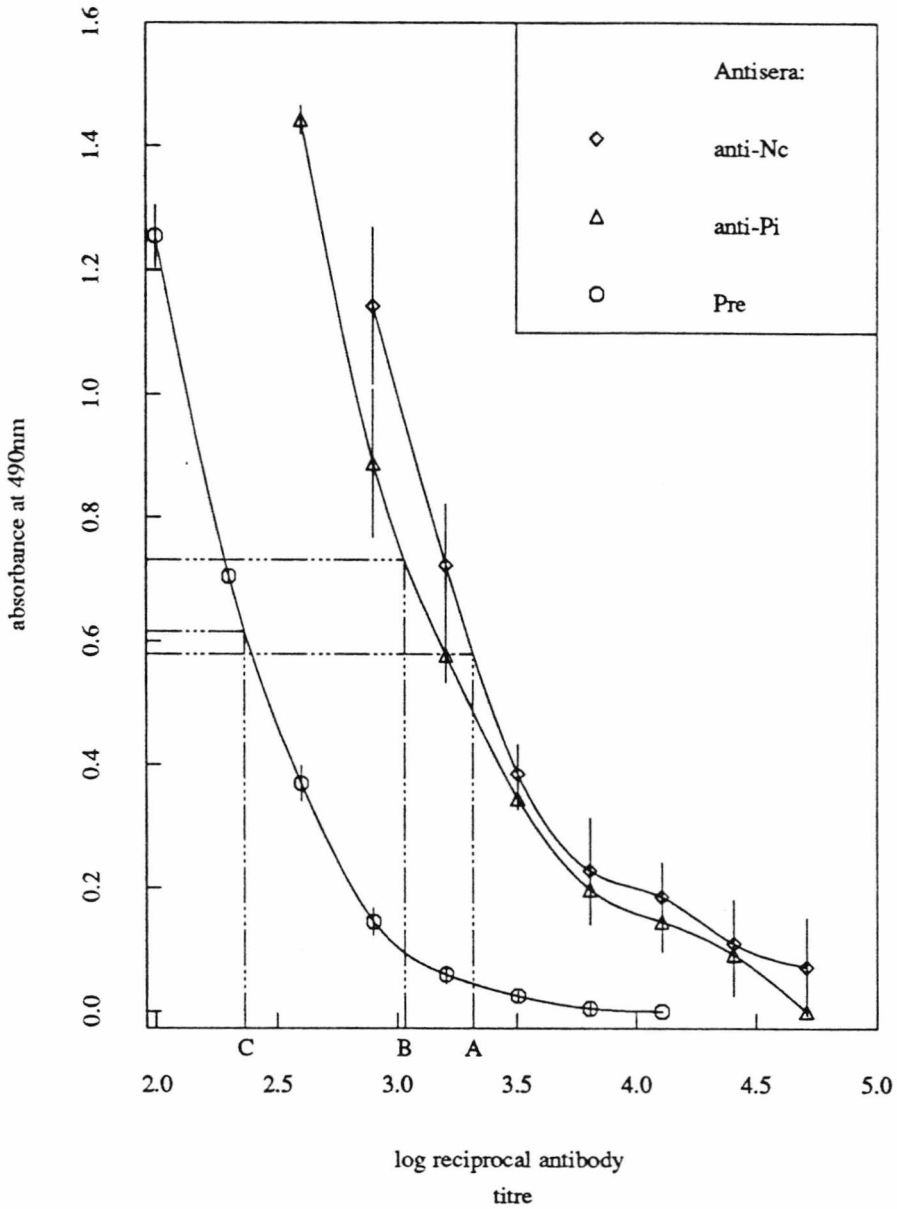
3.4.1. End-point titres.

Using the ELISA protocol described in Section 3.3.4 the end-point titre of the two polyclonal antisera were determined. The antisera were diluted in a two-fold series beginning at 1/100 and the results plotted graphically (Figs. 3.2 and 3.3). Antibody titres were plotted as the $\log_{10}$ of the reciprocal antibody dilution. When *N. crassa* was the antigen the end-point for pre-immune antiserum was 2.37 on the $\log_{10}$ scale which is equivalent to an antibody dilution of 1/230. The end-point was taken at 50% of the maximum recorded absorbance. The maximum absorbance value should be taken at saturation but this was not always possible as can be seen from the graphs and so the end point titres are an indication of titre rather than absolute values. The end-point antibody dilutions for anti-*N. crassa* and anti-*P. involutus* antisera against *N. crassa* were 1/2,075 and 1/1,080 respectively. When *P. involutus* cell walls were the antigen (Fig. 3.3) the homologous system had an end-point antibody dilution of 1/13,720, whereas the heterologous system had an end-point of 1/2,365. The pre-immune antiserum had an end-point of 1/310.

3.4.2. Pre-absorption of the polyclonal antisera.

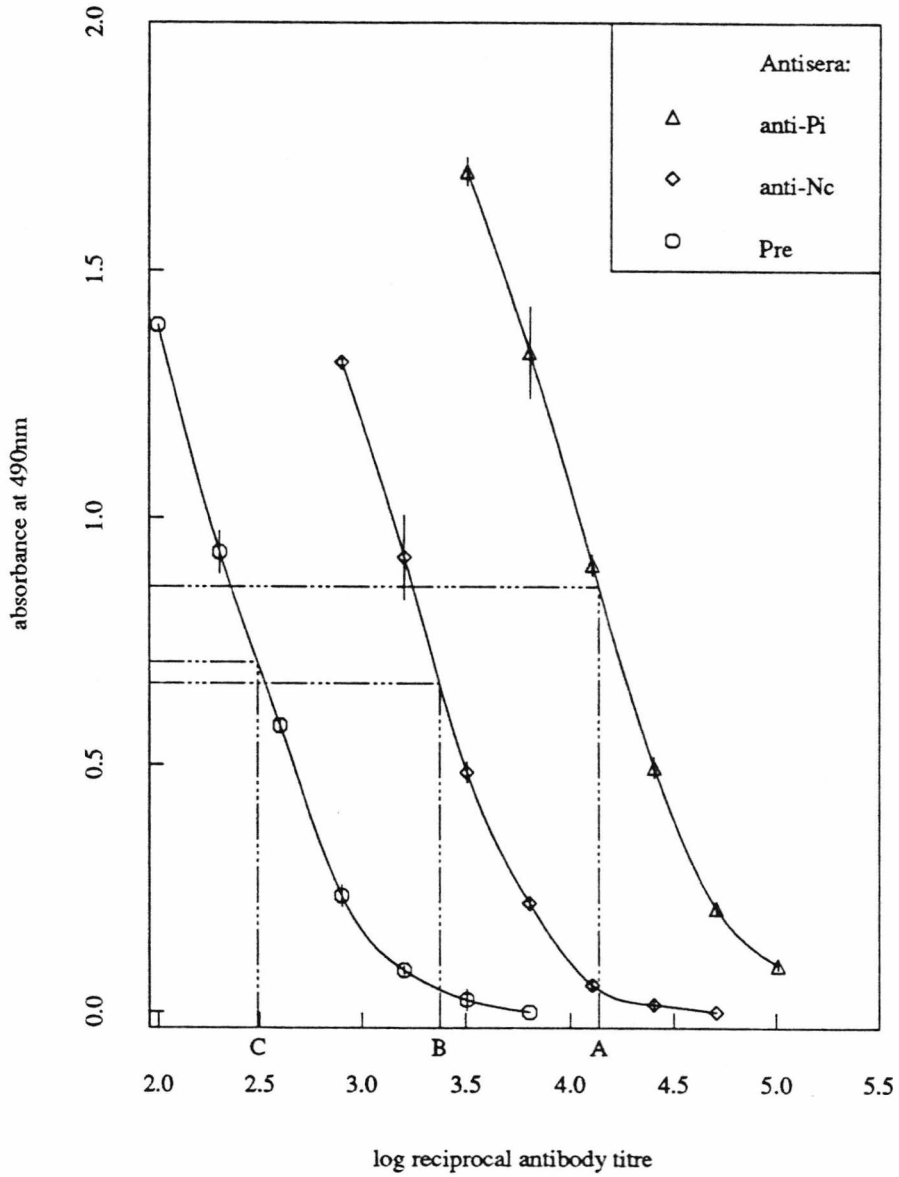
Antiserum raised against *N. crassa* was pre-absorbed with both *P. involutus* and *Pisolithus tinctorius* cell walls. Pre-absorption of an antiserum with cross-reacting

Fig. 3.2. The antibody titration curves of antisera reacted with *N.crassa* cell walls (10μg/well) in an ELISA.



Each point represents the mean of four values. Standard errors are shown for each point. Anti-Nc and anti-Pi represent the antisera raised against *N. crassa* and *P. involutus* respectively. Pre represents pre-immune serum from the rabbit injected with *N. crassa*. A, B and C indicate the end-point titres of anti-*N. crassa* antiserum (1/2,075), anti-*P. involutus* antiserum (1/1,075) and pre-immune serum (1/230), respectively.

Fig. 3.3. The antibody titration curves of antisera reacted with *P. involutus* cell walls (10μg/well) in an ELISA.



Each point represents the mean of four values. Standard errors are shown for each point. Anti-Pi and anti-Nc represent the antisera raised against *P. involutus* and *N. crassa*, respectively. Pre represents pre-immune serum from the rabbit injected with *P. involutus* . A, B and C indicate the end-point titres of anti-*P. involutus* antiserum (1/13,720), anti-*N. crassa* antiserum (1/2,365) and pre-immune serum (1/310), respectively.

Antigen	Antiserum		
	Anti-*N. crassa*	Anti-*P. involutus*	Pre-immune
N. crassa	1/2,075	1/1,080	1/230
P. involutus	1/2,365	1/13,720	1/310

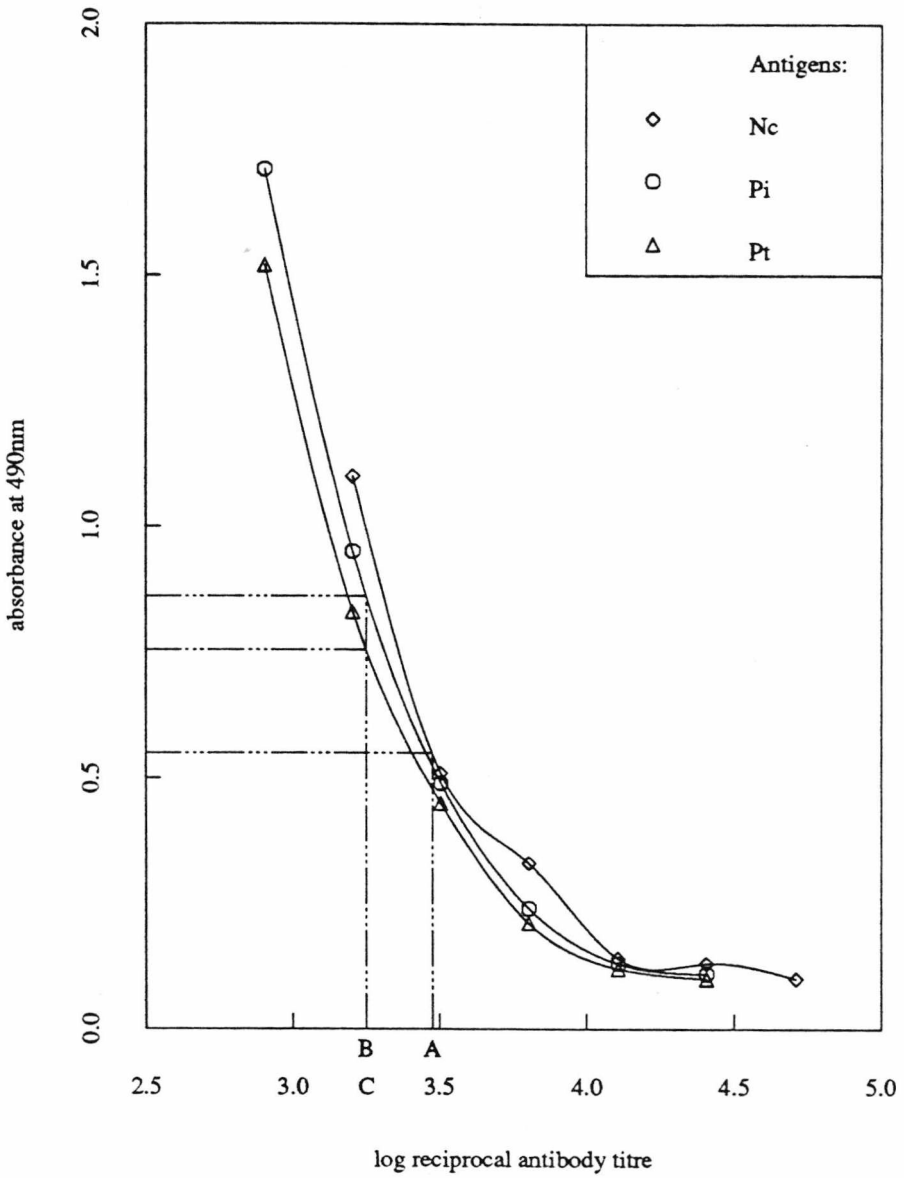
Table 3.10. A summary of the end-point titres for the rabbit polyclonal antisera with *N. crassa* and *P. involutus* cell wall antigens.

heterologous antigens can remove cross-reactivity of the antiserum with those antigens. The cross-reacting antibodies are adsorbed onto the heterologous antigen and so are removed when the antigen is removed. Similarly, antiserum raised against *P. involutus* was pre-absorbed with *N. crassa* and *Pisolithus tinctorius* cell walls. The pre-absorbed antisera were then tested for increased specificity using the ELISA protocol described in Section 3.3.4 and the results were plotted graphically to enable the end-points to be determined (Figs. 3.4, 3.5, 3.6 and 3.7).

Pre-absorption of anti-*N. crassa* antiserum with either *P. involutus* or *Pisolithus tinctorius* did not lessen the recognition of either basidiomycete species. The end-point titres were of a similar order to that prior to absorption (Table 3.11.) and so were not decreased by absorption.

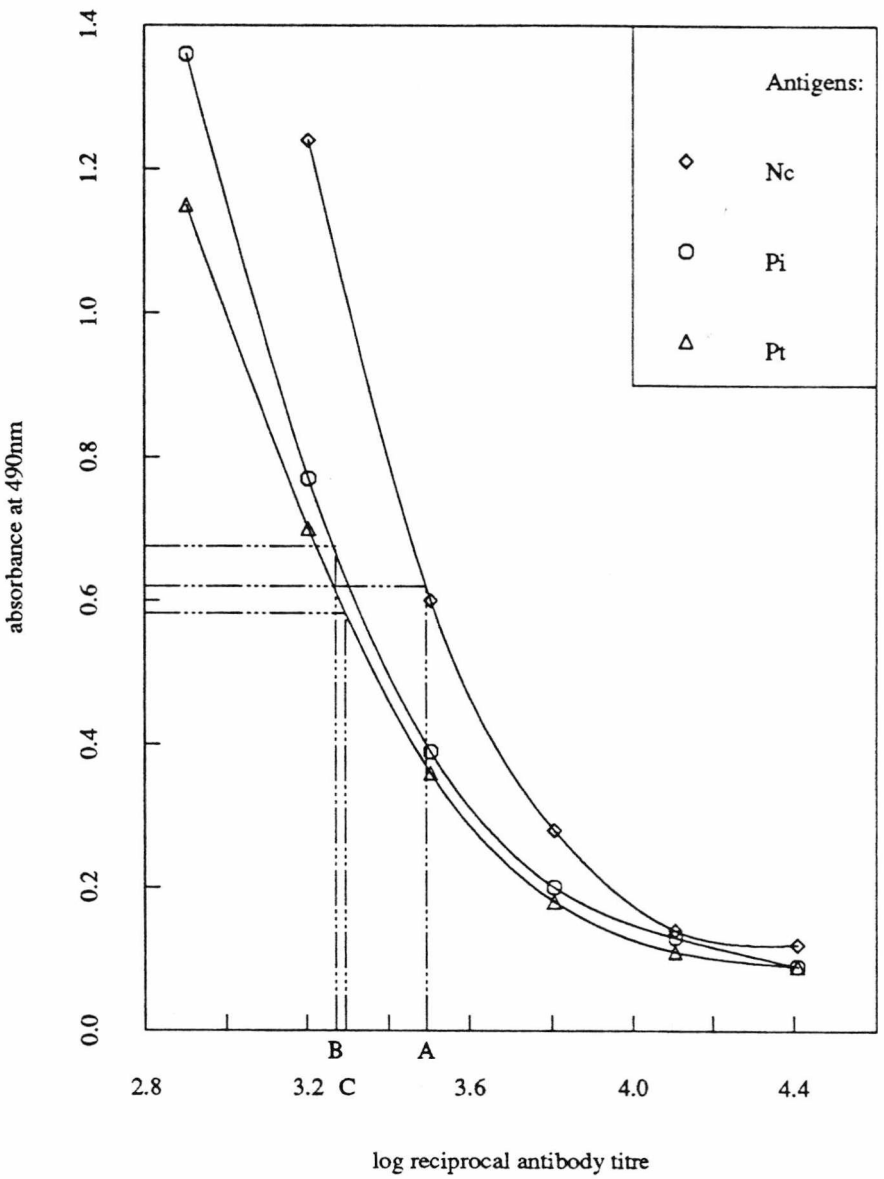
Pre-absorption of anti-*P. involutus* antiserum with *N. crassa* did not remove the cross-reaction with *N.crassa*, but this antiserum already showed a stronger reaction with its homologous antigen than anti-*N. crassa* antiserum showed with its homologous antigen. In this case the end-point titre of the antiserum was decreased by pre-absorption with *N. crassa* cell walls, (compare Fig. 3.6 with Figs. 3.2 and 3.3). Anti-*P. involutus* antiserum showed a high level of cross-reaction with *Pisolithus tinctorius* when pre-absorbed with *N. crassa* cell

Fig. 3.4. The antibody titration curves of the antiserum raised against *N. crassa* reacted with cell wall antigens from three fungi, following pre-absorption of the antiserum with *P. involutus* cell walls.



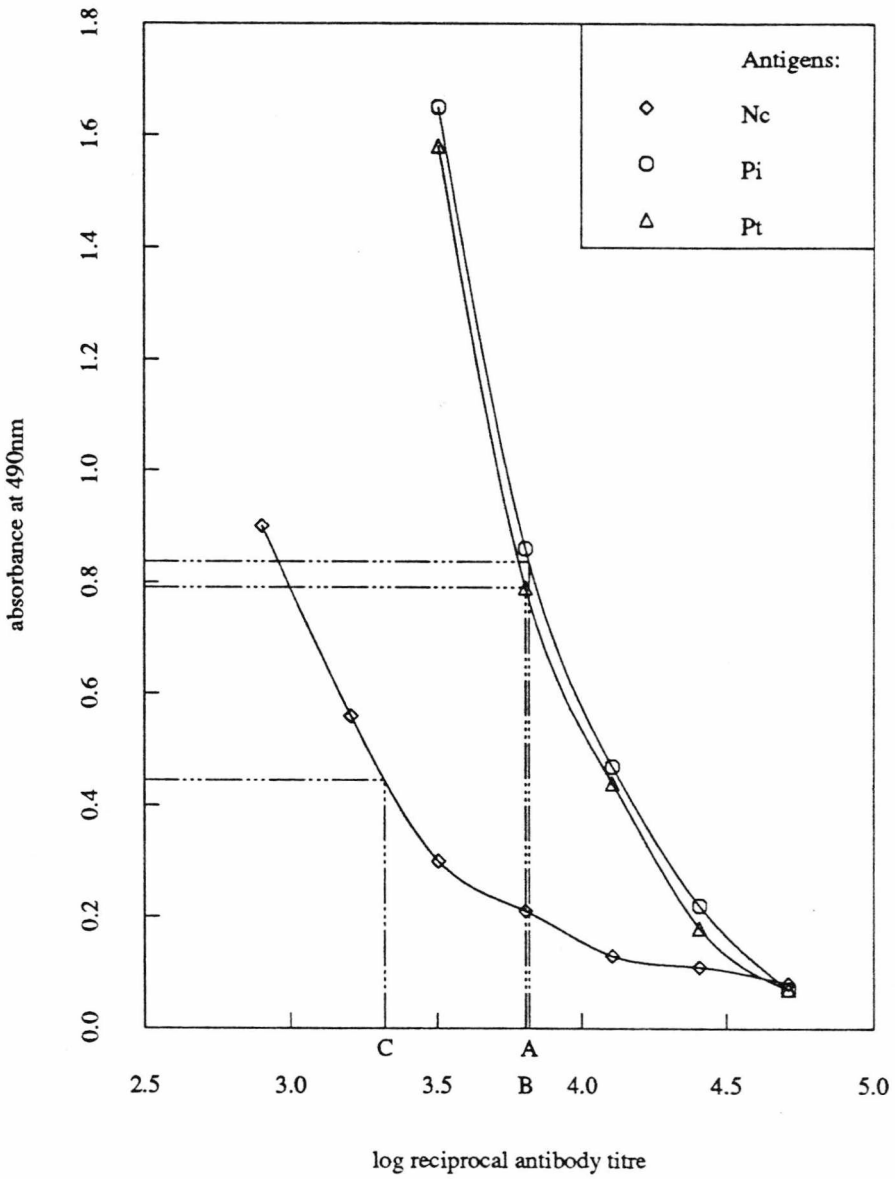
Each point represents the mean of duplicates. Nc, Pi and Pt represent the species of fungi from which the cell walls were isolated. The species were *N. crassa*, *P. involutus* and *P. tinctorius*, respectively. A, B and C indicate the end-point titres of the pre-absorbed anti-*N. crassa* antiserum with *N. crassa* (1/2986), *P. involutus* (1/1,775) and *P. tinctorius* (1/1,775), respectively.

Fig. 3.5. The antibody titration curves of the antiserum raised against *N. crassa* reacted with cell wall antigens from three fungi, following pre-absorption of the antiserum with *P. tinctorius* cell walls.



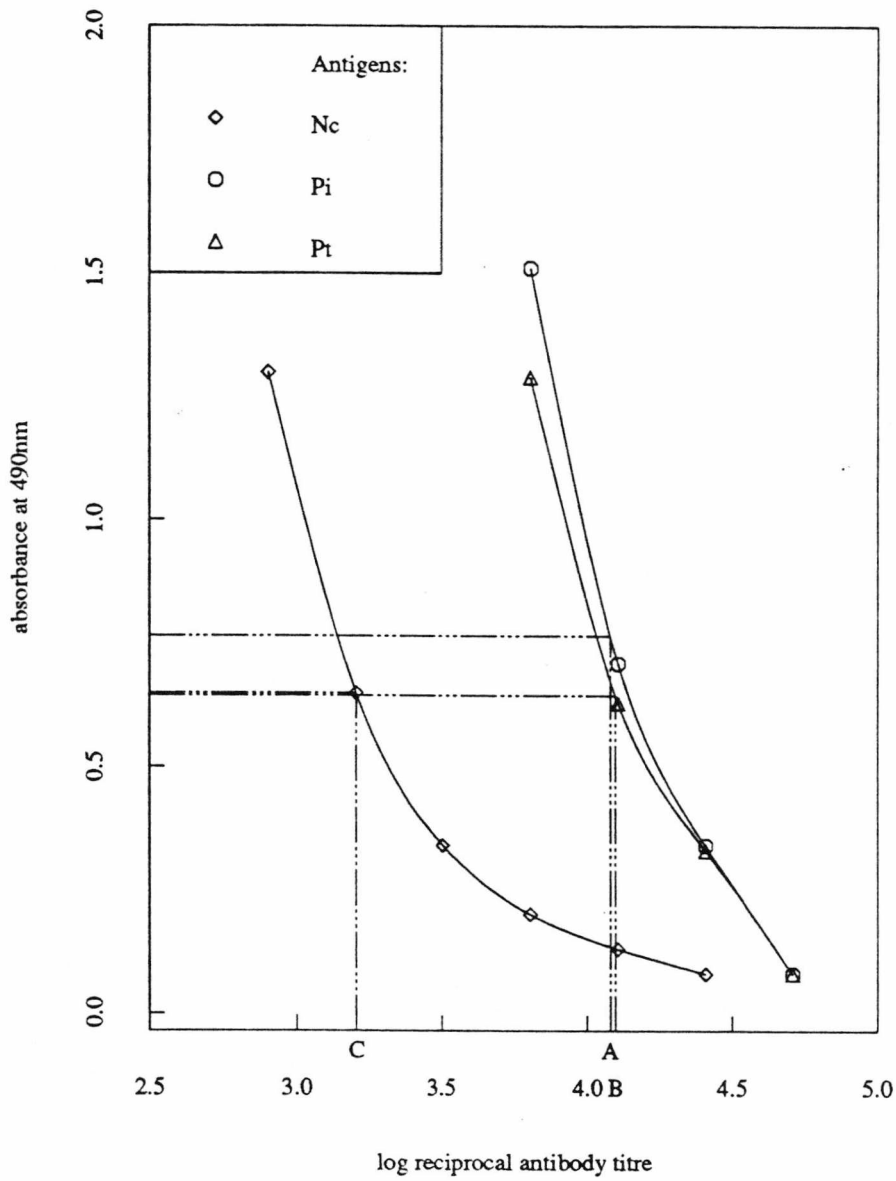
Each point represents the mean of duplicates. Nc, Pi and Pt represent the species of fungi from which the cell walls were isolated. The species were *N. crassa*, *P. involutus* and *P. tinctorius*, respectively. A, B and C indicate the end-point titres of the pre-absorbed anti-*N. crassa* antiserum with *N. crassa* (1/3,110), *P. involutus* (1/1,860) and *P. tinctorius* (1/1,970), respectively.

Fig. 3.6. The antibody titration curves of the antiserum raised against *P. involutus* reacted with cell walls from three fungi, following pre-absorption with *N. crassa* cell walls.



Each point represents the mean of duplicates. Nc, Pi and Pt represent the species of fungi from which the cell walls were isolated. The species were *N. crassa*, *P. involutus* and *P. tinctorius*, respectively. A, B and C indicate the end-point titres of the pre-absorbed anti-*P. involutus* antiserum with *P. involutus* (1/6,580), *P. tinctorius* (1/6,400) and *N. crassa* (1/2,075), respectively.

Fig. 3.7. The antibody titration curves of the antiserum raised against *P. involutus* reacted with cell walls from three fungi, following pre-absorption with *P. tinctorius* cell walls.



Each point represents the mean of duplicates. Nc, Pi and Pt represent the species of fungi from which the cell walls were isolated. The species were *N. crassa*, *P. involutus* and *P. tinctorius*, respectively. A, B and C indicate the end-point titres of the pre-absorbed anti-*P. involutus* antiserum with *P. involutus* (1/12,020), *P. tinctorius* (1/12,535) and *N. crassa* (1/1,600), respectively.

walls and pre-absorption with *Pisolithus tinctorius* cell walls did not remove this reaction (Fig. 3.7). When anti-*P. involutus* antiserum was pre-absorbed with *Pisolithus tinctorius*, the end-point titre was not decreased.

	Antigen		
	N. crassa	*P. involutus*	*Pis. tinctorius*
Antisera			
Anti-Nc/pre-abs Pi	1/2,985	1/1,775	1/1,775
Anti-Nc/pre-abs Pt	1/3,110	1/1,860	1/1,970
Anti-Pi/pre-abs Nc	1/2,075	1/6,580	1/6,400
Anti-Pi/pre-abs Pt	1/1,600	1/12,020	1/12,535

Table 3.11. A summary of the end-points of the two polyclonal antisera pre-absorbed with different fungi.

3.4.3. Immunofluorescence using the polyclonal antisera.

The immunofluorescence patterns exhibited by each antiserum show that both antisera react with their homologous cell wall fragments (Figs. 3.8 and 3.9); and react at higher titres than the corresponding pre-immune antisera with their homologous antigens (Fig. 3.10).

The antiserum raised against *N. crassa* reacts strongly with *N. crassa* cell wall fragments at titres lower than 1/32, but at a titre of 1/64 the level of fluorescence was similar to that shown by the pre-immune serum from the same rabbit at a titre of 1/8 (Fig. 3.10). At the 1/64 titre, however, the anti-*N. crassa* antiserum reacted strongly with some septa (arrowed, Fig. 3.8a) and, also with the ends of some fragments (arrows, Fig. 3.8c).

The pattern of fluorescence of the antiserum raised against *N. crassa* cell walls with *N. crassa* cell walls is shown in Fig. 3.8. The fluorescence shown by the cell wall fragments is

Fig. 3.8. The immunofluorescence pattern exhibited by *Neurospora crassa* cell wall fragments following reaction with the antiserum raised against *Neurospora crassa*.

(a) The fluorescence pattern with anti-*Neurospora crassa* antiserum at a 1/32 titre. The strong fluorescence of a transverse section of the wall is arrowed.

(b) Phase contrast of (a).

(c) The pattern of fluorescence with anti-*Neurospora crassa* antiserum at a 1/64 titre. Bright fluorescence at a septum and some fragments ends was seen (arrowed).

(d) Phase contrast of (c).

Scale bar represents 5μm. Refers to all micrographs

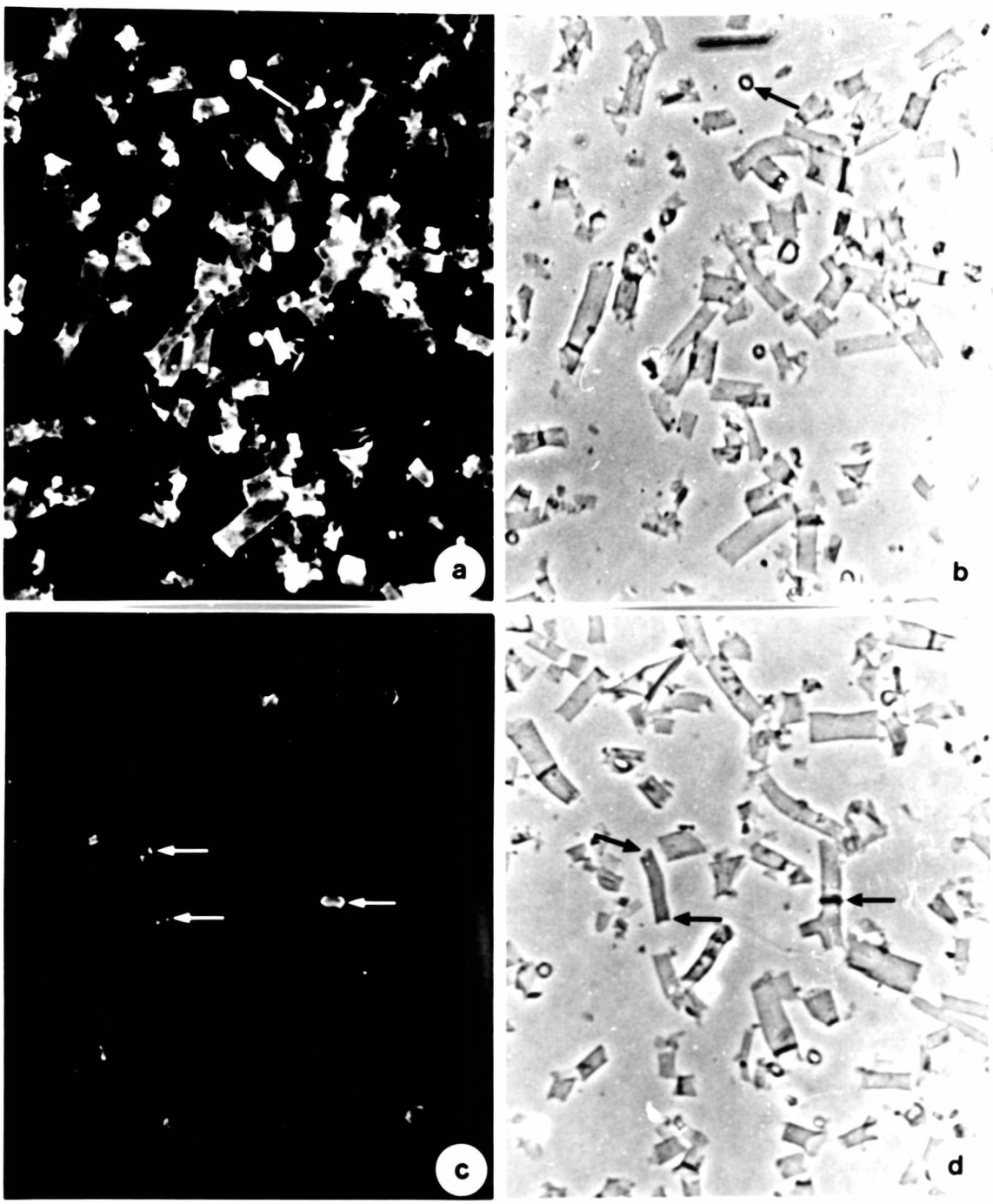

a
b
c
d

Fig. 3.9. The immunofluorescence pattern exhibited by *Paxillus involutus* cell wall fragments following reaction with the antiserum raised against *Paxillus involutus*.

(a) The fluorescence pattern with anti-*Paxillus involutus* antiserum at a 1/64 titre.

(b) Phase contrast of (a).

(c) The fluorescence pattern with anti-*Paxillus involutus* antiserum at a 1/128 titre.

(d) Phase contrast of (c).

Scale bar represents 5μm. Refers to all micrographs

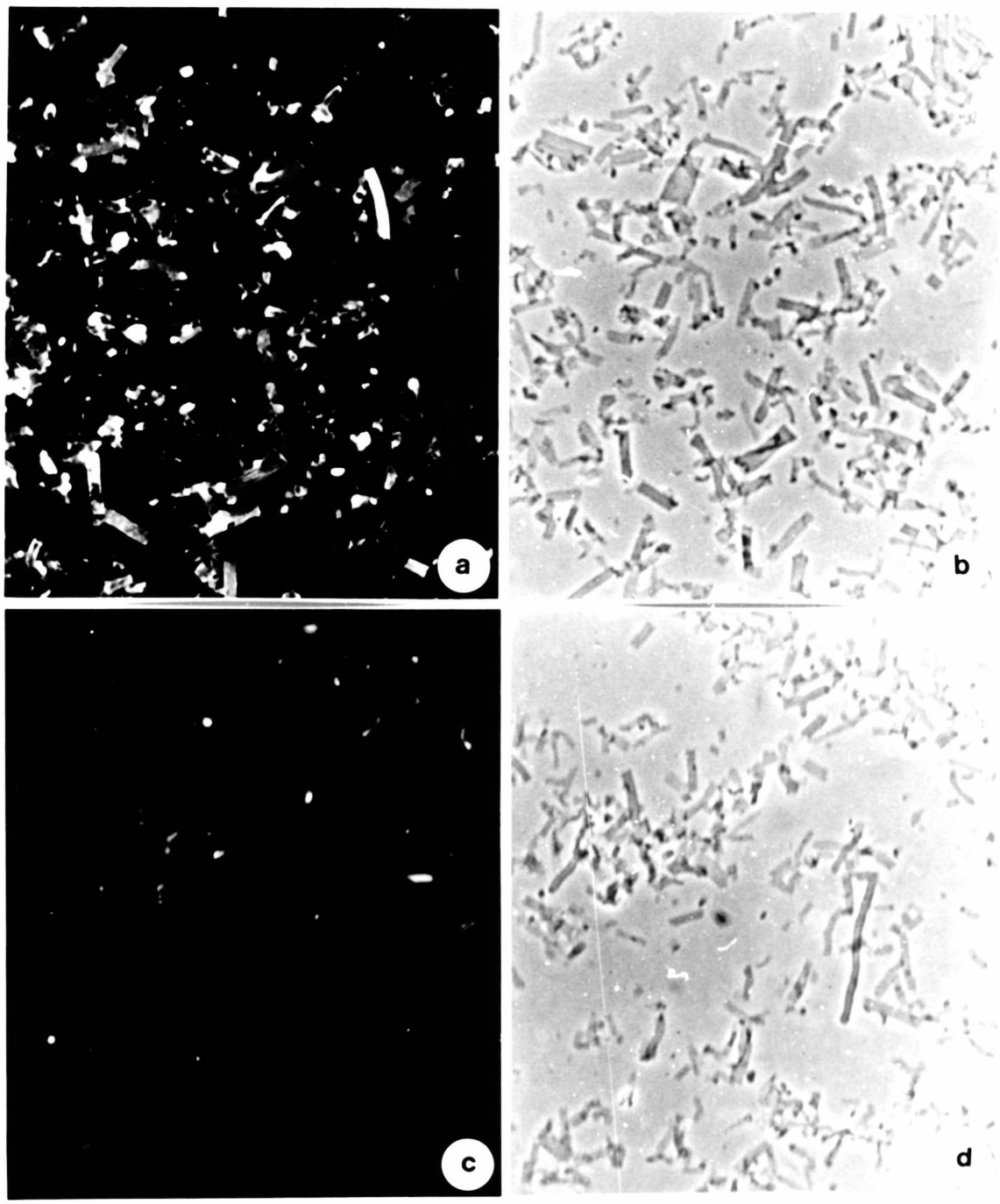

a
b
c
d

Fig. 3.10. The immunofluorescence pattern exhibited by *Neurospora crassa* and *Paxillus involutus* cell wall fragments following reaction with pre-immune antisera.

(a) The fluorescence pattern exhibited by *Neurospora crassa* cell wall fragments when reacted with pre-immune serum at a 1/2 titre.

(b) Phase contrast of (a).

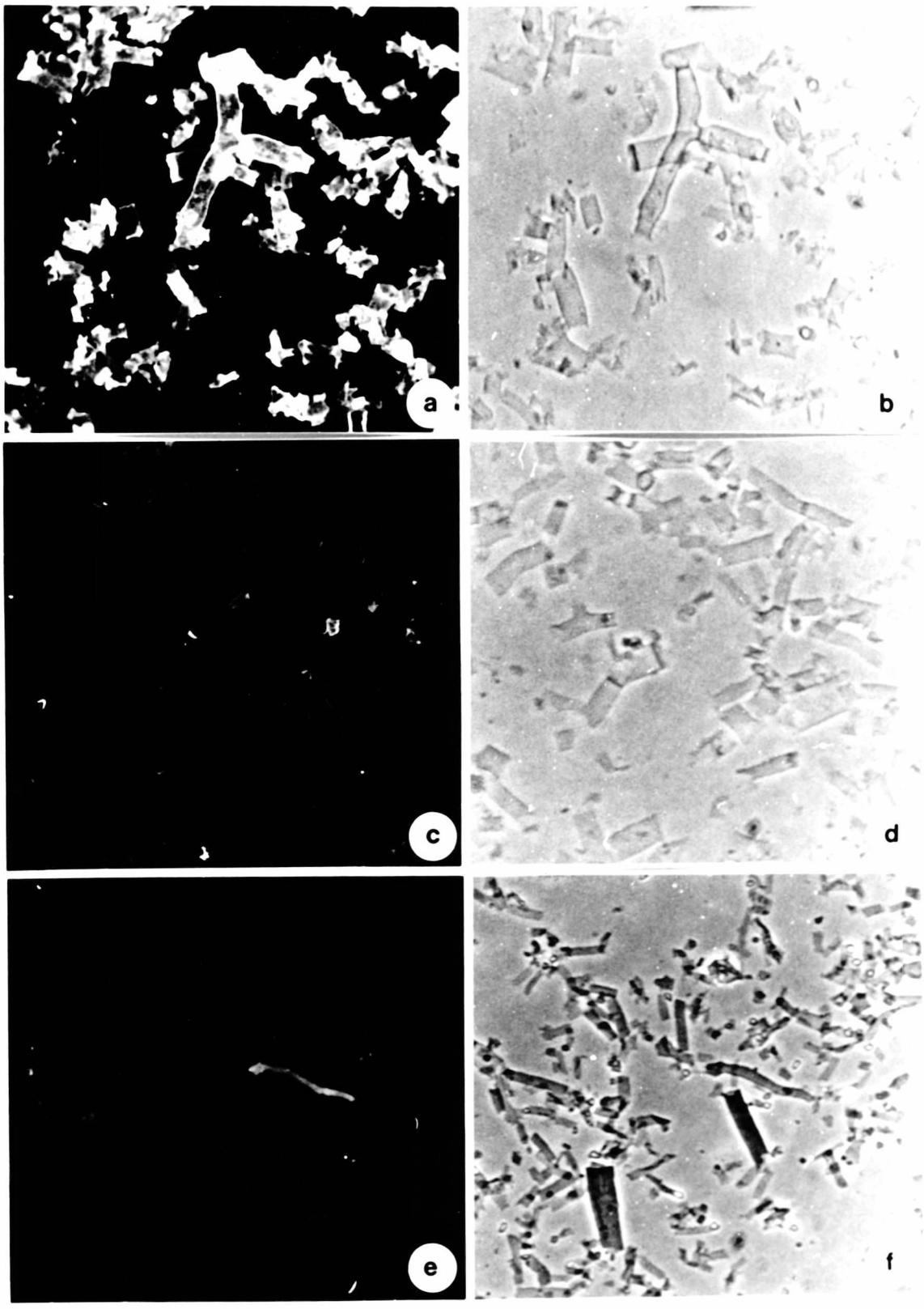
(c) The fluorescence pattern shown by *Neurospora crassa* cell wall fragments following reaction with pre-immune serum at a 1/8 titre.

(d) Phase contrast of (c).

(e) The fluorescence pattern shown by *Paxillus involutus* cell wall fragments when reacted with pre-immune serum at a 1/2 titre.

(f) Phase contrast of (e).

Scale bar represents 5μm. Refers to all micrographs


a
b
c
d
e
f

not uniform. Some fragments show fluorescence along their whole length particularly short fragments, whereas others fluoresce strongly at their ends but to a lesser degree along the rest of the fragment. Short fragments, which are end-on, show strong fluorescence of the wall cross-section (arrowed, Fig. 3.8c).

The pattern of fluorescence exhibited by anti-*P. involutus* antiserum on *P. involutus* cell wall fragments (Fig. 3.9) is similar to that of the anti-*N. crassa* antiserum on *N. crassa* cell wall fragments. No septa, however, were seen to exhibit fluorescence as clearly as those of *N. crassa* with the anti-*N. crassa* antiserum. The anti-*P. involutus* antiserum showed a higher specificity with its homologous antigen than anti-*N. crassa* antiserum with its homologous antigen, the anti-*P. involutus* antiserum reacted strongly with *P. involutus* cell walls at a titre of 1/64, and only at a 1/128 titre was very little fluorescence seen (Fig. 3.9b).

Pre-immune serum, from the rabbit which was subsequently immunised with *N. crassa* cell walls, showed a strong reaction in immunofluorescence tests with *N. crassa* cell walls at a 1/2 titre (Fig. 3.10a). At a 1/8 titre, however, the same pre-immune serum shown very little reaction with *N. crassa* cell walls (Fig. 3.10b). The antiserum from the rabbit injected with *N. crassa* cell walls, therefore, showed an increased specificity for *N. crassa* cell walls when compared with that of the serum before immunisation.

Pre-immune serum, from the rabbit subsequently injected with *P. involutus* cell walls, showed very little reaction with *P. involutus* cell walls even at a 1/2 titre (Fig. 3.10c). The antiserum from the rabbit immunised with *P. involutus* cell walls, therefore, showed an increased specificity for *P. involutus* cell walls than was shown prior to immunisation.

Both antisera showed relatively little cross-reaction with heterologous cell wall antigens when tested by immunofluorescence. When *N. crassa* cell wall fragments were reacted with the antiserum raised against *P. involutus* cell walls, very little fluorescence was seen with either antiserum at a 1/2 titre or antiserum at a 1/4 titre (Fig. 3.11). This level of

Fig. 3.11. The immunofluorescence pattern exhibited by *Neurospora crassa* cell wall fragments following reaction with the antiserum raised against *Paxillus involutus* cell walls.

(a) The fluorescence pattern with anti-*Paxillus involutus* antiserum at a 1/2 titre.

(b) Phase contrast of (a).

(c) The fluorescence pattern with anti-*Paxillus involutus* antiserum at a 1/4 titre.

(d) Phase contrast of (c).

Scale bar represents 5μm. Refers to all micrographs

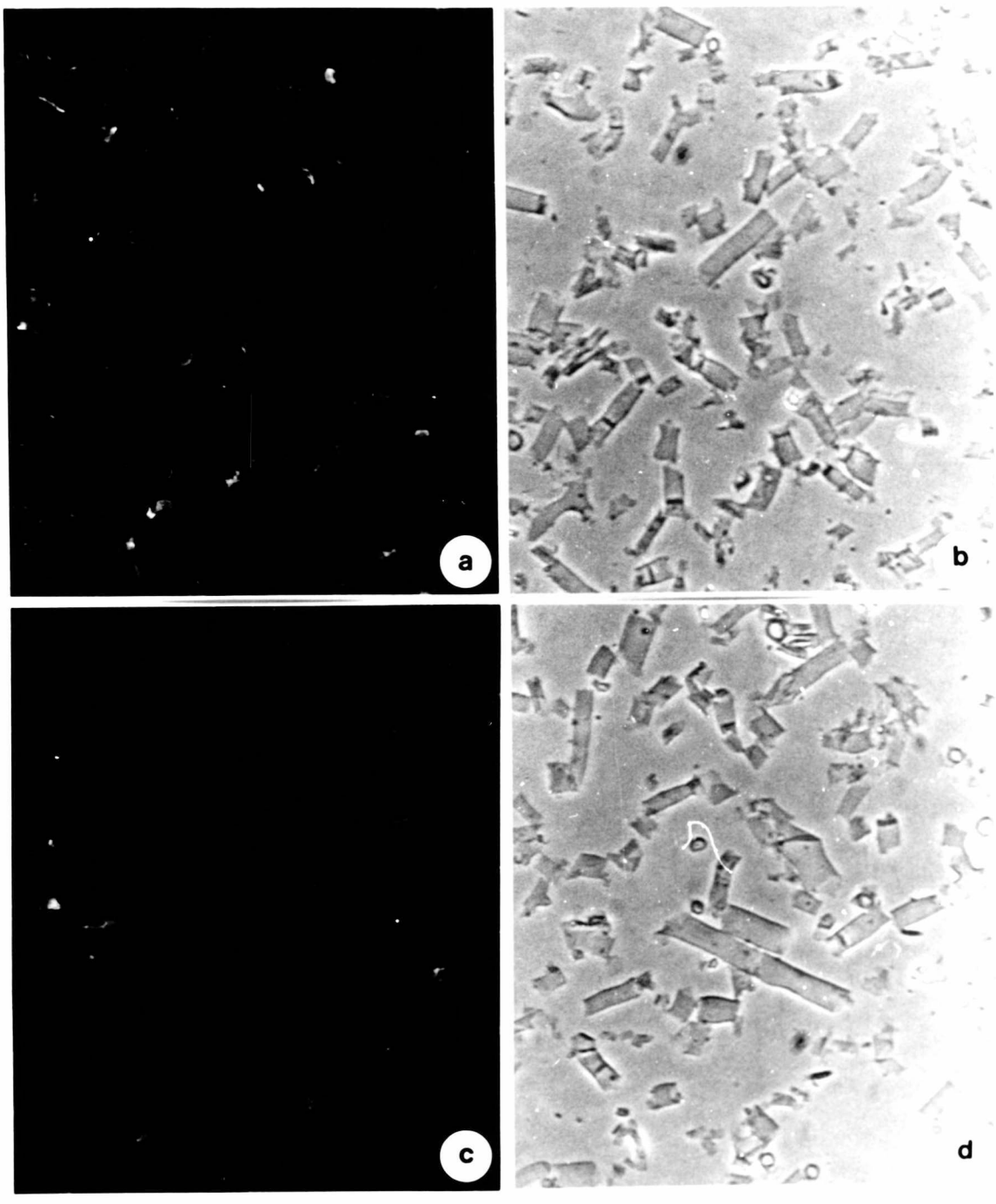

a
b
c
d

Fig. 3.12. The immunofluorescence pattern exhibited by *Paxillus involutus* cell wall fragments following reaction with the antiserum raised *Neurospora crassa*.

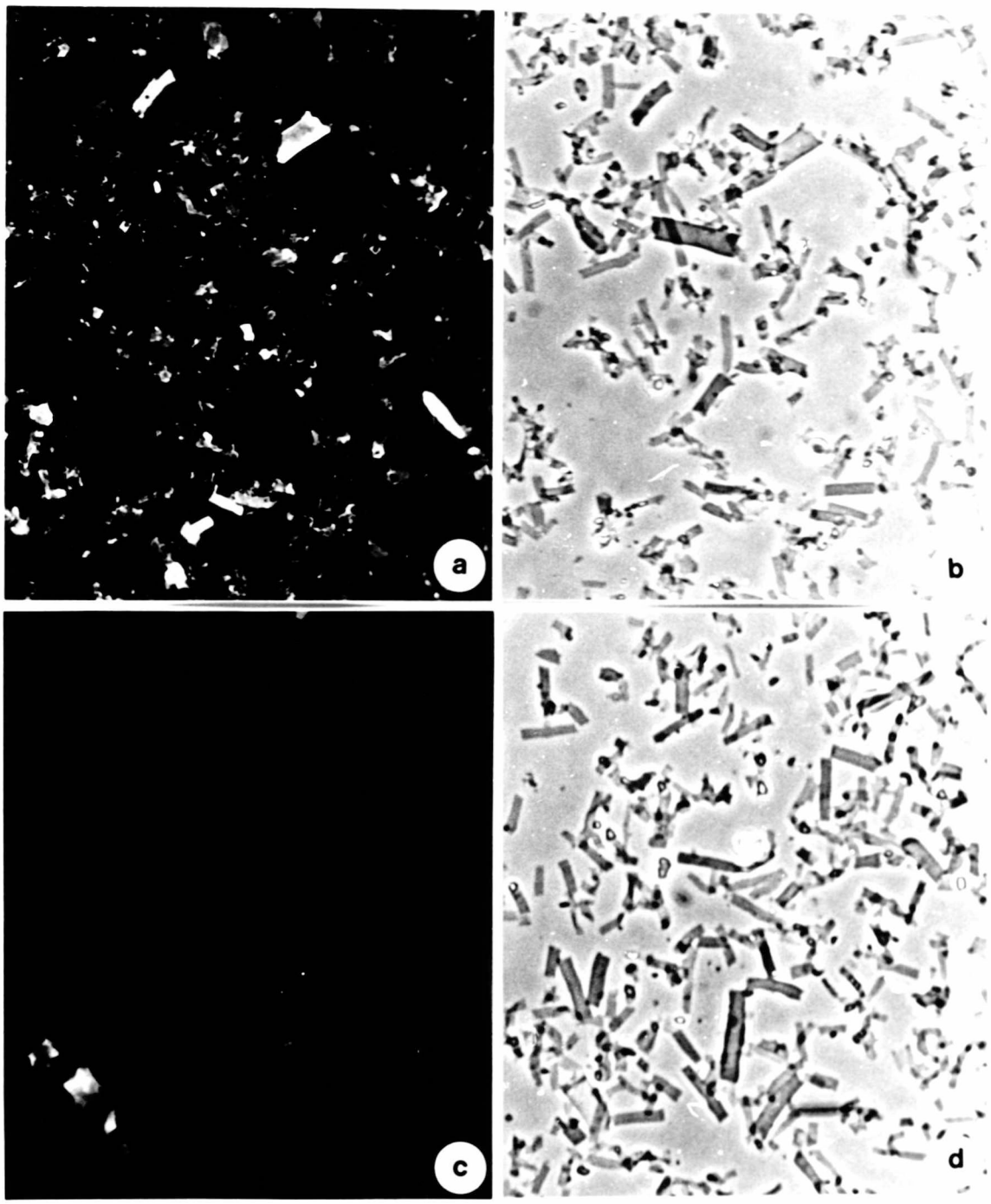
(a) The fluorescence pattern with anti-*Neurospora crassa* antiserum at a 1/4 titre.

(b) Phase contrast of (a).

(c) The fluorescence pattern with anti-*Neurospora crassa* antiserum at a 1/8 titre.

(d) Phase contrast of (c).

Scale bar represents 5μm. Refers to all micrographs


a
b
c
d

reaction was less than that of pre-immune serum with *N. crassa* cell walls. The antiserum raised against *N. crassa* cell walls reacted with *P. involutus* cell wall fragments at titres of 1/2 and 1/4. At 1/4 the fluorescence was quite strong (Fig. 3.12a), however, very little fluorescence was when the anti-*N. crassa* antiserum was diluted to 1/8 (Fig. 3.12b). This level of reaction was slightly higher than that of the pre-immune serum with *P. involutus* cell walls.

3.4.4. Electrophoretic studies and Western blotting.

3.4.4.1. Electrophoretic studies: 1-D PAGE.

Cell wall proteins of *N. crassa*, *P. involutus* and *Thelephora terrestris* were extracted using the different sample buffers and techniques outlined in 2.11.1. The extracted proteins were separated by 1-D PAGE.

Fig. 3.13 shows the patterns of proteins from *N. crassa* revealed by 1-D PAGE using Laemmli sample buffer, LSB, (Laemmli, 1970) following silver staining on 10, 12.5 and 15% polyacrylamide gels. A ladder of proteins from the cell walls of *N. crassa* is seen along the whole length of the gel lane (Fig. 3.13). The higher percentage acrylamide gels, 12.5 and 15%, were run to try to resolve the low molecular weight proteins. Some of these had molecular weights below 29Kd, the lowest molecular weight marker used. The two strongest bands occurred at estimated molecular weights of 49 and 30Kd, (Fig. 3.13). The proteins with molecular weight below 29Kd were also a relatively large component of the extracted proteins.

Treatments other than Laemmli sample buffer were carried out and the proteins extracted compared to those extracted with Laemmli sample buffer. Extraction of proteins using 1M NaOH followed by TCA precipitation and suspension in phosphate sample buffer (PSB) removed very few proteins from the isolated walls of *N. crassa*, similarly few proteins were extracted using 10M urea sample buffer (USB), (Fig. 3.14, lanes 1 and 2, respectively).

Fig. 3.13. Cell wall proteins from *Neurospora crassa* separated by 1-D PAGE in 10, 12.5 and 15% gels.

Notes: Molecular weights of markers are labelled adjacent to appropriate marker. The molecular weights are given in kilodaltons (Kd). The major protein bands from *Neurospora crassa* at approximately 49 and 30Kd are arrowed. To prepare the cell wall proteins 25mg (freeze-dried weight) of *N. crassa* cell wall material was treated with 1ml of 3x LSB. The loadings (in µl) are shown below. The loadings were the same for all three gels.

Lane no.	Loading (µl)
1	10
2	20
3	30
4	40
5	50
6	60
7	10
8	20
9	30
10	40
11	Molecular weight markers, 5µl

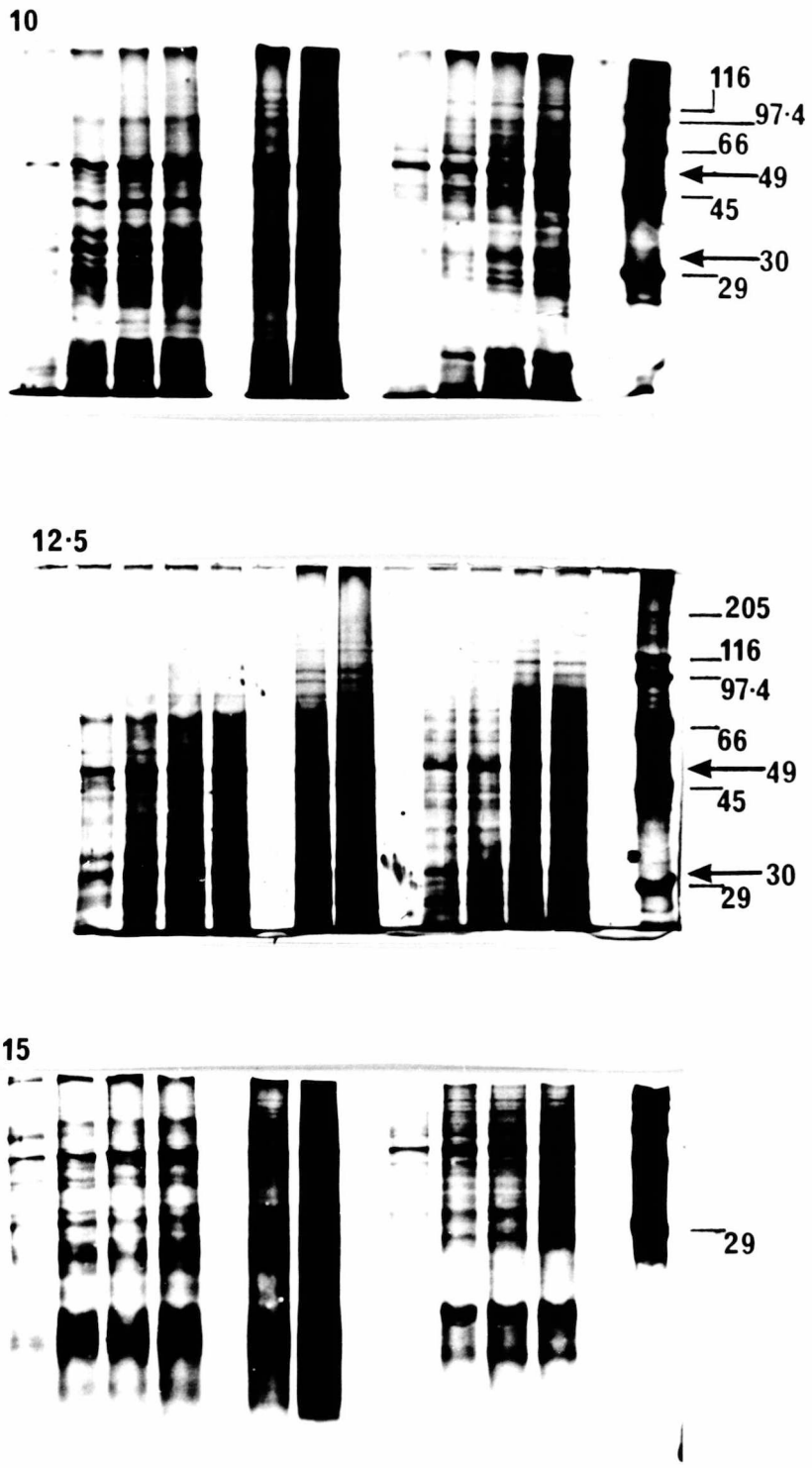
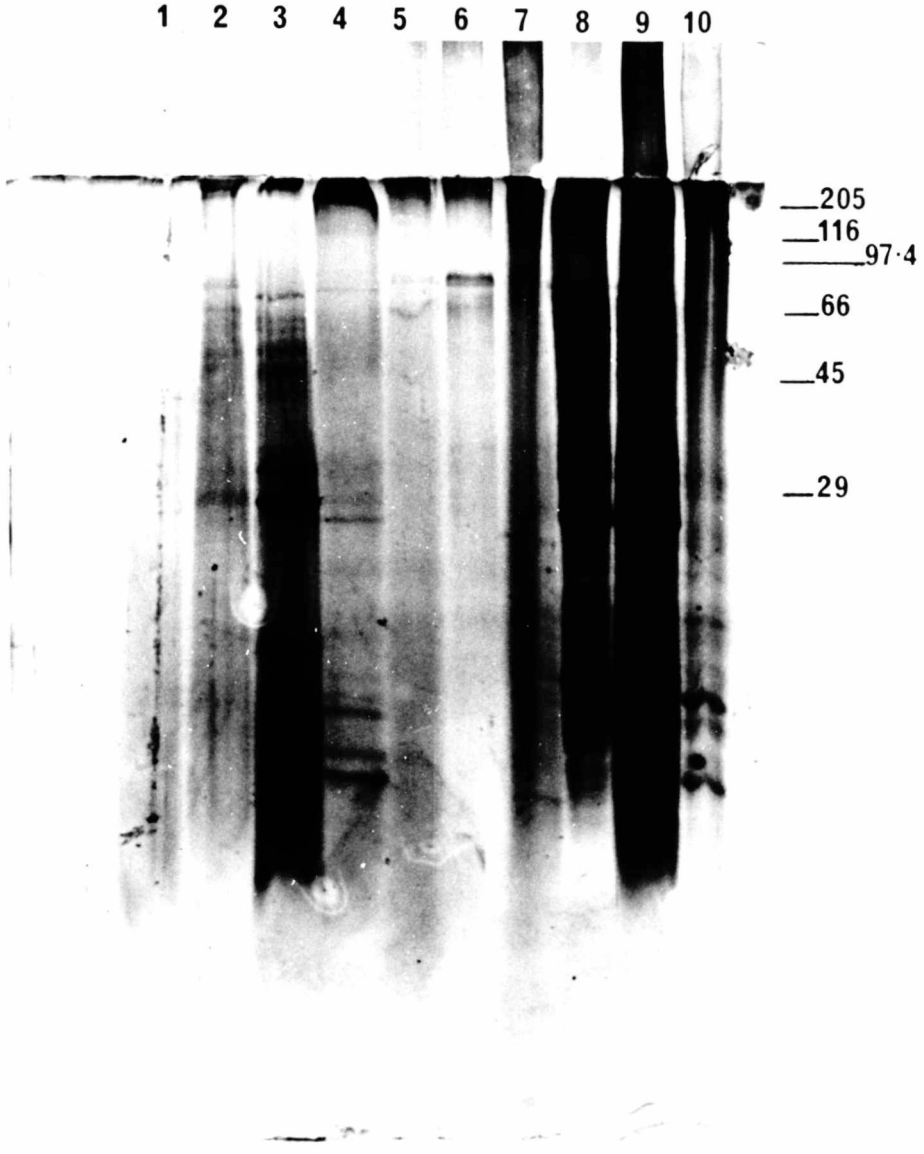

10
116
97·4
66
49
45
30
29
12·5
205
116
97·4
66
49
45
30
29
15
29

Fig. 3.14. Cell wall proteins from *Neurospora crassa, Paxillus involutus* and *Thelephora terrestris* extracted by different buffers separated by 1-D PAGE in a 15% gel.

Lane no.	Fungus	Method of extraction
1	*N. crassa*	1M NaOH and phosphate sample buffer (PSB)
2	*N. crassa*	0.5% SDS and PSB
3	*N. crassa*	Laemmli sample buffer (LSB)
4	*N. crassa*	Urea sample buffer, 10M (USB)
5	*P. involutus*	0.5% SDS and PSB
6	*P. involutus*	LSB
7	*P. involutus*	USB
8	*T. terrestris*	0.5% SDS and PSB
9	*T. terrestris*	LSB
10	*T. terrestris*	USB

Molecular weights, in kilodaltons, of markers (not shown) are labelled.


1
2
3
4
5
6
7
8
9
10
205
116
97·4
66
45
29

Extraction of proteins from *P. involutus* proved to be difficult, not only were very few proteins apparently extracted (Fig. 3.14, lanes 5, 6, and 7) by any of the treatments tried; but also the material that was extracted coloured the whole of the gel lane in a pale tan smear. This is best illustrated in Fig. 3.14, lane 7. It can also be seen that much of the extracted material did not enter the gel.

Proteins were more readily extracted from the cell walls of *T. terrestris* and both LSB and USB extracted a large number of proteins (Fig. 3.14, lanes 9 and 10, respectively). *T. terrestris* cell wall proteins were also extracted with 0.5% SDS, followed by TCA precipitation and treatment in PSB. More proteins were extracted from *T. terrestris* cell walls than from *N. crassa* cell walls (Fig. 3.14, compare lanes 1, 2, 3 and 4 with lanes 8, 9 and 10, respectively).

3.4.4.2. Western blotting.

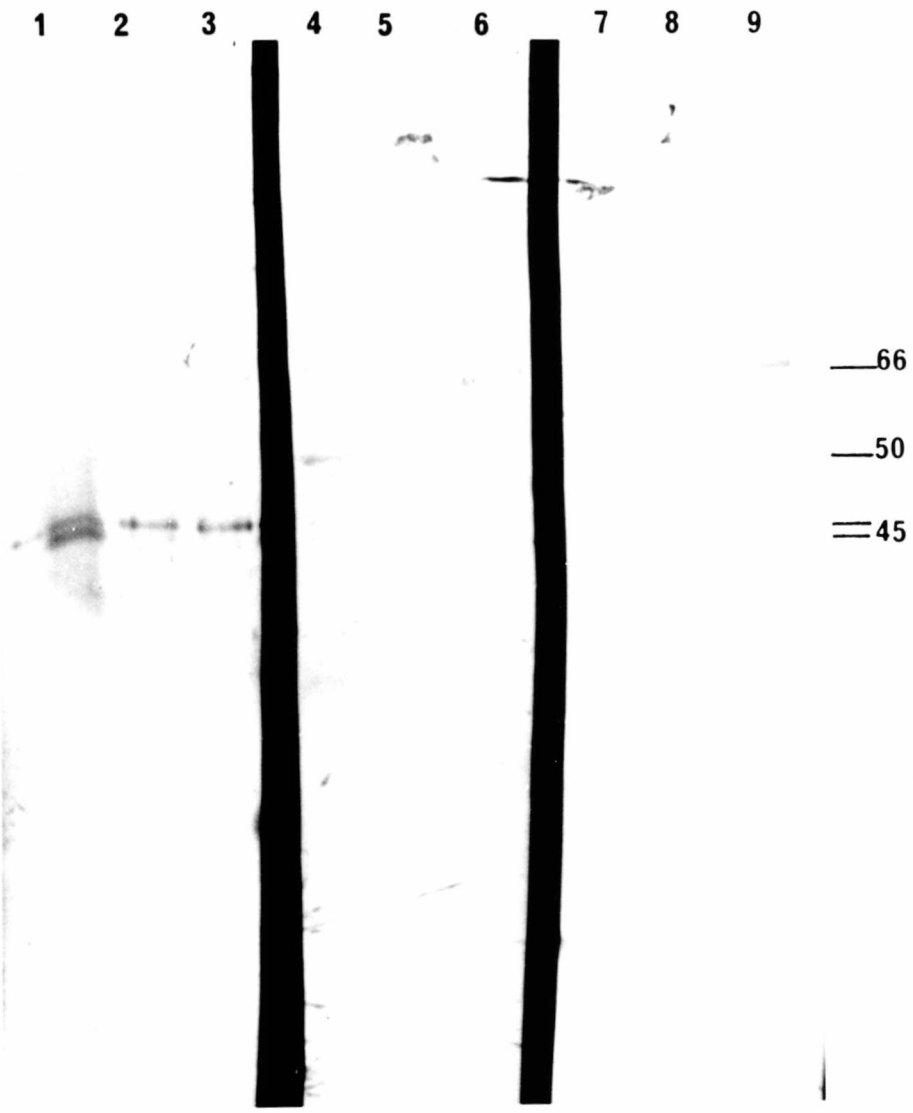
Anti-*N. crassa* antiserum reacted with proteins which had been extracted from *N. crassa* isolated cell walls and with proteins from *N. crassa* conidia (Fig. 3.15, lanes 1 and 2). The antiserum reacted with a doublet band of proteins, around 45Kd, in the cell wall extracts. The anti-*N. crassa* antiserum showed a similar intensity of reaction with both bands in the doublet on cell wall extracts, but reacted more strongly with the upper band of the conidial protein doublet. Some reaction with the molecular weight marker bovine albumin occurred with all three antisera (Fig. 3.15, lanes 3, 6 and 9). Anti-*N. crassa* antiserum also showed some cross-reaction with egg albumin (Fig. 3.15, lane 3).

Anti-*P. involutus* antiserum also reacted with *N. crassa* cell wall proteins. A weak reaction with a doublet band, at a similar molecular weight as that which reacted with anti-*N. crassa* antiserum, was seen (Fig. 3.15). The strongest reaction of anti-*P. involutus* with a *N. crassa* protein, however, was with a protein band, which had an estimated molecular weight of 50Kd from the blot. This band corresponded in estimated molecular weight to the strong

Fig. 3.15. Western blot showing the reaction of *Neurospora crassa* cell wall proteins with three antisera, anti-*N. crassa*, anti-*Paxillus involutus* and pre-immune.

Lane no.	Sample	Antiserum
1	*N. crassa* hyphal wall proteins	Anti-*N. crassa*
2	*N. crassa* conidial surface proteins	Anti-*N. crassa*
3	Molecular weight markers	Anti-*N. crassa*
4	*N. crassa* hyphal wall proteins	Anti-*P. involutus*
5	*N. crassa* conidial surface proteins	Anti-*P. involutus*
6	Molecular weight markers	Anti-*P. involutus*
7	*N. crassa* hyphal wall proteins	Pre-immune
8	*N. crassa* conidial surface proteins	Pre-immune
9	Molecular weight markers	Pre-immune

Notes: All three antisera reacted with the molecular weight marker, bovine albumin (indicated at 66Kd). Anti-*N. crassa* showed a strong reaction with a doublet band at 45kd (labelled), lanes 1 and 2. Anti-*P. involutus* reacted with a hyphal wall protein of approximately 50Kd (labelled), lane 4. Anti-*N. crassa* also reacted wih the marker, egg albumin (M.W. 45Kd).


1
2
3
4
5
6
7
8
9
66
50
45

band seen on the gels (Fig. 3.13), which had an estimated molecular weight of 49Kd.

The smearing seen on the blots in the cell wall extract lanes with both antisera may have been due to either binding of other antibodies in the antisera to these other cell wall proteins, or, since it occurred on the blot reacted with pre-immune serum, to non-specific binding of either first or second antibody molecules. Very litle reaction between *N. crassa* cell wall proteins and pre-immune serum was observed (Fig. 3.15, lanes 7, 8 and 9). All three antisera, however, reacted with bovine serum albumin.

3.5 Discussion.

3.5.1. Development of an indirect non-competitive ELISA.

The optimum conditions for consistent repetition of an indirect non-competitive ELISA with isolated fungal cell walls as the antigen were determined. Indirect non-competitive ELISA is the most commonly used ELISA technique for the screening of hybridoma supernatants. Usually the antigen in the indirect non-competitive ELISA is soluble and is bound to the plate by non-covalent binding following coating of the plate with a carbonate buffer solution containing the antigen. The indirect non-competitive ELISA technique has been used successfully in the screening of hybridoma supernatants for specificity to soluble antigens from fungi (Wright *et al.*, 1987; Wycoff *et al.*, 1987; Dewey *et al.*, 1988, 1989). The use of non-soluble antigens in this type of ELISA has been less frequent. Hardham *et al.* (1985), for example, used glutaraldehyde to fix zoospores and cysts of *Phytophtora cinnamomi* to ELISA plates as did Banowetz *et al.* (1984) to fix teliospores of two species of *Tilletia*. The direct binding of cell wall fragments using drying overnight at 37°C is a more simple technique and omits any non-specific binding of antibody by glutaraldehyde.

The high background absorbances due to non-specific binding of antibodies, especially noticeable at high concentrations of pre-immune antiserum, were significantly reduced by

the blocking and washing procedures adopted here following comparison of different blocking and washing regimes.

The ELISA protocol devised in this investigation was suitable for determination of end-point titres of the polyclonal antiserum and for discrimination of non-specific supernatants from hybridoma cell lines.

The determination of a threshold value for discriminating between hybridoma supernatants which contain specific antibodies and those which do not is very important in the screening of fusion products. The choice of a threshold value of the mean of the background absorbance when non-specific MAbs were the first antibody plus four standard deviations of that mean was based on the conclusion of Tijssen (1985) that 99% of negatives should be below this value. The work of Tijssen (1985) was based on the findings from screening polyclonal antisera, but this threshold value can be used to discriminate any antibodies from one another. Other threshold values that have been used for screening antibodies with specific activity against fungi have included three times the mean of the tissue culture fluid control absorbance (Dewey *et al.*, 1988; 1989a and b). Other workers have not disclosed the threshold values they used in ELISA screens of hybridoma supernatants.

If three times the mean absorbance of the tissue culture supernatant (3x SDMEM = 0.45) had been chosen as the threshold value, BS7 would have been chosen as a positive cell line with specific activity against *N. crassa*. Reaction between *P. involutus* and BS7 gave an absorbance reading of 0.39. This was the same value as three times the mean absorbance of the tissue culture supernatant. Other threshold values which would have excluded the inclusion of BS7 from positive selection included the mean of the non-specific MAb background plus three standard deviations of that mean and three times the mean of the non-specific MAb background. Any of the threshold values described here that were based

on the mean of the background from the non-specific MAbs excluded all of the non-specific MAbs which were screened. Any of the threshold values calculated in the same way, but based on the background readings from the tissue culture medium did not exclude all of the non-specific MAbs. A threshold value of three times the mean of the tissue culture supernatant, however, did exclude all of the non-specific MAbs except BS7. These findings are summarised in Table 3.12.

In retrospect a threshold value based on three times the mean of the background of non-specific MAbs would have proved just as useful as the threshold value for discriminating negatives for the non-specific MAbs which were screened. This value may also have proved to be a better threshold value than the mean plus four standard deviations if the standard deviation had been a smaller value, as was in the case with the tissue culture medium controls. Based on the results from the the tissue medium the threshold value of three times the mean would have been 0.45 and 0.39 for *N. crassa* and *P. involutus*, whereas threshold values based on the mean of this absorbance plus four standard deviations of the mean would have been 0.19 for *N. crassa* and 0.21 for *P. involutus*. These latter threshold values would have led to more false positives than the use of three times the mean.

From this small study the threshold value was shown to exclude all non-specific MAbs screened when the threshold value was based on the background absorbances recorded when non-specific, control, MAbs were used in the ELISA as the first antibody. Any of the three methods used for calculating the threshold value, *i.e.* the mean plus four standard deviations, the mean plus three standard deviations or three times the mean, proved able to distinguish all of the control antibodies as having no specific reaction against the cell walls. Threshold values calculated by these same methods but based on the background absorbances when tissue culture medium was used as the equivalent of first antibody, did not distinguish the control MAbs as having no specific reaction against the cell wall in all cases. Dewey *et al.*

	Antigen	
	N. crassa	*P. involutus*
3x TCS mean	0.45 (+)	0.39 (±)
TCS mean + 3xS.D.	0.18 (+)	0.19 (+)
TCS mean + 4xS.D.	0.19 (+)	0.21 (+)
3x MAb mean	0.84 (-)	0.69 (-)
MAb mean + 3xS.D.	0.73 (-)	0.44 (-)
MAb mean + 4xS.D.	0.88 (-)	0.51 (-)

Table 3.12. A summary of the threshold values calculated from the background absorbance readings in the ELISA (see Table 3.9) and the relationship of these to the selection of positive cell lines.

Notes:

TCS: tissue culture supernatant background reading

MAb: background reading form nine non-specific MAbs

(+), (-) refer to selection of BS7 as a positive or negative cell line with reference to the calculated threshold values.

The absorbance readings for BS7 in reaction with *N. crassa* and *P. involutus* were 0.66 and 0.39, respectively.

(1989a and b) used a threshold value based on three times the tissue culture supernatant, and produced Mabs with relatively high specificities for the desired fungi, *Humicola lanuginosa* and *Ophiostoma ulmi*. The data here suggest that this threshold value will not exclude antibodies with relatively strong non-specific cross-reactions and should not be relied upon. In work where the threshold value is not given but two antibodies with a similar absorbance reading are defined as negative in one case and positive in another the absence of the threshold value brings into question the specificity of the reaction. Fox & Hahne (1988), as an example, claimed they were able to separate isolates of *Armillaria mellea* using a panel of MAbs, but the absorbances quoted did not vary significantly between the isolates, and the values were very low (<0.2 in some cases).

3.5.2. The polyclonal antisera.

The polyclonal antisera raised against *N. crassa* and *P. involutus* both reacted against their respective fungi. They proved very useful in the development of the ELISA as discussed previously in Section 3.5.1 and for testing the immunofluorescence technique described in Section 2.8.

They both, however, showed reactivity with heterologous fungi in ELISA screens, even when they were pre-absorbed with heterologous antigens before reaction. Cross-reactivity with heterologous fungi has been a well documented phenomenon of polyclonal antisera raised against fungi (El-Nashaar *et al.*, 1986; Chard *et al.*, 1983, 1985b; Barker & Pitt, 1988). The use of cell wall material as the immunogen has been found by some workers to produce antisera with fewer cross-reactions (El-Nashaar *et al.*, 1986; Kough *et al.*, 1983; Fitzell *et al.*, 1980). Conversely, other workers (Gerik *et al.*, 1987; Aldwell *et al.*, 1983, 1985) have found that the use of cytoplasmic fractions as immunogens produced more specific antisera. There is, therefore, no fungal immunogen that has been shown to enable species-specific antisera to be readily produced. The polyclonal antisera raised in this

project, using cell wall material as the immunogen, were not species-specific. Having shown that the antisera cross-react with species relatively distantly related taxonomically, a study of the pattern of cross-reactivity shown by the antisera with an extensive cross-section of fungal species was not undertaken, since the value of yet another discussion of the cross-reactivity of polyclonal antisera with heterologous fungi is of limited value if no variation from the protocols of other workers has been tried.

The level of cross-reactivity of the antisera with heterologous fungi varied depending on the method used for testing the antibody-antigen reaction. The antisera were shown to have a higher level of cross-reactivity with heterologous antisera as judged by the end-point titres from antibody dose-response curves established by ELISA, than by immunofluorescence. When tested by ELISA the antiserum raised against *N. crassa* showed only a two-fold greater level of reactivity against its homologous antigen, *N. crassa*, than against *P. involutus*, an heterologous antigen, comparing the end-point titres. In immunofluorescence the anti-*N. crassa* antiserum reacted eight times more strongly with *N. crassa* than with *P. involutus*, comparing the last titres at which the fluorescence was above that seen with pre-immune serum. Anti-*P. involutus* antiserum showed a 32-fold stronger reaction with *P. involutus* than with *N. crassa* in immunofluorescence tests as compared to only a six-fold greater reaction in the ELISA.

Antisera raised against fungi have been previously shown to exhibit different levels of cross-reaction in different types of antibody-antigen testing techniques. Chard *et al* (1985b) found that an antiserum that showed few cross-reactions with heterologous fungi using an immunodiffusion technique showed more cross-reactions when the heterologous reactions were tested using immunofluorescence.

Pre-absorption of the antisera with heterologous antigens did not lessen the level of cross-reaction observed with heterologous antigens. In the case of anti-*P. involutus*

antiserum absorbed with *N. crassa* cell wall fragments the end-point titre was reduced. Different degrees of success with pre-absorption have been reported. On the one hand, Iannelli *et al.* (1982) were able to remove cross-reactions of antisera raised against different formae speciales of *Fusarium* to produce specific antisera. Similarly, Polonelli *et al.* (1988) raised an antiserum which specifically recognised *Rhizopus microsporus* following pre-absorption with other closely related *Rhizopus* spp.. Other workers have found that pre-absorption failed to increase antiserum specificity (Schmidt *et al.*, 1974; Dewey *et al.*, 1984 and Chard *et al.*, 1985b). Gerik *et al.* (1984) were able to increase specificity of the antiserum raised against *Verticillium dahliae*, but the antibody titre was considerably reduced.

P. involutus and *Pisolithus tinctorius* both belong to the Agaricales, an order of the Basidiomycotina. One of the original aims of this project was to use immunological reagents to distinguish between closely related fungi living in such close proximity to one another. In axenic culture the two fungi produce different colonial growths on PDA and MMN media, but the individual hyphae have no distinguishing characteristics. The finding that an antiserum raised against *P. involutus* also cross-reacts with *Pisolithus tinctorius* is not surprising, and the failure of pre-absorption to remove any of the cross-reaction between anti-*P. involutus* antiserum and *Pisolithus tinctorius* highlights the problem faced when using a complex of antigens as the immunogen. The cell walls of *P. involutus* and *Pisolithus tinctorius* have not been the subjects of detail study, but they may both be considered to contain chitin and glucans, the characteristic, and major, components of the walls of fungi in the Basidiomycotina (Bartnicki-Garcia, 1968). The major wall components of *P. involutus* and *Pis. tinctorius* appear to be very similar, as shown by the failure of pre-absorption to decrease significantly the level of cross-reaction between anti-*P. involutus* antiserum and *Pisolithus tinctorius*.

The use of polyclonal antisera to identify cell wall proteins that were not shared by other fungi was investigated. The extraction of proteins from the cell walls of *N. crassa* revealed a variety of proteins varying in molecular weight from 205Kd to less than 29Kd. The protein content of *N. crassa* cell walls has been estimated to be 8.6% by weight (Wrathall & Tatum, 1973); therefore the proteins form a small minority of the wall components. They were estimated in this project make up 10% of dry weight of isolated cell walls of *N. crassa*. The inclusion of sodium dodecyl sulphate (SDS) in the extraction buffers, however, can interfere with the Lowry protein assay, and although allowance was made for this, this value must be taken as an estimate only.

The extraction of proteins from *P. involutus* cell walls was not achieved consistently. This suggests different binding of the proteins in the wall and it has been found in *S. commune* (Wessels & Sietsma, 1981) that protein is tightly bound to carbohydrate components in the wall and is, therefore, difficult to separate by conventional protein extraction techniques. The melanin, or other pigment, which was extracted, shown by the colouring of the gel lanes, may also have contributed to the difficulties experienced in extracting proteins from *P. involutus*. It was, however, possible to extract and resolve proteins from the ectomycorrhizal fungus, *T. terrestris*, which also has pigmented hyphal walls. Proteins from *T. terrestris* cell walls were extracted using LSB, USB and 0.5% SDS.

The difficulties experienced in the extraction of proteins from *P. involutus* meant that it was not possible to compare the reactions of the two antisera with homologous and heterologous antigens, but the reaction of the antisera with *N. crassa* proteins showed that majority of the antibodies present in the antisera do not react with protein epitopes. Given the nature of the cell wall and the small percentage of the cell wall components that are proteins this was perhaps not surprising.

The reaction of the two antisera with proteins from *N. crassa* corroborates the cross-reactivity of both antisera with *N. crassa* cell walls shown by ELISA. The reaction of the two antisera with different proteins highlights the probability that antisera, raised against complex fungal cell wall antigens, will react with heterologous fungi. The two antisera reacted with different *N. crassa* wall proteins. The reaction of anti-*P. involutus* antiserum with a *N. crassa* protein, which anti-*N. crassa* antiserum did not recognise, suggests that similar proteins may be antigenic in one type of fungal cell wall but not another. Studies of medically important fungi have revealed that different components have different antigenic properties (Sundstrom & Kenny, 1984; Sundstrom *et al.*, 1988; Matthews *et al.*, 1987). The finding that a similar protein was antigenic in one fungus but not in another has not been reported before, It is perhaps very important to bear this finding in mind if a fungal antigens are to be screened for species-specificity; as an example, a polyclonal antiserum can reveal which fungal components are antigenic but it cannot be used alone to find a species-specific component. A comparison of the fungal components from a wide range of fungi must be made to find such a component.

Differences in protein patterns between closely related fungi have been shown to exist and have been used to differentiate fungi (Bielenin *et al.*, 1988; Kawchuk *et al*, 1988; Tariq *et al.*, 1985), however, in these cases the proteins have been cytoplasmic proteins. It was also not shown whether the different proteins were specific to one fungus, or whether they occurred in fungi outside the narrow taxonomic range of fungi screened, although they allowed differentiation of the fungi within the closely related groups. It is possible that an antibody which recognised such a discriminatory protein may show that the protein is not present in the closely related fungi, but is present in distantly related fungi.

An immunisation regime that used a purified and unique protein may, however, enable a species-specific antiserum to be raised. In event of such a protein, or other type of antigen,

being found, the production of a MAb would offer advantages over a polyclonal antiserum because an indefinite and constant supply of species-specific antibody would be achieved. Chard *et al.* (1985a) found a precipitin line (Line A) on immunodiffusion gels which was specific to *Mycena galopus*. The antiserum raised against "Line A" material did show less cross-reactivity than other antisera raised against *M. galopus*. Other antisera raised against fungi, which have been reported to have the least cross-reactivity, have been those raised against other single fungal components. For example, Wood (1979) found that only two other *Agaricus* spp. reacted with an antiserum raised against laccase from *Agaricus bisporus*. Similarly, Benhamou *et al.* (1985) produced a MAb, derived from a mouse injected with a glycoprotein toxin from *O. ulmi*, which only reacted with this toxin and could be used to follow the toxin during Dutch elm infections of seedlings. It may, therefore, be possible to use such an approach; however, the choice of the fungi with which to compare the proteins from the test fungus would need to be considered carefully.

The study of the polyclonal antisera raised against *N. crassa* and *P. involutus* highlighted the most common problem found with antisera raised against a complex fungal antigen, such as a cell wall, namely cross-reactivity with other fungal species. The attempt to identify immuno-reactive species-specific proteins showed that the cell wall proteins are not the immunodominant components of the cell wall. To raise an antiserum which specifically recognise the proteins, a protein extract would need to be used for immunisations to avoid the immunodominance of other wall components. An antiserum raised in such a way could then be used to identify the antigenic proteins and compared with data from gel electrophoresis to find a species-specific protein.

The immunodominance of antigens common to both *N. crassa* and *P. involutus* highlights the difficulty of using complex antigens to raise specific antisera. MAb production offers an alternative approach which might overcome this problem if careful screening of the antibodies produced by the hybridomas was carried out. Progress using this approach is discussed in the following chapters.

Chapter Four

Production of monoclonal antibodies which recognise fungi.

4.1. Introduction.

In this Chapter the development of methods used for the production of the four MAb cell lines described in Chapters 5, 6, 7 and 8 is described and discussed.

4.2. Development of an immunisation schedule.

Initial immunisations with 200μg (freeze-dried weight) of mycelial homogenate from *N. crassa* were made. Following five intraperitoneal (i.p.) injections, at five day intervals, 100μl aliquots of blood were taken from three mice by bleeding from the tail vein. Dilutions of the plasma prepared were screened for their reaction with *N. crassa* cell walls using the immunofluorescence technique. The intensity of fluorescence of the cell walls at a 1/10 dilution was no greater when immune sera were used as the first antibody than when the corresponding dilution of pre-immune serum was used. Only this set of mouse tailbleeds were screened by immunofluorescence as the parameters in the ELISA were being optimised at this time.

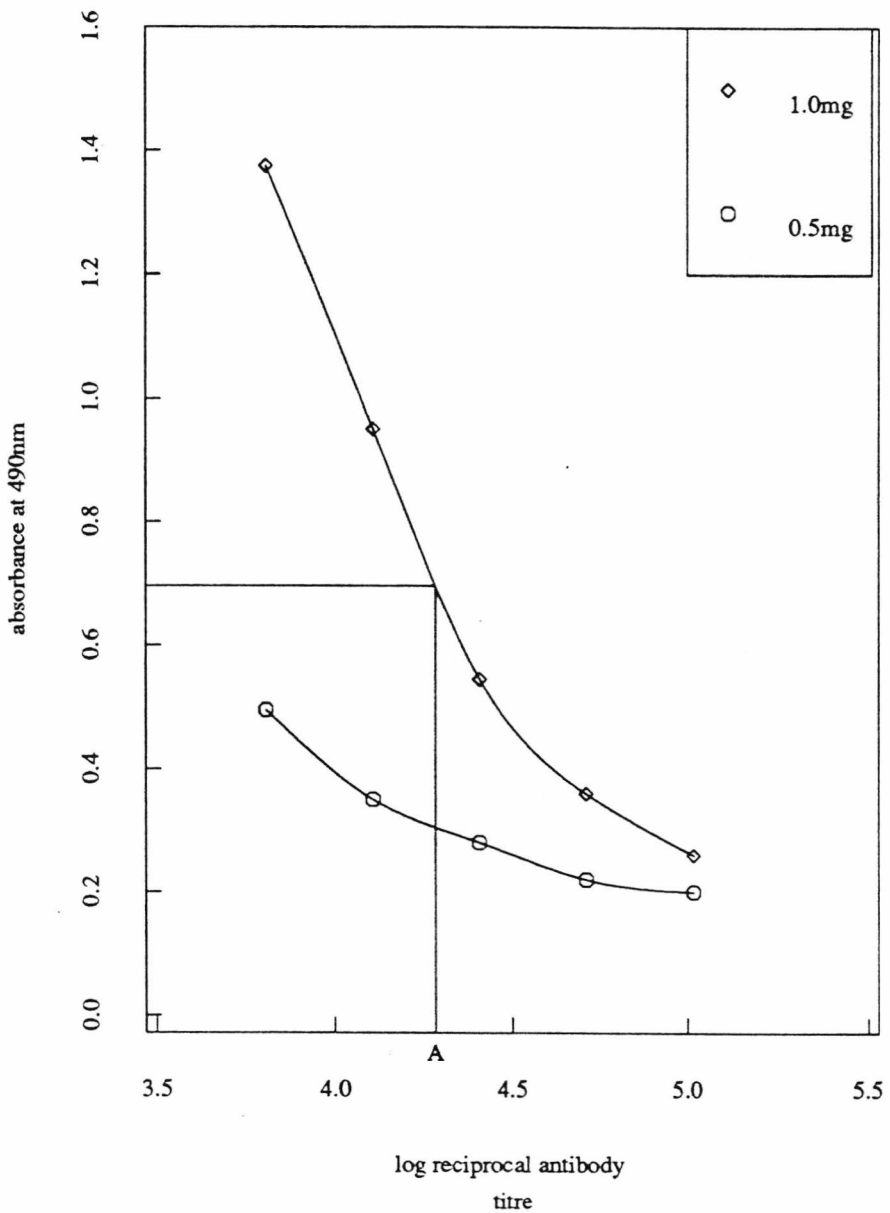
To overcome this problem, and to determine the appropriate level of immunogen to be used, five further sets of Balb/c mice were given i.p. injections with 10, 5, 2.5, 1 and 0.5mg (freeze-dried weight) *N. crassa* cell walls respectively. Two sets of four mice were injected with 10mg and 5mg doses, respectively, and three sets of three mice were injected with 2.5,

1 and 0.5mg doses, respectively. Prior to injection the cell walls were suspended in sdH_2O and sonicated to produce a homogenous suspension.

The higher concentrations of cell walls, 10, 5 and 2.5mg, proved fatal for one mouse in each set of mice injected with these concentrations. For subsequent injections the nine remaining mice in these three sets were given 1mg doses. These and the mice in the other sets were immunised on four further occasions with 1 or 0.5mg doses as appropriate. The timing of the injection was such that each mouse was given one injections of the appropriate dose on days 1, 7, 14, 28 and 49, five injections in total. All of the mice (14) were bled from the tail vein on day 59, 10 days after the fifth injection. Plasma from each mouse was tested for its reaction with *N. crassa* cell walls using the ELISA protocol outlined in 3.3.4. This test of the tailbleed titre compared 1mg and 0.5mg doses of *N. crassa* cell walls. The 1mg dose produced a much higher end-point titre than the 0.5mg dose (Fig. 4.1). The mouse plasmas compared in Fig. 4.1 were from mice in the original 1mg and 0.5 mg sets of mice. The end-point titre for the mouse injected with 1mg doses of *N. crassa* cell walls was 1/18,000, whereas that for the 0.5mg mouse was below 1/6,400. The two other mice in the 1mg dose set also had tailbleeds with end-point titres above 1/12,800 and the other 0.5mg mice had titres below 1/6,400. Plasma from mice in the original 10, 5 and 2.5mg sets also showed end-point titres in excess of 1/10,000, one had an end-point titre estimated to be 1/39,000.

Injections of 1mg were subsequently used for all injections, with approximately 14 days between each. A booster injection (1mg) was given four days before a mouse was sacrificed for fusion. No increase in the amount of immunogen given in a booster injection was made because of the traumatic effect observed in mice that were given doses higher than 1mg.

Fig. 4.1. The antibody titration curves for two polyclonal antisera, raised in mice against *N. crassa*, reacted with *N. crassa* cell wall antigens.



Two Balb/c mice were immunised with *N. crassa* cell walls, one was given 1.0mg (freeze-dried weight) injections of cell walls and the other 0.05mg injections. A indicates the end-point titre (1/19,180) for the antiserum raised against 1.0mg injections of *N. crassa* cell walls. The end-point of the antiserum raised against 0.05mg injections of *N. crassa* cell walls occurs at a titre lower than tested in the ELISA (1/6400).

4.3. Fusions.

In an initial fusion, a suspension of spleen cells was divided into two equal aliquots, one aliquot was fused 5:1 with Sp2.0 myeloma cells and the other 5:1 with X63/Ag-8.653 cells. The spleen cells were obtained from a Balb/c mouse injected with *N. crassa* cell walls. The end-point titre of plasma from this mouse was 1/39,000. Two sets of 476 wells were each seeded with one of the fusion mixtures. Hybridomas grew in the majority of wells (90%). Supernatants from all of the wells were screened by ELISA, including supernatants from the four control wells, two seeded with Sp2.0 cells only and two with spleen cells only. The supernatant was tested when the clumps of hybridoma cells could be seen macroscopically. This occurred approximately 10-14 days after fusion.

53 supernatants from the Sp2.0 fusion showed a positive reaction with *N. crassa* cell walls in the ELISA. The threshold value of 0.88 was used in all screens (see 3.5.1 for discussion). 31 supernatants from the X63/Ag8.653 fusion showed a positive reaction. These 84 positives were tested by immunofluorescence with *N. crassa* cell walls. Six of the supernatants from the Sp2.0 fusion showed bright fluorescence of *N. crassa* cell walls and two from the X63/Ag8.653 fusion.

Only one of these positive hybridoma cell lines was successfully cloned. This hybridoma cell line was from the fusion with Sp2.0 myeloma cells. When tested against conidia of *N. crassa* by immunofluorescence showed a characteristic fluorescence pattern at the ends of conidia. The loss of hybridomas at this stage is not uncommon and although all the reasons are not known (Campbell, 1985), genetic instability probably plays a major role in hybridoma loss.

Fewer positive hybridomas were found in the fusion in which X63/Ag8.653 myeloma cells were used, thus it was deemed that Sp2.0 myeloma cells were the better fusion partners by this criterion; as well as the fact that the only hybridoma cell line successfully cloned was

a cell line from the Sp2.0 fusion. It was, therefore, decided to choose Sp2.0 myeloma cells as the partners for further fusions. This was an empirical decision based on the results from this initial fusion. Other myeloma cell lines have, however, been successfully used as fusion partners in the production of MAb cell lines which recognise fungi. For example, Ianelli *et al.* (1983) and Wright *et al.* (1987) used NS1-Ag4.1 myeloma cells; Callow *et al.* (1987) used NSO/U myeloma cells and Takeuchi *et al.* (1988) used X63/Ag8.653 myeloma cells.

The observation that this positive hybridoma cell line secreted an antibody which recognised the ends of conidia of *N. crassa* and the septal region of hyphae led, subsequently, to the use of conidia and germlings in immunofluorescence screens of hybridoma supernatants. It is easier to screen the hybridoma supernatants for different patterns of fluorescence using conidia and germlings than cell walls fragments (see Fig. 4.2) This enables the observer to recognise an antibody with a fluorescence pattern of particular interest and to select it for further study.

Immunofluorescence screening does, however, have its drawbacks. It takes longer to perform than ELISA and, in the screening of 480 hybridoma supernatants, time and ease of the assay are important considerations. It was, therefore, decided to screen the hybridoma supernatants using the ELISA initially and, then to screen the hybridomas which were found to be positive using the ELISA by immunofluorescence. In the case of hybridomas raised against *N. crassa*, conidia and germlings were used as the antigen to enable discrimination of hybridomas which showed different patterns of fluorescence. In the absence of permanent supply of *P. involutus* basidiospores, hybridomas raised against *P. involutus* were screened using cell wall fragments. This approach was also particularly suitable for *P. involutus* since it was mycelial antigens that were of most interest.

It can be seen from the results of the fusions (Table 4.1) that a number of positives found using ELISA were not positive in immunofluorescence screens. This may have been

Fig. 4.2. The immunofluorescence pattern exhibited by conidia and hyphal wall fragments of *Neurospora crassa* following reaction with MAb S3B3.

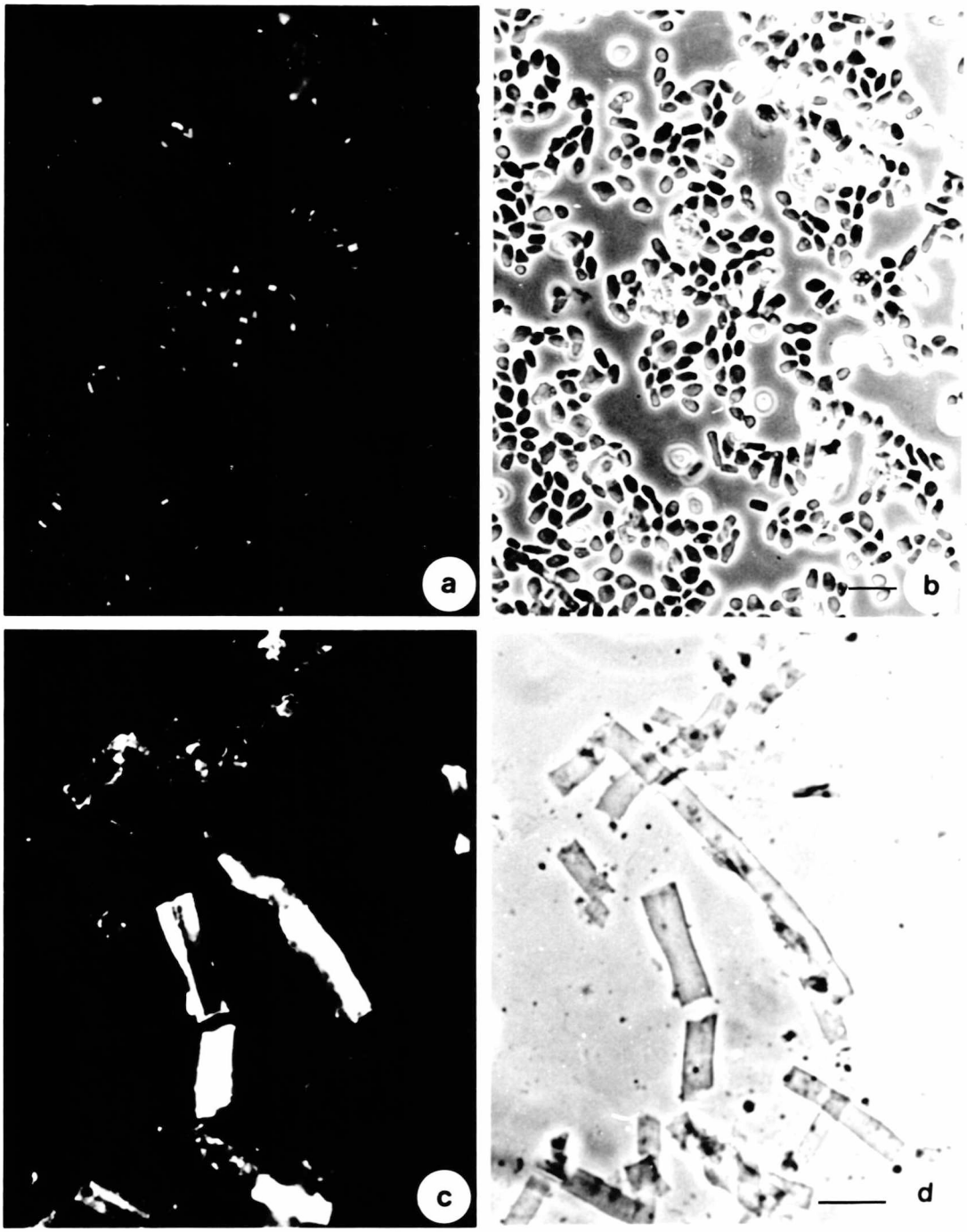
(a) The immunofluorescence pattern exhibited by conidia

(b) Phase contrast of (a)

(c) The immunofluorescence pattern exhibited by hyphal wall fragments

(d) Phase contrast of (c)

Scale bars represent 5μm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due to three reasons: 1. false positives from the ELISA; 2. loss of the secretion of the reactive antibody by the hybridoma; and 3. inability of the antibody to bind to the antigen in the immunofluorescence test.

Antigen used for immunisation	No. of wells tested	No. of +s ELISA	No. of +s Immunofluorescence
N. crassa	3360	286 (8.5%)	102 (3%)
P. involutus	1440	108 (7.5%)	29 (2%)

Table 4.1. A summary of the results from all of the fusions.

The different reaction of MAbs with their antigen in different screening methods has been reported (de Boer & Wieczorek, 1984). This two stage screening process, therefore, enabled choice of antibodies which reacted with the antigen in two different types of immunological tests.

4.4. The positive hybridomas.

Fusions of spleen cells from Balb/c mice immunised with *N. crassa* cell walls produced positive hybridomas which fell into four major groups when classified by their fluorescence patterns, based on antibodies which reacted primarily with:

1. conidial ends and septa
2. hyphae and conidia
3. hyphae only
4. spores only

Fig. 4.3. The immunofluorescence patterns exhibited when antibodies from the four postive hybridoma cell lines were reacted with their homologous antigens.

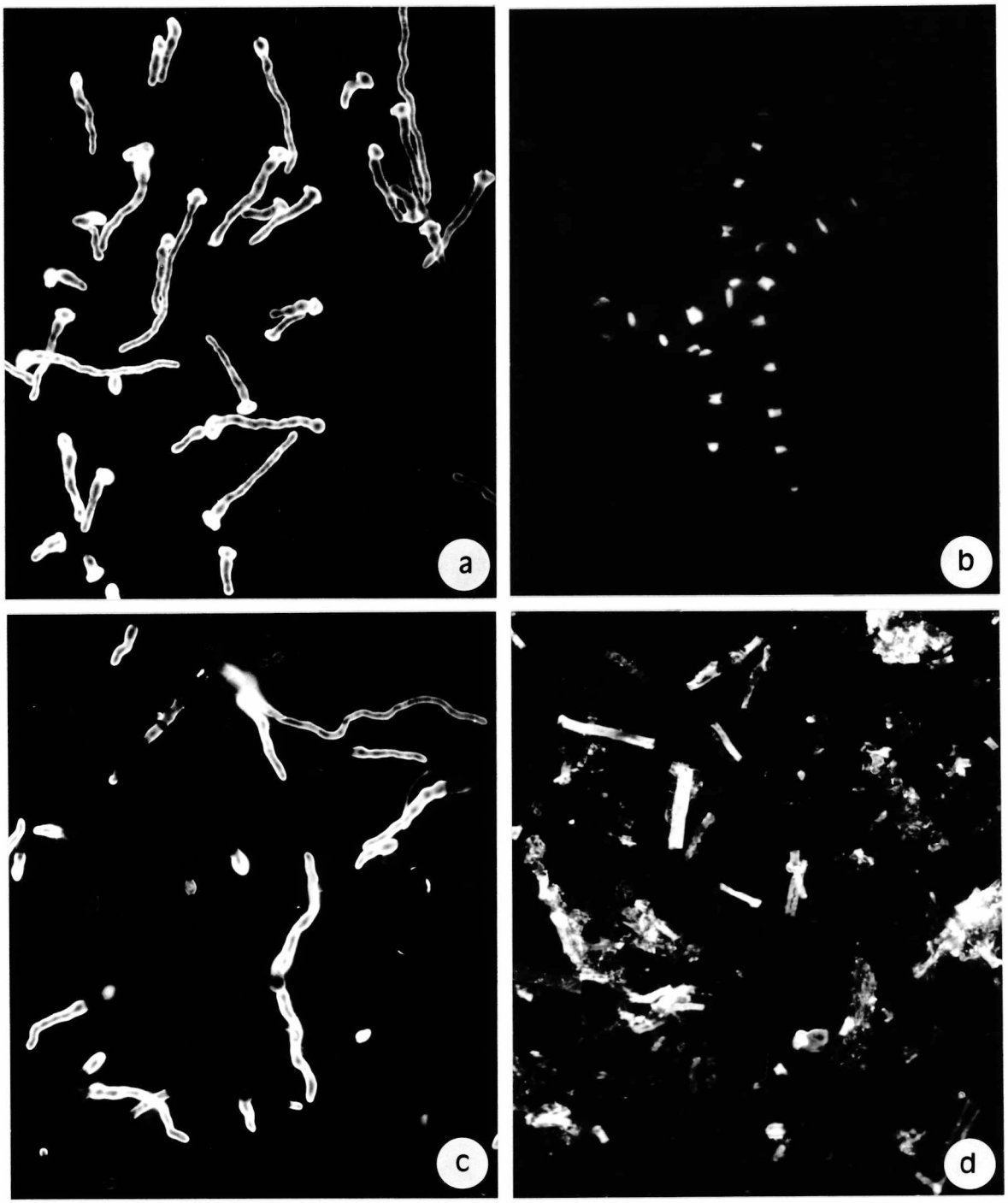
(a) Germlings of *Neurospora crassa* and S4D1

(b) Conidial chain of *N. crassa* and S3B3

(c) Germlings of *N. crassa* and S1E5

(d) Cell wall fragments of *Paxillus involutus* and Pax-1

Scale bar represents 5μm and refers to all micrographs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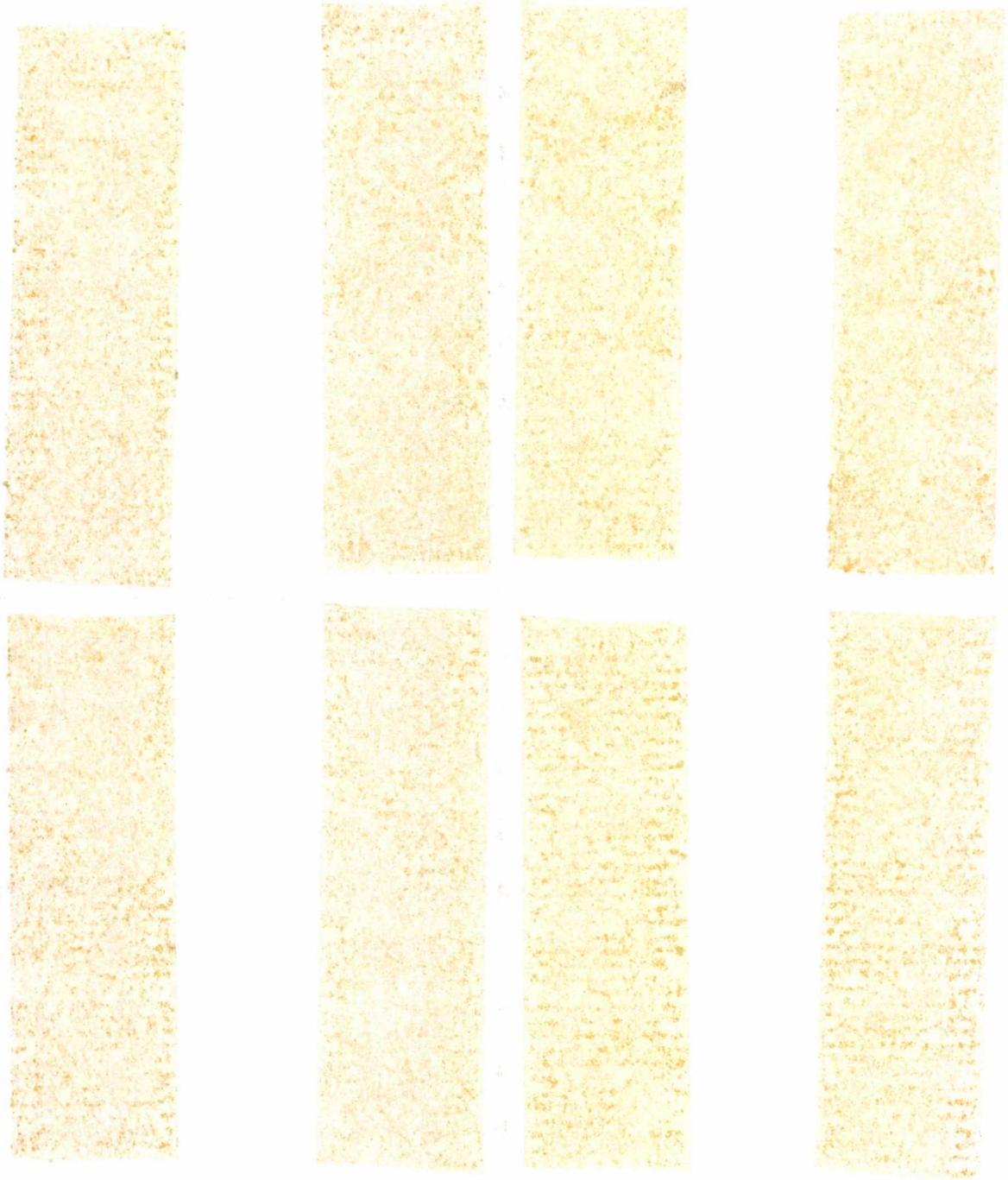

Hybridoma cell lines which fall into the first three categories were successfully cloned and are represented by MAbs S3B3, S4D1 and S1E5, respectively (Fig. 4.3a, b, and c). These three MAbs are described in more detail in the Chapters 5, 6 and 7. A MAb representative of the final category was identified in first screens of hybridoma supernatants but was not successfully cloned.

Hybridomas from *P. involutus* injected mice only showed one immunofluorescence pattern, that of MAb Pax-1 (Fig. 4.3d). From three fusions and 108 positives by ELISA this was the only clear immunofluorescence pattern that occurred.

4.5. Discussion.

A successful immunisation protocol was established which produced a "good" immune response when measured by end-point titres of tailbleeds in an ELISA. End-point titres from the antiserum have no direct relationship with the production of hybridomas secreting the specific antibody required, but a bench-mark of 1/200 is generally considered to correlate with the production of specific hybridomas (Campbell, 1985). No significant correlation was found when end-point titres and the number of positive hybridomas were compared during this project. No papers which describe the production of MAbs to recognise fungi have given the end-point titres of the immunised mouse antisera. The assumption is that if a MAb, or MAbs, is produced then the immunisation protocol has been successful, and there is no need to give such preliminary data. Different immunisation protocols may all produce an immune response to the immunogen and no one protocol is more successful than others. When, however, immunogens, different to those used successfully by other workers, are being used for immunisation, it helpful to use the end-point of the antiserum as a measure of the success of the immunisation protocol.

Hybridomas were produced using Sp2.0 myelomas cells as the fusion partners and a large number of hybridomas secreting antibodies which recognised *N. crassa* and *P. involutus* cell walls were obtained, following fusions using spleen cells from mice with high tailbleed titres *e.g.* in excess of 1/10,000. The relative lack of variation in the immunofluorescence patterns suggests that immunodominance by some wall components occurs.

Four MAb cell lines were successfully established using the protocols worked up during this study. The antibodies they secrete show specific recognition of fungi. The characteristics of the four MAbs are described in the following chapters.

Chapter Five

The monoclonal antibody S4D1

5.1 Introduction.

This Chapter describes the characterisation of the MAb S4D1: the reactions of the MAb with different fungi using immunofluorescence; the localisation of the epitope as shown by immunogold electron microscopy; the reaction of the MAb with different wall fractions from fungal cell walls; and the estimation of fungal biomass using the MAb in an ELISA assay.

5.2. Antibody subclass.

MAb S4D1 was characterised by Ouchterlony immunodiffusion as an IgM antibody. Some cross-reactivity between MAb S4D1 and anti-mouse IgG_{2b} was also observed.

5.3 The immunofluorescence reactions of MAb S4D1.

5.3.1. With its homologous fungus, *N. crassa*.

S4D1 recognises an epitope which is present on the surface of *N. crassa* hyphae and conidia (Fig. 5.1). The epitope is distributed over the entire surface of both the hyphae and conidia and does not appear to be preferentially located in any particular area of these surfaces.

The epitope MAb S4D1 recognises is present on the conidium before the germ tube emerges (Fig. 5.2), and as the germ tube emerges from a conidium the epitope is already

Fig. 5.1. The immunofluorescence patterns exhibited by germlings and conidia of *Neurospora crassa* following reaction with MAb S4D1.

(a) Germlings of *Neurospora crassa* show fluorescence along their whole length and fluorescence was seen on the conidia.

(b) Phase contrast of (a)

(c) Conidia of *N. crassa* show fluorescence over their whole surface

(d) Phase contrast of (c)

Scale bar represents 5μm

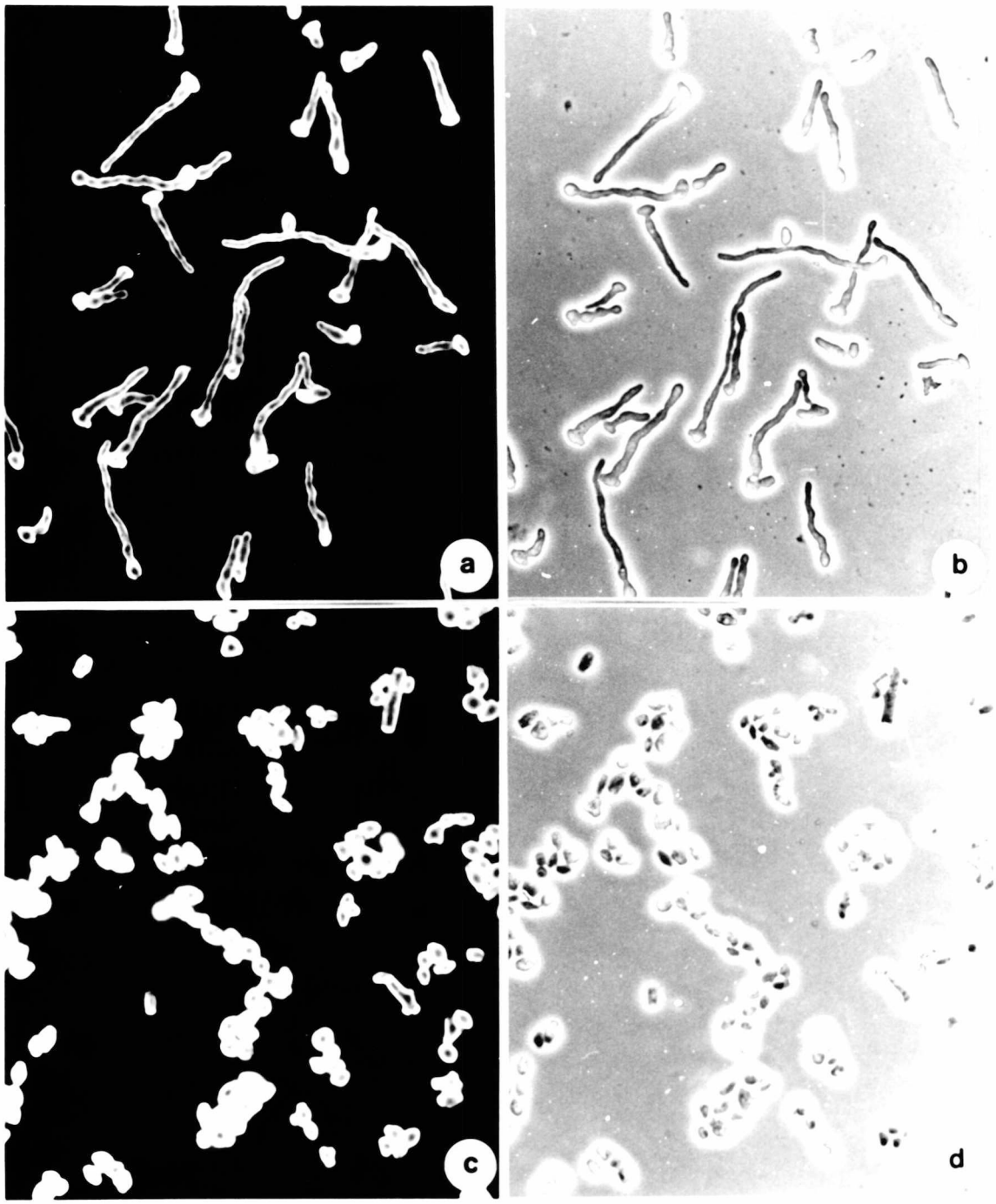
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Fig. 5.2. The immunofluorescence pattern seen on germ tubes of *Neurospora crassa* following reaction with MAb S4D1.

(a) Fluorescence was seen over the whole surface of conidia incubated for 1h in germination medium.

(b) Phase contrast of (a)

(c) Fluorescence was seen on the germ tube surface as it emerges from the conidium. Fluorescence was seen on the surface of the conidium during germ tube emergence.

(d) Phase contrast of (c)

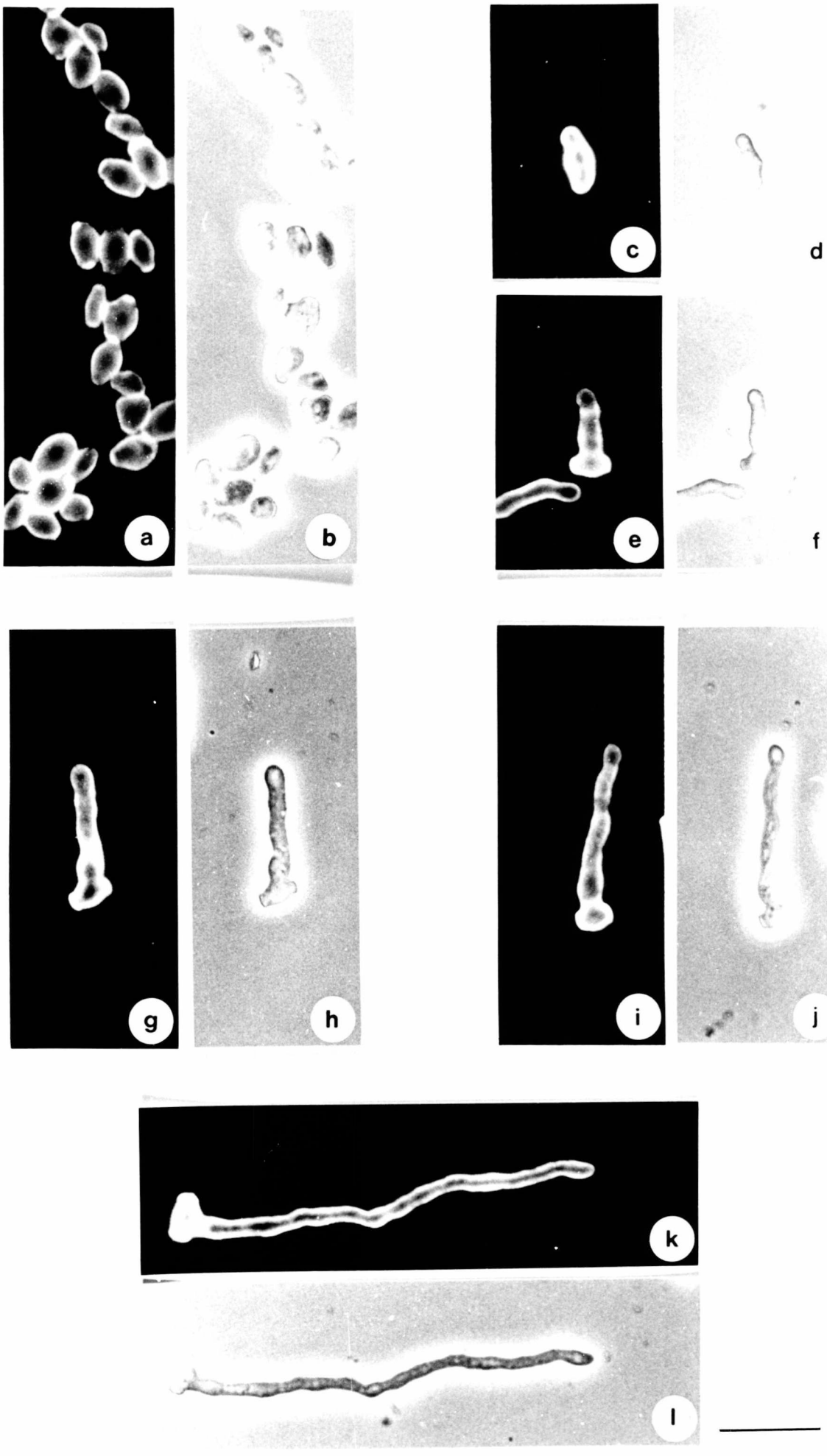
(e) Fluorescence was seen along the entire germ tube surface as it extends.

(f) Phase contrast of (e)

(g), (i) and (k) Fluorescence was seen along the germ tube as it extends

(h), (j) and (l) Phase contrast micrographs of (g), (i) and (k), respectively.

Scale bar represents 5μm and refers to all micrographs.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l

present on the hyphal surface (Fig. 5.2c). The epitope is present along the whole length of the germ tube, as it elongates, and the epitope is evenly distributed along the length of the germ tube throughout its early stages of development (Fig. 5.2). Fluorescence of conidia does not alter during germination as can seen in Fig. 5.2c-l.

5.3.2. With heterologous fungi.

S4D1 was raised against *N. crassa* CMI 142819, but showed the same pattern of reaction with another *N. crassa* isolate, CMI 53420. It also showed the same pattern of fluorescence with two other members of the genus *Neurospora*, *N. sitophila* and *N. tetrasperma* (Fig. 5.3).

S4D1 reacted to a more limited extent with *Sordaria fimicola*, a fungus classified in the same family as *Neurospora*, the Sordariaceae. Weak fluorescence was seen on the asci and ascospores. Some fluorescence can be seen on the hyphae but this does not extend along their whole length (Fig. 5.4a).

S4D1 showed no reaction with the hyphae of *Podospora paucieta* (Fig. 5.4c), a member of the family Lasiosphaeriaceae which also belongs to the order Sordariales. Nor did S4D1 cross-react with *Chaetomium globosum*, a member of the family Chaetomiaceae, another family of the Sordariales.

No fluorescence was seen on arthroconidia of *Geotrichum candidum*, a member of the Endomycetales, another order of the Ascomycotina. If *N. crassa* germlings and *G. candidum* arthroconidia are mixed together and screened for reaction with MAb S4D1 by immunofluorescence; the MAb clearly differentiates between the two species (Fig 5.5).

The reaction of S4D1 with fungi from genera outside the Ascomycotina was also studied. S4D1 showed a reaction with three members of the Deuteromycotina, *Penicillium chrysogenum, Aspergillus nidulans* and *Colletotrichum gloeosporioides*. The fluorescence

Fig. 5.3. The immunofluorescence patterns exhibited by *Neurospora sitophila* and *Neurospora tetrasperma* following reaction with MAb S4D1.

(a) Fluorescence was seen across the whole surface of conidia of *N. sitophila*.

(b) Phase contrast of (a)

(c) Bright fluoresence was seen on hyphae and conidial initials of *N. tetrasperma*. Some parts of the hyphal surface fluoresced less strongly.

(d) Phase contrast of (c)

Scale bar represents 5μm and refers to all micrographs.

a
b
c
d

Fig. 5.4. The fluorescence patterns exhibited by *Sordaria fimicola* and *Podospora paucieta* following reaction with MAb S4D1.

(a) Fluorescence was seen on some hyphal surfaces of *S. fimicola*, but not all. The surface of the ascus showed weak fluorescence.

(b) Phase contrast of (a)

(c) No fluorescence was seen on the surface of hyphae of *P. paucieta*.

(d) Phase contrast of (c).

Scale bar represents 5μm ans refers to all micrographs.

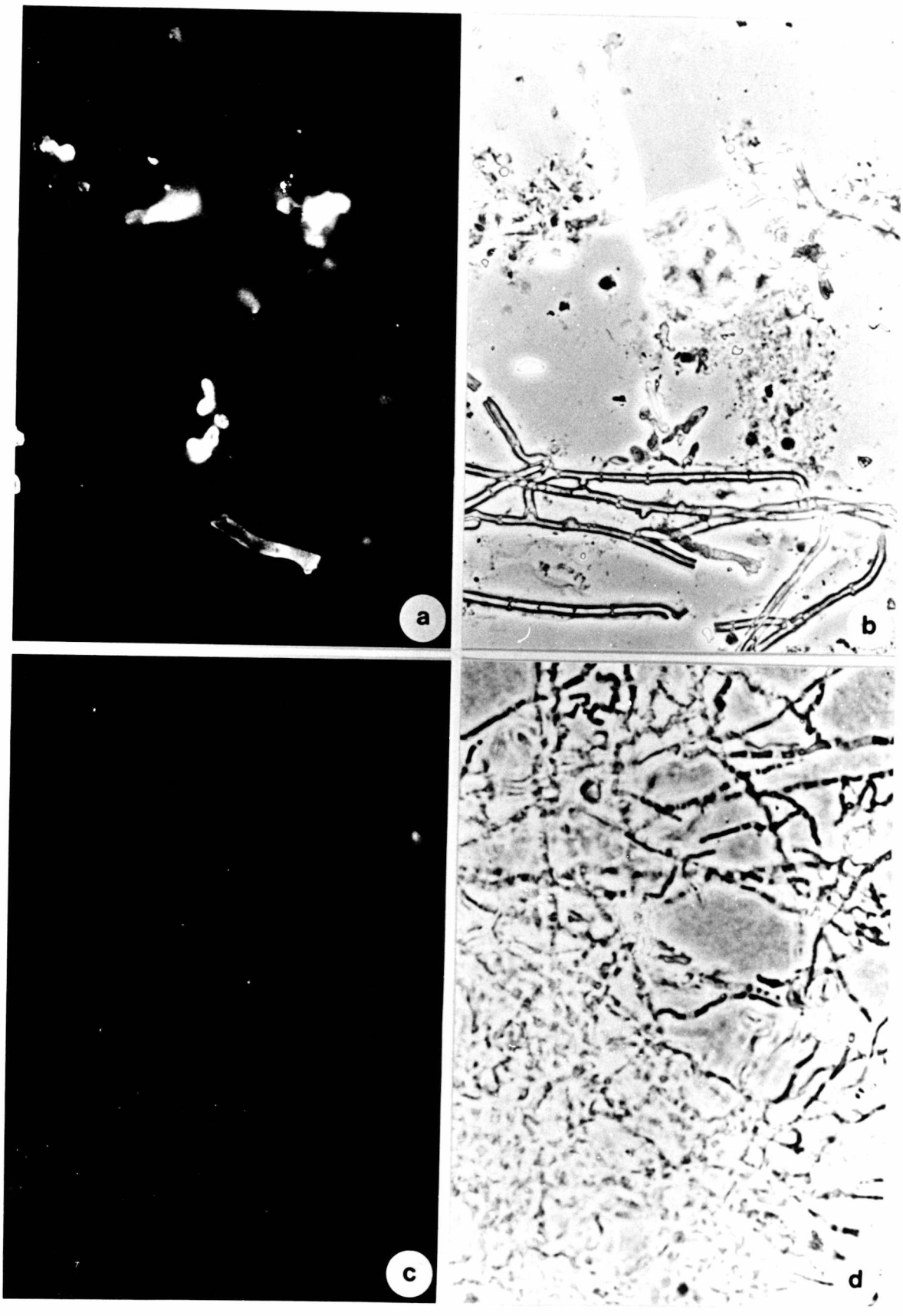
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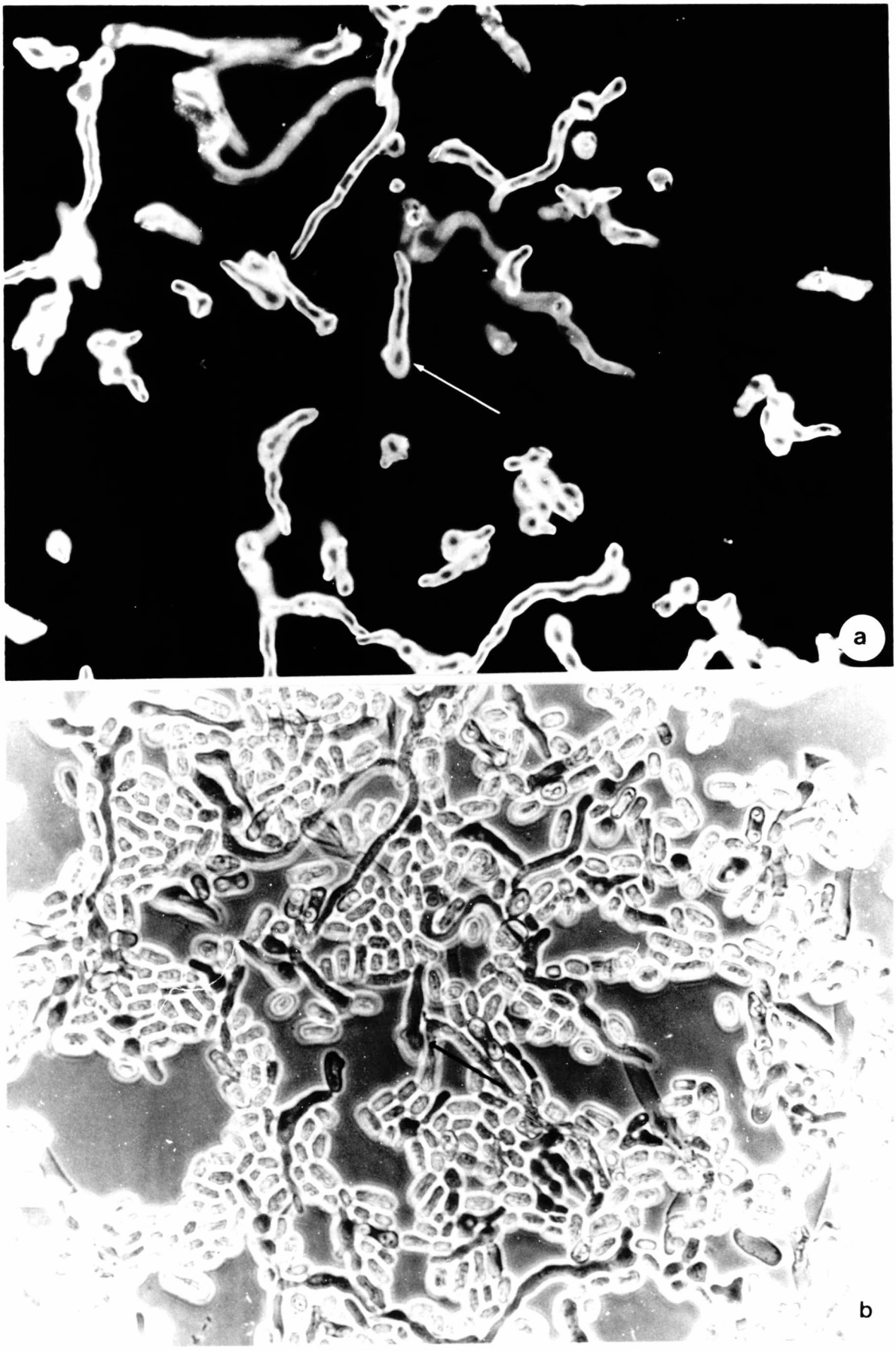
Fig. 5.5. The differentiation of germlings of *Neurospora crassa* and arthroconidia of *Geotrichum candidum* using the MAb S4D1 in an immunofluorescence technique..

(a) MAb S4D1 reacted with the surface of the germlings of *N. crassa* enabling them to be seen clearly in a mixture containing arthroconidia of *G. candidum*, with which S4D1 did not react.

(b) Phase contrast of (a).

Scale bar represents 5μm.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patterns exhibited by these three fungi show differential localisation of the S4D1 epitope on the surface of the structures from different parts of their life cycles.

With the Hyphomycete fungus, *P. chrysogenum*, as an example, fluorescence was exhibited along the surface of the hyphae and conidiophores, but not on the surface of the phialides or conidia (Fig. 5.6). With *A. nidulans* the fluorescence seen on the surface of the conidia varied in intensity on different conidia (Fig. 5.7). Bright fluorescence was seen on the hyphae (not shown), as on *P. chrysogenum* hyphae. The pattern of fluorescence seen on the Coelomycete fungus, *C. gloeosporioides*, shows that the S4D1 epitope is present on the surface of germ tubes, but is not present on the appressorium (Fig. 5.8). The epitope does not occur across the entire surface of the conidia, but does occur where there is a septum (Fig. 5.8a). The epitope is present at the surface of the germ tubes as they elongate (Fig. 5.8a-h).

When the reaction of some members of the Basidiomycotina with S4D1 were tested differential localisation of the epitope on structures from different parts of the life cycle was again seen. For example, S4D1 showed no reaction with the hyphae from a fruiting body of *Amanita muscaria* but fluorescence was observed on the surface of the basidiospores (Fig. 5.9).

In reactions with other members of the Basidiomycotina the S4D1 epitope was found to be present on the hyphal surface, unlike the situation in *A. muscaria*. As an example, S4D1 reacted with the hyphae of *Schizophyllum commune*. Fluorescence was seen particularly along the surface of the long, unbranched skeletal hyphae, which have thick walls (Fig. 5.10a). Some fluorescence was also seen on fragments of thin walled generative hyphae (Fig 10a, arrowed). MAb S4D1 also reacted with hyphal cell walls from a fruit body of *P. involutus* (Fig. 5.10c).

Fig. 5.6. The fluorescence patterns exhibited by different morphological forms of *Penicillium chrysogenum* following reaction with Mab S4D1.

(a) The hyphae and conidiophores of *P. chrysogenum* exhibited fluorescence along their surfaces, but the phialides (arrowed) did not. Nor was fluorescence seen on the surface of conidia.

(b) Phase contrast of (a)

Scale bar represents 5μm.

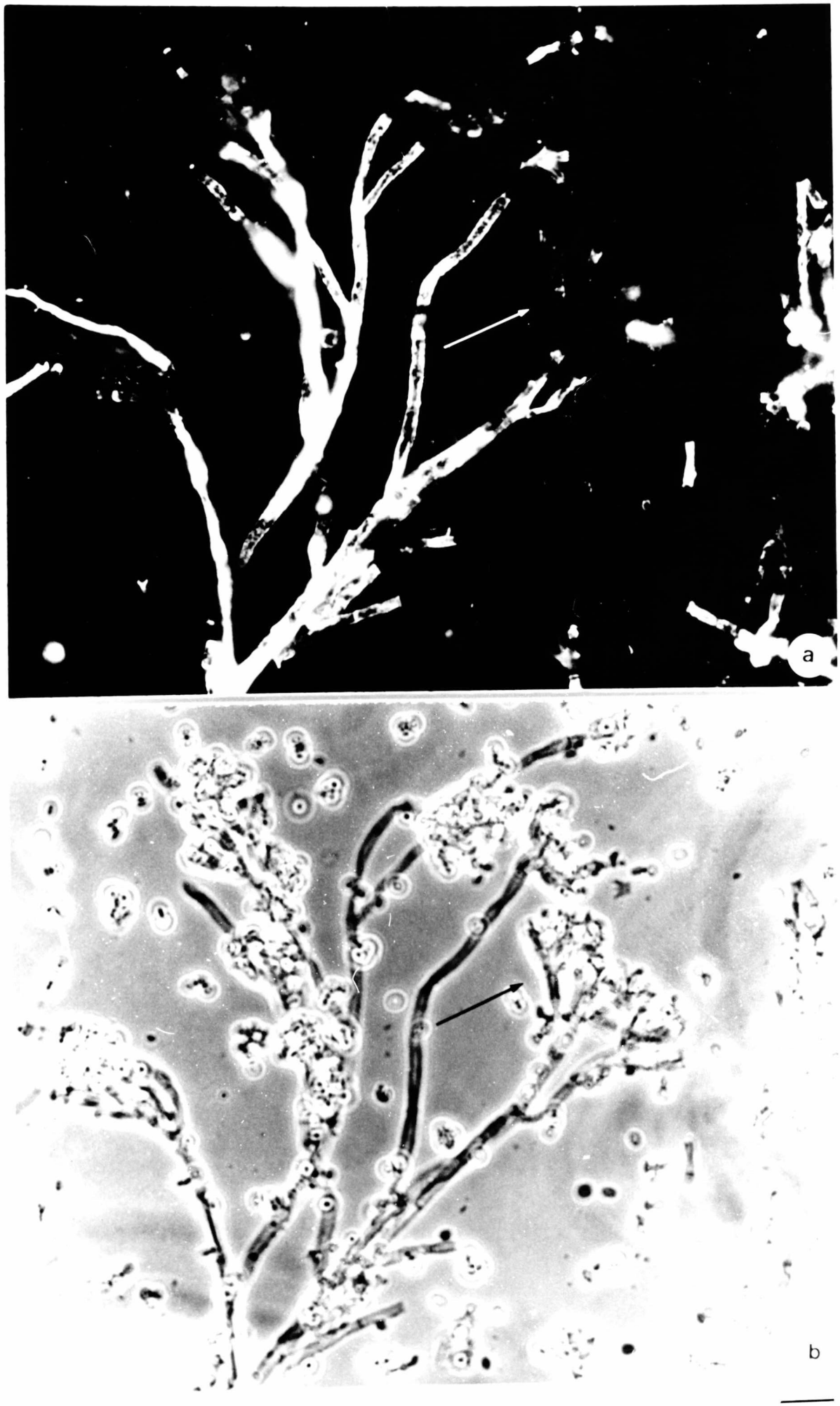
a
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Fig. 5.7. The fluorescence pattern exhibited by the conidia of *Aspergillus nidulans* following reaction with MAb S4D1.

(a) Fluorescence was seen on the surface of conidia of *A. nidulans*. The fluorescence was not as strong as that seen on conidia of *Neurospora crassa* (see Fig. 5.1c). Fluorescence was also seen on the hyphal surface of *A. nidulans* (not shown).

(b) Phase contrast of (a).

Scale bar represents 5μm.

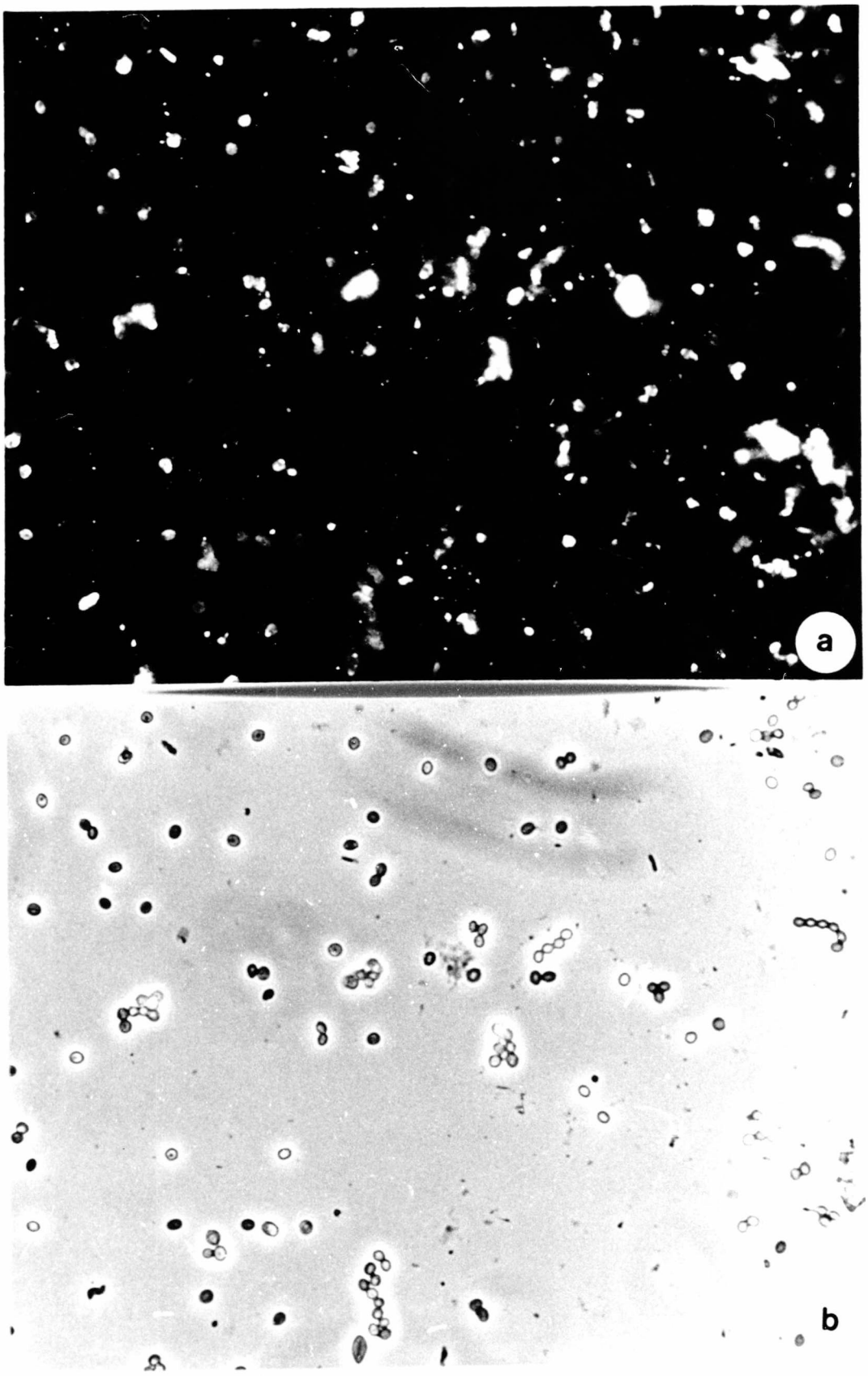
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Fig. 5.8. The fluorescence pattern exibited by conidia, appressoria and developing germ tubes of *Colletotrichum gleosporioides* following reaction with MAb S4D1.

(a) Fluorescence was seen on the germ tube as it emerged from the conidium. Weak fluorescence was seen on the conidium at the point where the germ tube emerged and across the septum.

(b) Phase contrast of (a).

(c) Bright fluorescence was seen on the germ tube surface as it extended. No fluorescence was seen on the surface of the hypha from which the appresorium developed.

(d) Phase contrast of (c).

(e) Fluorescence was seen on the surface of two germ tubes in different stages of elongation. No fluorescence was seen on the surface of the appresorium.

(f) Phase contrast of (e)

(g) Fluorescence was seen on the surface of the germ tube as further elongation occurred.

(h) Phase contrast of (h).

Scale bar represents 5μm and refers to all micrographs.

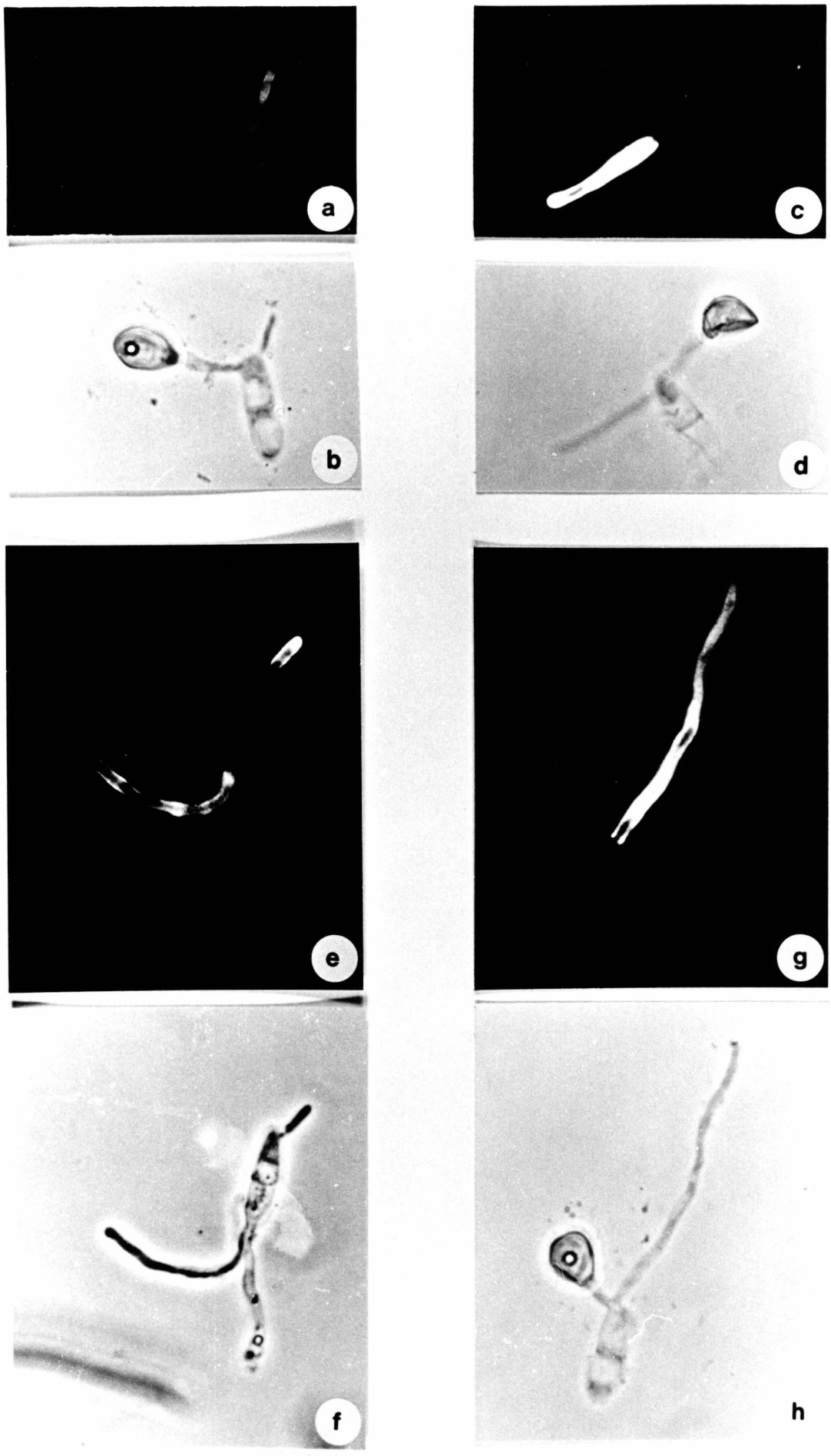
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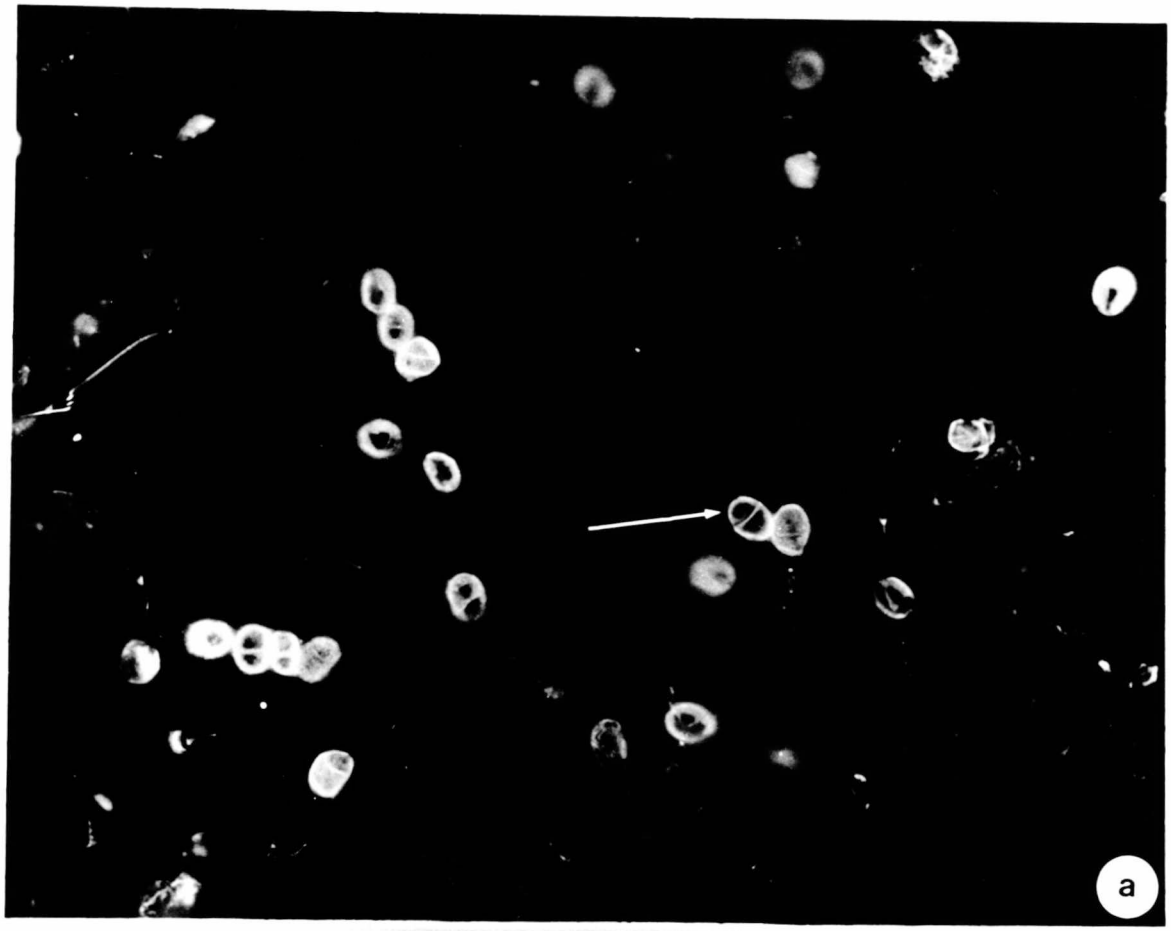
Fig. 5.9. The fluorescence patterns exhibited by different structures present within the fruiting body of *Amanita muscaria* following reaction with MAb S4D1.

(a) Fluorescence was seen on the surface of basidiospores present in a crude preparation from the fruiting body of *A. muscaria*, but fluorescence was not seen along the surface of the hyphae.

(b) Phase contrast of (a).

Scale bar represents 5μm.


a

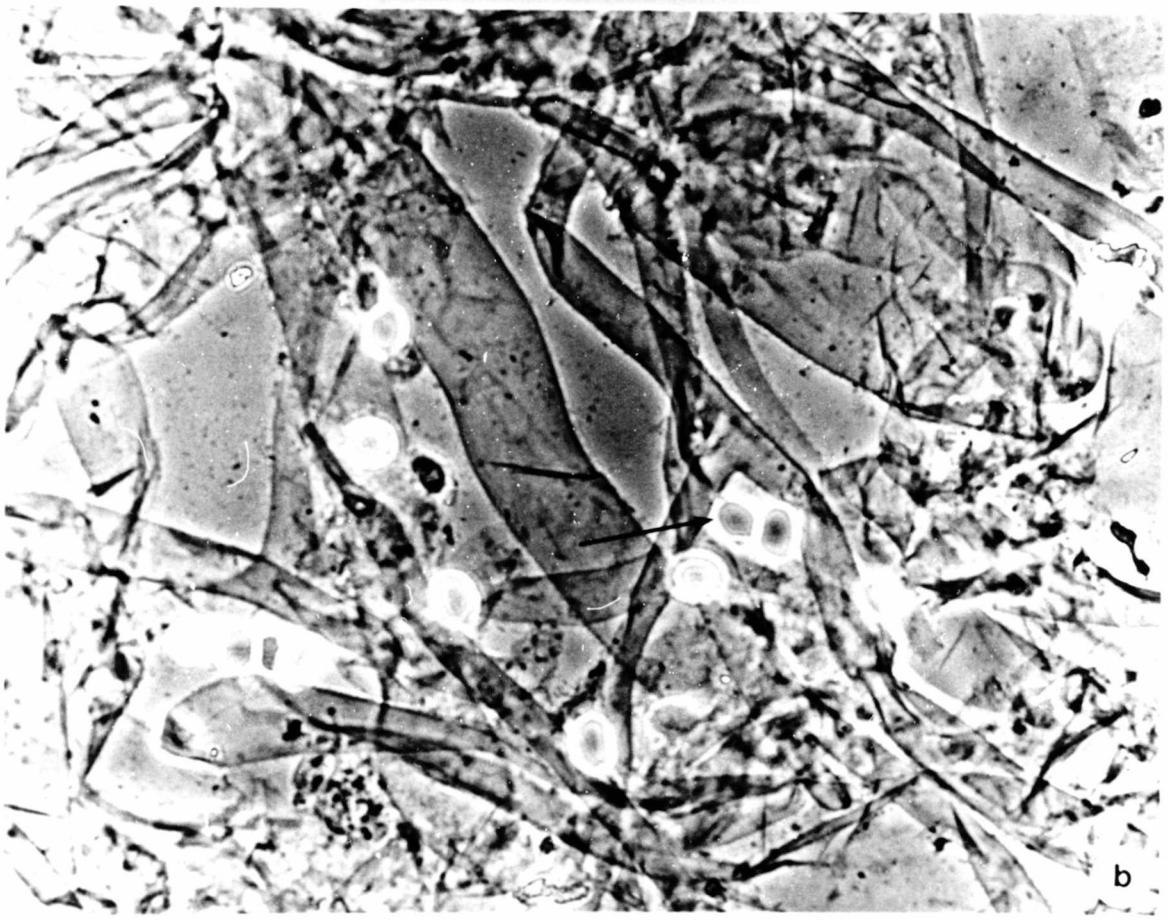

b

Fig. 5.10. The fluorescence patterns exhibited by *Schizophyllum commune* and *Paxillus involutus* following reaction with MAb S4D1.

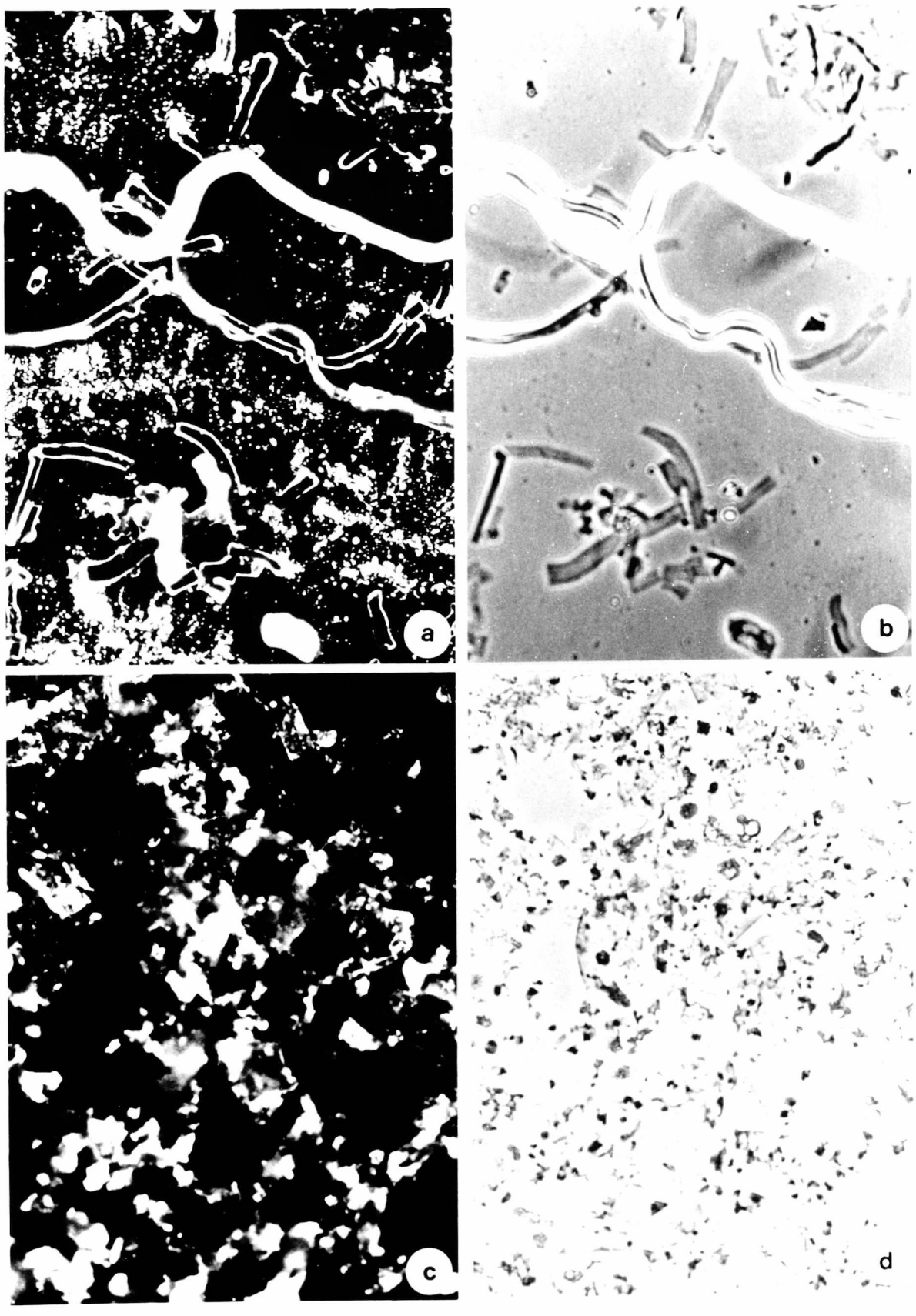
(a) Bright fluorescence was seen on the surface of the skeletal hyphae, whereas bright fluorescence was seen in a discrete halo around hyphal fragments of vegetative hyphae. Bright fluorescence was also seen which did not appear to correspond to any structures. This was not seen in other preparations and was brighter than background fluorescence seen in any other preparations.

(b) Phase contrast of (a).

(c) Bright fluorescence was seen on the surface of hyphal fragments extracted from a fruiting body of *P. involutus*. Bright fluorescence was not seen on the surface of the basidiospores.

(d) Phase contrast of (c).

Scale bar represents 5μm and refers to all micrographs.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5.4. Localisation of the S4D1 epitope in *N. crassa* as revealed by immunogold electron microscopy.

Thin sections of three day old *N. crassa* conidia have a distinct, thin outer wall layer which appears to be detached from the inner wall and the plasmalemma during preparation for electron microscopy (Fig. 5.11a). The inner wall material is only lightly stained and remain close to the plasmalemma (Fig. 5.11a). When the outer layer becomes detached an electronlucent area is seen which varies in width, whether this electronlucent middle wall region contains wall components or is an artefact of the fixing and embedding was not established. The outer wall layer appears to consist of two thin sheets with a central lumen.

The S4D1 epitope is present throughout the wall layers of the *N.crassa* conidium (Fig. 5.11b). The gold particles show heaviest labelling at the ends of the conidia rather than along the sides. This is illustrated in Fig. 5.11c. The gold labelling is clearly associated with the wall rather than the plasmalemma, but the gold label does not differentially distinguish outer wall material from inner wall material, nor is the label localised in discrete layers..

The epitope occurs throughout the septal region in sections observed (Fig. 5.12a), and is present as the conidia are formed in chains (Fig. 5.12b). The epitope is clearly present when the end conidial wall is formed (Fig. 5.11), but insufficient sections were observed to view the distribution of the epitope as the double septum develops.

The hyphal wall was observed to be much thinner than the conidial wall in the thin sections of three day old hyphae (Fig. 5.13). On a transverse section of hyphal wall (Fig. 5.13a) the labelling is present on all parts of the wall, although the labelling is not heavy. In a hyphal septum the labelling appears to be associated particularly with material in the central part of the septum.

Fig. 5.11. The localisation of the S4D1 epitope in the conidial walls of *Neurospora crassa* using immunogold electron microscopy.

(a) The epitope was located in the conidial wall, few gold particles were seen in the cytoplasm. Scale bar represents 0.5μm.

(b) The epitope was found throughout the wall and was not localised in one layer. Scale bar represents 0.1μm.

(c) The heaviest labelling was seen at the ends of the conidia, where the wall was at its thickest. Scale bar represents 0.5μm.


a
b
c

Fig. 5.12. The localisation of the S4D1 epitope in the septal region of conidial chains of *Neurospora crassa* using immunogold electron microscopy.

(a) Low magnification electron micrograph showing a conidial chain and its septal regions. Scale bar represents 1.0μm.

(b) The epitope was found in the septal region of developing conidia. Scale bar represents 0.5μm.

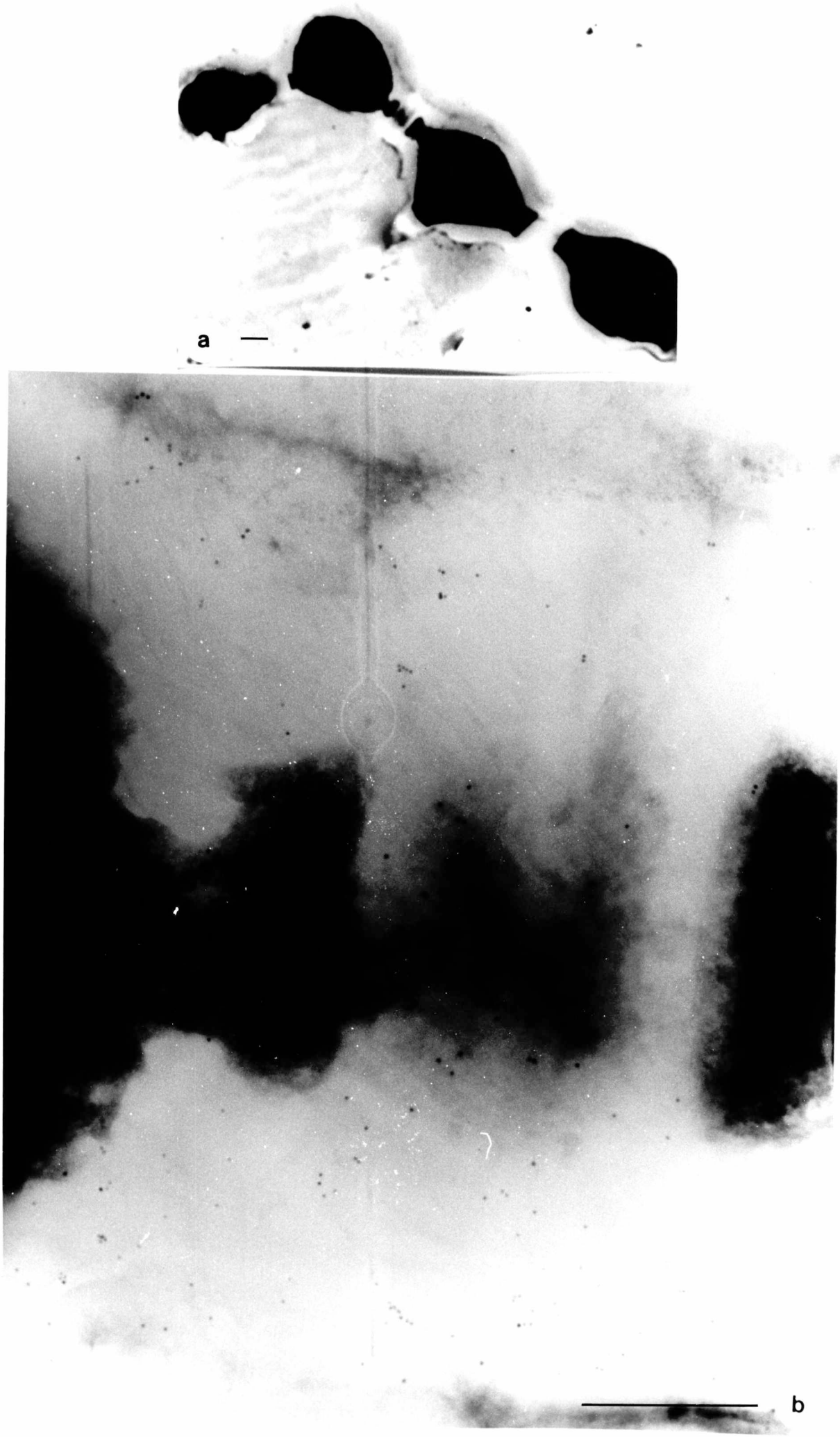
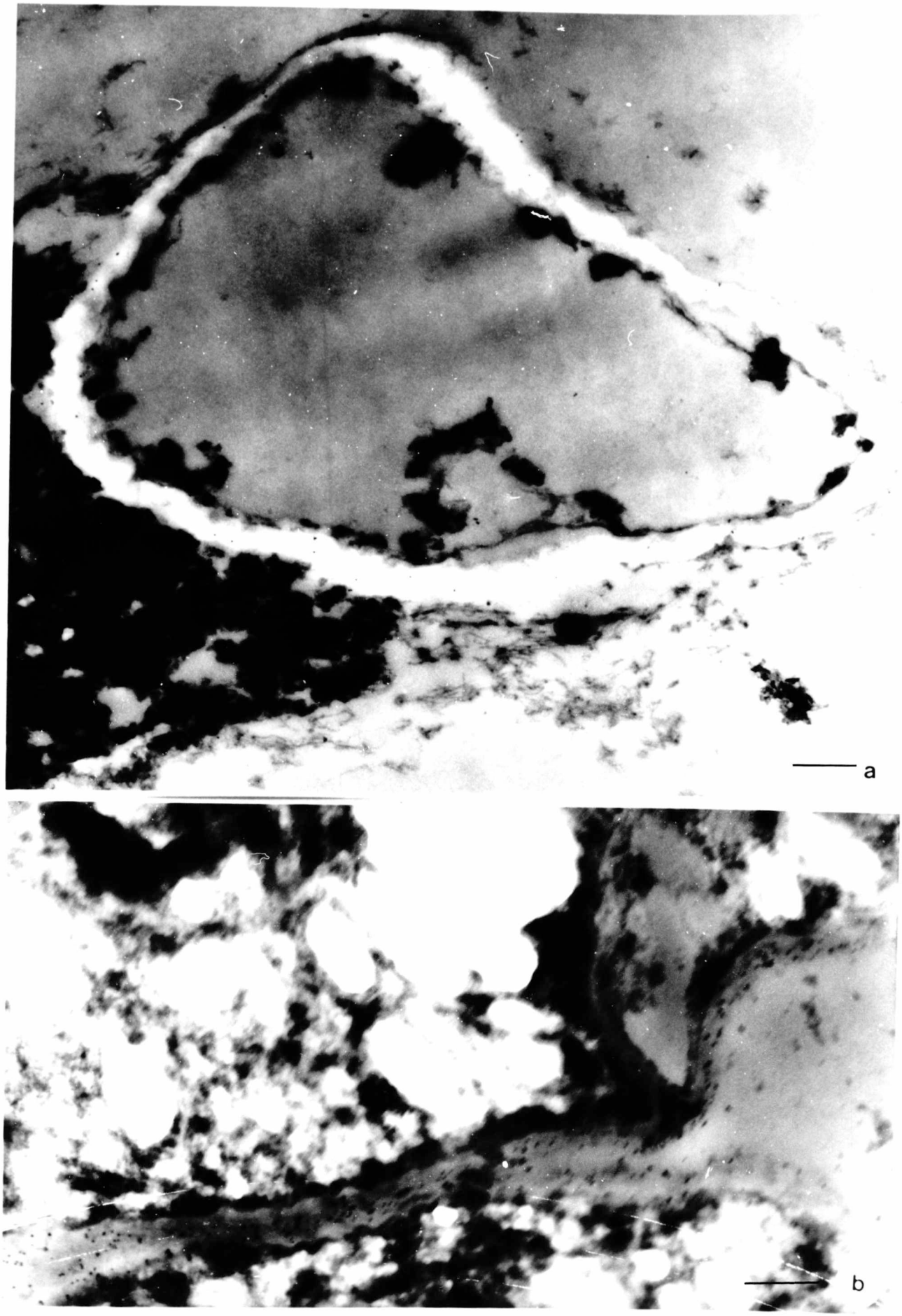

a
b

Fig. 5.13. The localisation of the S4D1 epitope in the hyphal walls of *Neurospora crassa* using immunogold electron microscopy.

(a) The epitope was located throughout the hyphal wall. Scale bar represents 0.5μm.

(b) The epitope was found in the septal region of hyphae, and throughout the wall region near the septum. Scale bar represents 0.5μm.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5.5. The presence of the S4D1 epitope in fungal wall fractions.

The cell walls of fungi can be differentiated into different groups depending on their components (see Table 1.2). The different components of the cell wall can be extracted from the wall by different methods, although very rarely are extracted fractions free from contamination with other wall components. The cell wall of *N. crassa* cell wall was fractionated following the method of Mahadevan & Tatum (1975). The resulting fractions, I, II, III and IV, were tested for their reaction with S4D1 in an ELISA. MAb S4D1 reacts differentially with different wall fractions (Table 5.1). The MAb showed no reaction with Fraction II, but reacted with the other three fractions. MAb S4D1 reacted most strongly with Fraction III, and with the other two fractions, I and IV, to a lesser extent.

Mahadevan & Tatum (1965) have analysed the principal components of each fraction. Fraction IV consisted mainly of chitin. MAb S4D1 was, therefore, tested for its reaction with chitin. S4D1 did not react with chitin, when a pure sample of chitin was used in an ELISA (Table 5.2); nor did it react with N-acetyl-glucosamine, the chitin subunit. The reaction of MAb S4D1 with Fraction IV was, therefore, not due to a reaction with chitin, but to a reaction between S4D1 and a wall component that is also extracted in Fraction IV. The major component of Fraction III is glucan (Mahadevan & Tatum, 1965). Fraction III reacted strongly with S4D1, suggesting that the antigen is a major part of this wall fraction. S4D1 also cross-reacts with Fraction I which contains glucan, galactosamine and peptides. The antigen may, therefore, be any one of these; and Fraction III may contain one of these components as well as the glucan. MAb S4D1 was tested for its reaction with galactosamine, a component of Fraction I. It did not react (Table 5.2).

Cell wall fractions have been isolated from the cell walls of other fungi. Several of these contain glucan, the major component of *N. crassa* Fraction III. As MAb S4D1 reacted with Fraction III, the reactivity of the MAb with these other glucans was tested in an ELISA.

Fraction	Absorbance (410nm)
I	0.83
II	0.12
III	1.31
IV	0.57
Wcw	1.10

Table 5.1. The reaction of MAb S4D1 with wall fractions from *N. crassa* cell walls in an ELISA. Results expressed as absorbance values read at 410nm.

Notes:

I	Galactosamine-peptide-glucan
II	Sugar residues
III	Glucan
IV	Chitin

Wcw: Whole cell walls

The absorbance values given are the mean of four replicates

Cell wall component	Absorbance (410nm)
A. nidulans α-1,3 glucan	0.88
S. commune α-1,3 glucan (S-glucan)	0.70
S. commune β-1,4, β-1,6 glucan (R-glucan)	0.24
Claviceps glucan	0.00
Chitin	0.00
N-acetyl-glucosamine	0.00
Glucosamine	0.00
Galactosamine	0.02
Yeast mannan	0.04

Table 5.2. The reaction of MAb S4D1 with molecules known to be components of fungal cell walls in an ELISA.

Notes:

The absorbance values given are the means of four replicates.

A. nidulans α1,3 glucan and the glucans from *S. commune* were gifts to Prof. K. Gull from Prof. B. J. M. Zonneveld and Prof. J. G. H. Wessels, respectively.

Fig. 5.14. Cell wall material extracted from *Neurospora crassa* using Laemmli sample buffer was reacted with MAbs S4D1 and S3B3 in an immunoblotting procedure.

Slot No.	Treatment	Reaction
1	SDMEM	None
2	S3B3	None
3	S4D1	Positive reaction, purple colour of positive substrate seen
4,5 and 6	Amido black staining	Indicated the presence of cell wall material on the nitrocellulose filter.

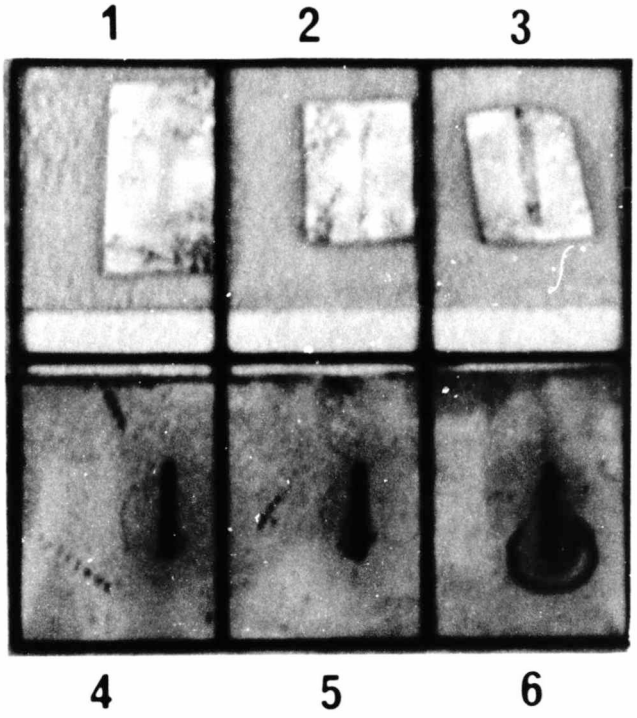

1
2
3
4
5
6

Fig. 5.15. The immunofluorescence patterns of cell wall fragments from *Neurospora crassa* before and after treatment with Laemmli sample buffer (LSB) and following reaction with MAb S4D1.

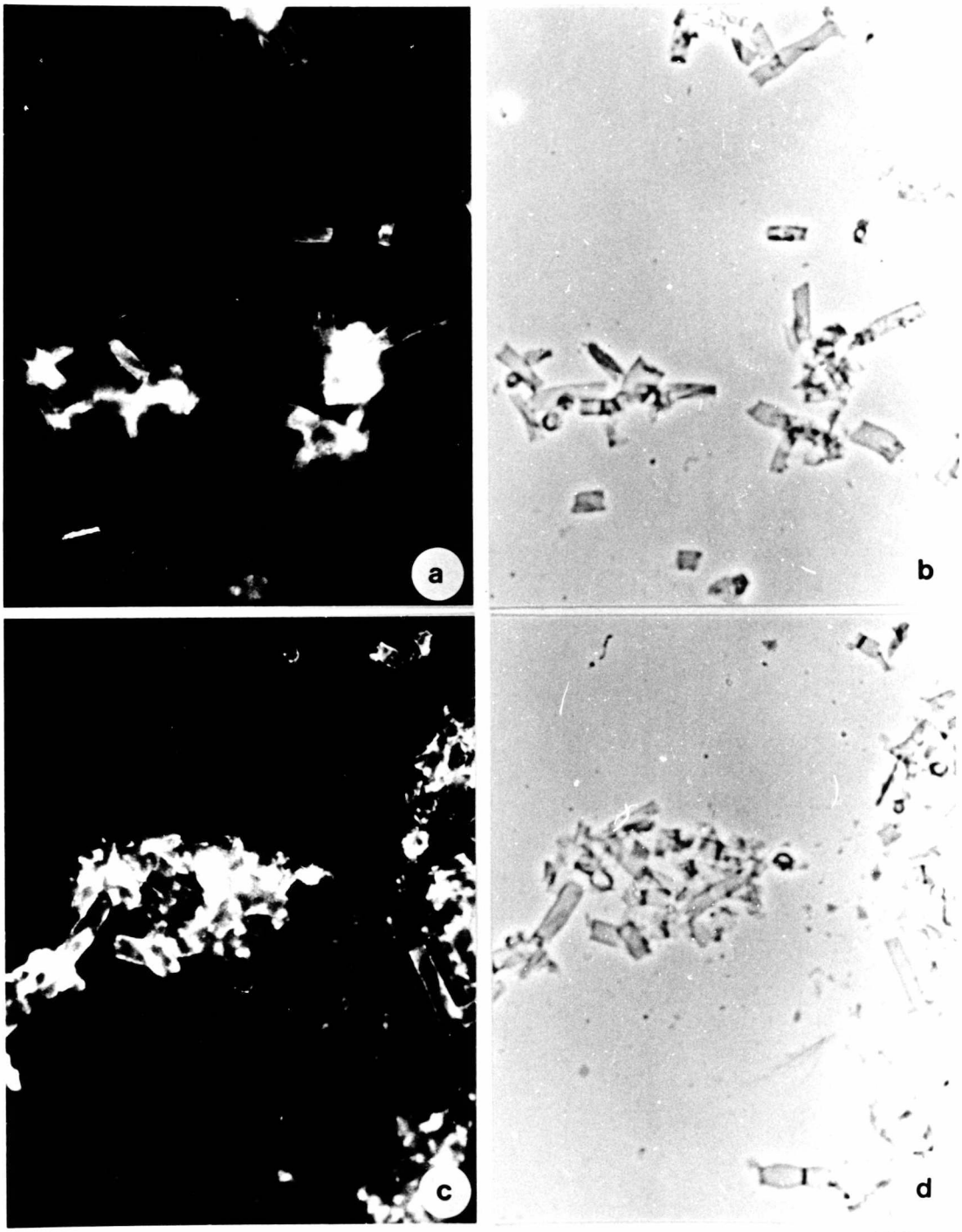
(a) The fluoresence pattern seen before treatment with LSB.

(b) Phase contrast of (a).

(c) The fluorescence pattern seen after treatment with LSB.

(d) Phase contrast of (c).

Scale bar represents 5μm and refers to all micrographs.


a
b
c
d

The MAb reacted with with α-1,3 glucan isolated from *A. nidulans* cell walls and α-1,3 glucan (or S-glucan) isolated from *S. commune* cell walls (Table 5.2). The MAb did not react with *Claviceps* cell wall glucan and only very weakly with β-1,3, β-1,6 glucan (or R-glucan) from *S. commune* cell walls.

The peptides present in Fraction I are extracted from the cell wall by 2M NaOH. In Section 3.4 it was shown that Laemmli sample buffer (LSB) extracted polypeptides from *N. crassa* cell walls more efficiently than NaOH for gel electrophoresis. Polypeptides extracted by LSB were, therefore, transferred onto a nitrocellulose filter and tested for their reaction with MAb S4D1 by Western blotting; no reaction occurred. The wall material extracted using LSB did, however, react with MAb S4D1 when a slot-blotting method was used (Fig. 5.14). This suggests that the wall component with which S4D1 reacts does not enter a 10% SDS-polyacrylamide gel, but is extracted by LSB.

Following treatment of *N. crassa* cell walls with LSB the cell walls themselves were washed with dH_2O and tested for their reaction with MAb S4D1 by immunofluorescence. The cell walls reacted strongly with S4D1 showing bright fluorescence on all of the fragments and no difference could be seen between the reaction of S4D1 with cell walls before or after extraction of cell wall material using LSB (Fig. 5.15).

5.6. The quantification of *N. crassa* biomass using MAb S4D1.

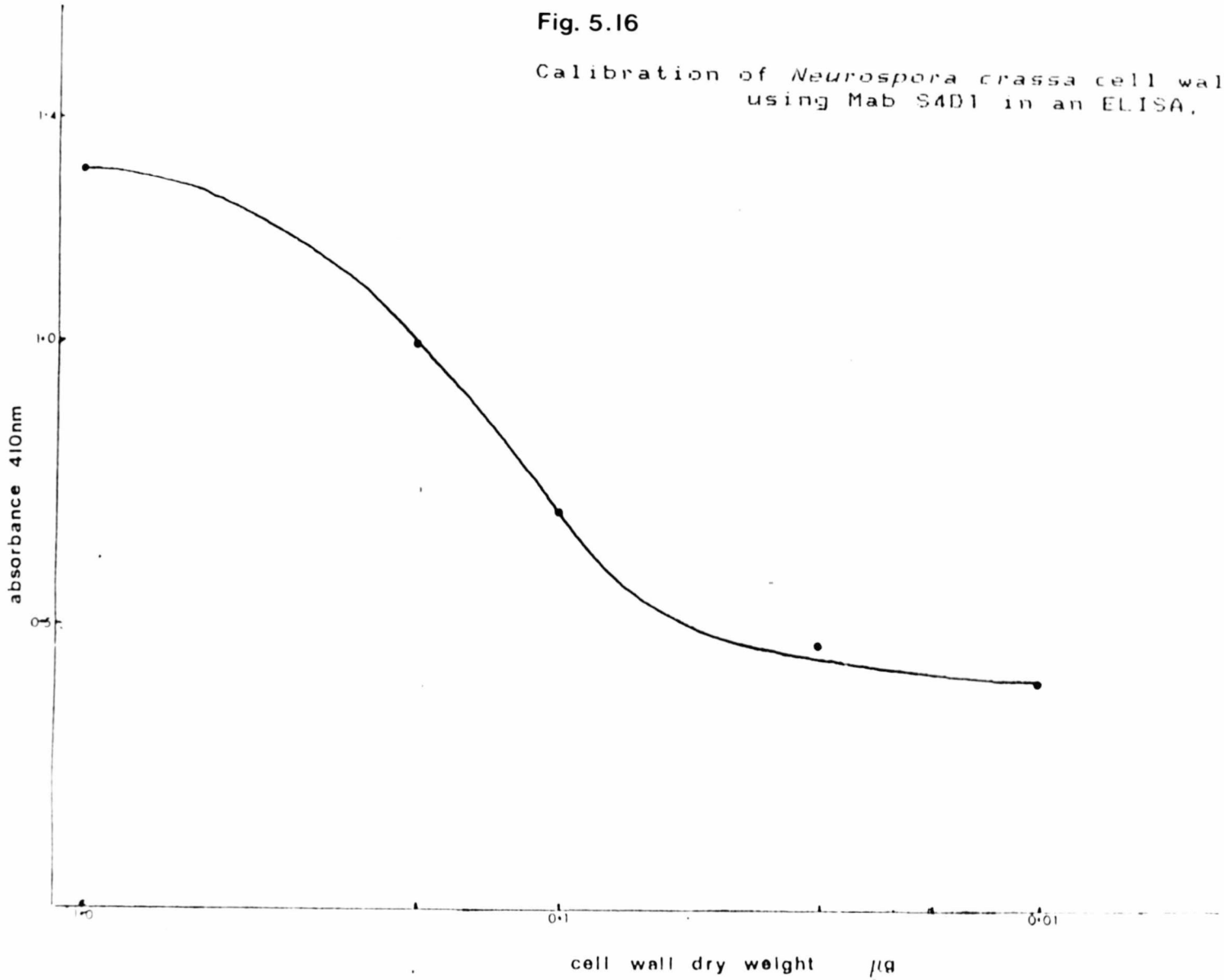
5.6.1. The calibration curve.

The use of an ELISA to quantify biomass using a MAb has potential applications in both plant pathology and fungal ecology. This applications are dependent on a linear relationship between the ELISA response (absorbance) and the biomass of the fungus.

Fig. 5.16 shows the calibration curve obtained using MAb S4D1 in an ELISA to detect varying biomasses of *N. crassa* cell walls. The antibody concentration was not itself

Fig. 5.16

Calibration of *Neurospora crassa* cell wall dry weight using Mab S4D1 in an ELISA.



determined; instead the protein concentration of the hybridoma supernatant containing S4D1 was measured. The protein concentrations of the two supernatants tested were 1.4mg ml^{-1} and 2.7mg ml^{-1}. The supernatant with the concentration of 2.7mg ml^{-1} was diluted with sdH_2O and used at concentrations of 2, 1 and 0.5mg ml^{-1}.

The reaction of the cell walls with the MAb at concentrations of 1 and 2mg ml^{-1}, as measured by absorbance were not different, but with the MAb at a concentration of 0.5mg ml^{-1} the absorbance values were less. A sigmoid dose response curve was seen, however, in both cases. The linear range of the curve occurred in the biomass range of 0.1 to 1µg freeze-dried weight of *N. crassa* cell walls.

5.6.2. Test using spiked vermiculite samples for extraction and quantification of *N. crassa* cell walls.

1mg (freeze-dried weight) of *N. crassa* cell walls was added to 1g of vermiculite. Before being added to the vermiculite the cell walls were lightly ground in a pestle and mortar to ensure that the cells would be dispersed in the vermiculite. The cell walls and vermiculite were mixed together in a large glass boiling tube by stirring. 10ml of sdH_2O was added to the mixture. The vermiculite floated to the top and was removed from the tube using a spatula. A two-fold dilution series of a 0.5ml aliquot from the extraction suspension was made. 100µl aliquots from the undiluted extraction suspension and each of the seven dilutions made were placed in duplicate wells of an ELISA plate. If, therefore, 100% recovery occurred 10, 5, 2.5, 1.25, 0.63, 0.31, 0.15 and 0.075 µg, respectively, of *N. crassa* cell walls would have been placed in wells on the plate. In this case the four lower concentrations of cell walls would have fallen within the linear range of the calibration curve. If 100% recovery was not achieved, as might be expected, the higher concentrations would have still, hopefully, fallen within the linear range of the calibration curve. As a control 1g of vermiculite was mixed with 10ml of sdH_2O. The vermiculite was removed

from the sdH_2o in the same way as for the test samples and a two-fold dilution series prepared. 100µl aliquots of the control dilutions were dispensed into wells on the ELISA plate. The ELISA protocol described in 3.3.3 was followed.

All of the absorbance values including those from vermiculite which had not been spiked gave absorbance readings in excess of 2 at 410nm. This placed even the sample dilutions from the unspiked vermiculite off the top end of the scale. This strongly suggested that a large degree of non-specific binding of either of both antibodies was occurring during the antibody incubation steps of the ELISA. This work was not followed further.

5.7. Discussion.

The discussion of the reactions of the MAb S4D1 in this section will be confined to points which relate specifically to this MAb. Points which relate to all four of the MAbs described in this thesis will be discussed in Chapter 9.

MAb S4D1 was successfully derived from a mouse injected with *N. crassa* cell walls. The MAb recognises an epitope which is present at the cell surface of both hyphae and conidia of *N. crassa*. The epitope is present at the surface of these structures throughout their development; and is present on hyphae which develop into conidiophores. Other workers have described MAbs which react with different parts of the fungal life cycle. These will be discussed in Chapter 9 as all of the MAbs produced during this project show such differential reactions.

The epitope which S4D1 recognises is present in the cell wall of *N. crassa*, as shown by the reaction of S4D1 with isolated cell walls in ELISA tests. In localisation studies using immunogold the epitope was shown to be present throughout the cell wall of hyphae and conidia, but was not found in the cytoplasm. In the conidial cell wall the epitope is not associated with the thin outermost layer of the conidia. The appearance of this outermost

layer corresponded with that of the rodlet layer described by Dempsey & Beever (1979). This rodlet layer was reported to be present only on the conidia (Dempsey & Beever, 1979; Beever *et al.*, 1979) and this was observed to be the case in this work. Bearing this in mind, the immunofluorescence reaction of MAb S4D1 had already suggested that the epitope was unlikely to be localised in the rodlet layer. Any MAb which specifically recognised the rodlet layer would only react with the surface of conidia in immunofluorescence studies.

As the rodlet layer is present at the surface of the conidia, how does the MAb S4D1 gain access to its epitope in conidia during immunofluorescence? One explanation may be that the rodlet layer is removed during the immunofluorescence procedure and the S4D1 epitope is exposed at the surface of the conidia. Dempsey & Beever (1979) and Beever *et al.*(1979) reported that the rodlet layer was easily removed from the conidia by simply washing the surface of the conidia with water. The sections observed by electron microscopy showed that the rodlet layer remained in association with the conidia. The conidia prepared for immunofluorescence were fixed in the same way as the conidia prepared for electron microscopy; therefore, it is not possible to assume that the removal of the rodlet layer serves as an adequate explanation. An alternative explanation may be that, although the rodlet layer remains associated with the conidia, the MAb is able to gain access to the epitope through this layer. This may be due to the natural properties of the rodlet layer allowing access of molecules to the cell, rather than the fixative causing changes in the rodlet layer, as unfixed conidia also reacted with MAb S4D1.

MAb S4D1 reacted with cell wall fractions I, III and IV extracted from *N. crassa* cell walls. Subsequent reactions suggested that the MAb reacted with either a peptide or a glucan. The reaction of the MAb with α-1,3 glucan isolated from other fungi strongly suggested that the epitope is present in an α-1,3 glucan. α-1,3 glucan has been shown to be present in the cell walls of *N. crassa* by Bull (1970) and Cardimil & Pincheira (1979).

Mahadevan & Tatum (1965) reported that they found β linked glucan, but made no mention of α glucan.

The cross-reactivity of MAb S4D1 with other fungi therefore showed that α-1,3 glucan is present in other fungi besides *N. crassa*. This had already been shown to be the case by other workers: for example; Bull (1970) and Zonneveld (1971) showed that α-1,3 glucan is present in *A. nidulans* cell walls; Wessels *et al.* (1972) showed that it is present in the walls of *S. commune*; Bacon *et al.* (1968) found the glucan in the walls of *Cryptoccocus*, *Schizosaccharomyces* and *Polyporus*; and Kreger (1954) showed it is present in the walls of *Agaricus bisporus*.

When germlings of *N. crassa* are reacted with S4D1 fluorescence is exhibited at the hyphal tip. This is in contrast to the finding of Hunsley & Kay (1976) that an antiserum raised against Fraction III reacted with the hyphal walls, but not the hyphal tip. Mahadevan & Tatum (1965) and Hunsley & Burnett (1970) reported β-glucan to be present in this Fraction. The results presented here suggests that α-1,3 glucan is also present in this Fraction and that it is present at the hyphal tip throughout growth, unlike the antigen/s recognised by the antiserum raised by Hunsley & Kay (1976).

The immunogold labelling pattern of seen on the walls of *N. crassa* did not correspond to any of the wall layers proposed by Hunsley & Burnett (1970). They proposed a model for hyphal walls rather than conidial walls, but α-1,3 glucan appears to occur throughout the wall of both hyphae and conidia, rather than in a discrete layer. Zonneveld (1972) proposed that α-1,3 glucan is a major storage carbohydrate and showed that the glucan content in the of walls of *A. nidulans* decreased if only 0.8% glucose was present in the medium. No change in the labelling pattern was found during this work between fungi grown on minimal and full media, but the minimal medium contained 2% (w/v) sucrose and this is above the glucose content used by Zonneveld (1972). As reviewed in Section 1.8 wall composition is

very important to the morphology of the fungus and with a MAb such as S4D1 the changes within the cell wall could be studied by immunofluorescence and immunogold labelling to reveal more extensive data. If α-1,3 glucan is a storage carbohydrate rather than a functional wall component in terms of morphology S4D1 may help to reveal this.

The MAb was used to localise α-1,3 glucan in other fungi besides *N. crassa* and revealed that it showed differential labelling in these fungi as described in Section 5.3.2. This reveals two aspects: one is the surface distribution of α-1,3 glucan in different fungi. As an example, distribution of the glucan at cell surface of *P. chrysogenum* compared to the distribution at the surface in *A. muscaria*. Secondly, it reveals the distribution of the glucan throughout the different fungal genera. The glucan was not present in fungi from the Zygomycotina, as might be expected (Bartnicki-Garcia, 1968), but it was present in fungi from all of the other major groups, the Asco-, Basidio- and Deuteromycotina.

For *N. crassa* the MAb appeared to be an ideal candidate for quantification. It recognised cells in all the different stages of the life cycle and it appeared to show relatively uniform distribution of the epitope. A relationship was found between the fungal biomass and the epitope recognised by MAb S4D1 when measured by absorbance. A linear range appeared to occur between 1.0 and 0.1μg freeze-dried weight, but further work is needed to confirm this result. The dose-response curves for antigen do not show the same range as antibody dose-response curves (Tijssen, 1985). A similar linear relationship was found using a MAb which recognised a Flavobacterium (Mason, pers. comm., 1989). Dose response curves have been reported for fungal antigens using antisera, (Johnson *et al.*, 1982; El-Nashaar *et al.*, 1986; Gleason *et al.*, 1987), but not for MAbs raised against fungi. The MAbs used in the Agri-Diagnostic kits have been related to fungal biomass, but this has not been reported in the literature. They give their data in terms of risk of disease in a green-amber-red colour code, in which red means that fungicide should be applied, amber it would

be better to spray and green no need for spraying. Similar linear relationships between fungal biomass (dry weight) and the measurement of a single fungal component have been reported. This was reviewed in Section 1.5 These techniques can only be used to quantify either the whole fungal population biomass, or the biomass of a single fungus when it is the only fungus present. Similarly quantification with antisera has been limited because of the non- specificity of the antiserum (see Section 1.4). It has been proposed by some workers that MAbs would be able to overcome this problem by specific recognition of one fungus (Matcham *et al.*, 1984), however, this is not a straight forward solution.

The first problem is the production of a species-specific MAb and this will be discussed in more detail in Chapter 9. The other problem is the production of a species-specific MAb which recognises an epitope with a linear relationship with fungal biomass. Although no evidence was found in this quantification study, or in any of the other work that the quantity of the S4D1 epitope varies in *N. crassa* further work is needed to establish whether the epitope content of *N. crassa* cell walls varies with nutritional status of the fungus, and whether any change is reflected in the relationship between the epitope and biomass as expressed by a dose-response curve.

In conclusion, MAb S4D1 recognises α-1,3 glucan which is widespread throughout the fungal kingdom. The distribution of the glucan at the cell surface was found to be different in different fungi. This might be related to differences in wall ultrastructure in different fungi, or it might be that in different fungi the glucan has different functions within the wall. MAb S4D1 can be used to quantify *N. crassa* biomass, but this has not yet been successfully carried out for cell walls extracted from a solid substrate.

Chapter Six

The monoclonal antibody S3B3

6.1. Introduction.

This chapter describes the characterisation of the MAb S3B3: its reactions with different fungi and the localisation of the epitope in cell walls by immunogold electron microscopy.

6.2. Antibody subclass.

Using Ouchterlony immunodiffusion Mab S3B3 was characterised as an IgM immunoglobulin.

6.3. The immunofluorescence reactions of MAb S3B3.

6.3.1. With its homologous fungus, *N. crassa*.

S3B3 recognises an epitope present in the cell walls of *N. crassa* (see Fig. 4.2). From immunofluorescence studies it can be seen that the epitope is also present at the ends of conidia isolated from 3 to 30 day old cultures (Fig. 6.1). The fluorescence pattern reveals that this epitope is not present at the ends of all conidia. On conidia which do show fluorescence, different patterns of fluorescence are seen. Some conidia exhibit fluorescence at one end only, some at both ends of the conidia or, in the case of conidia which have been at a branching point in a conidial chain, at three points around the conidium. These different patterns of fluorescence are shown in Figs. 6.2 and 6.3.

Fig. 6.1. The fluorescence pattern exhibited by conidia of *Neurospora crassa* following reaction with MAb S3B3.

(a) fluorescence was seen at the ends of conidia, but not all conidia in the population showed fluorescence. Fluorescence was seen at both ends of some conidia, but only at one end of others (arrowed).

(b) Phase contrast of (a)

Scale bar represents 5μm.

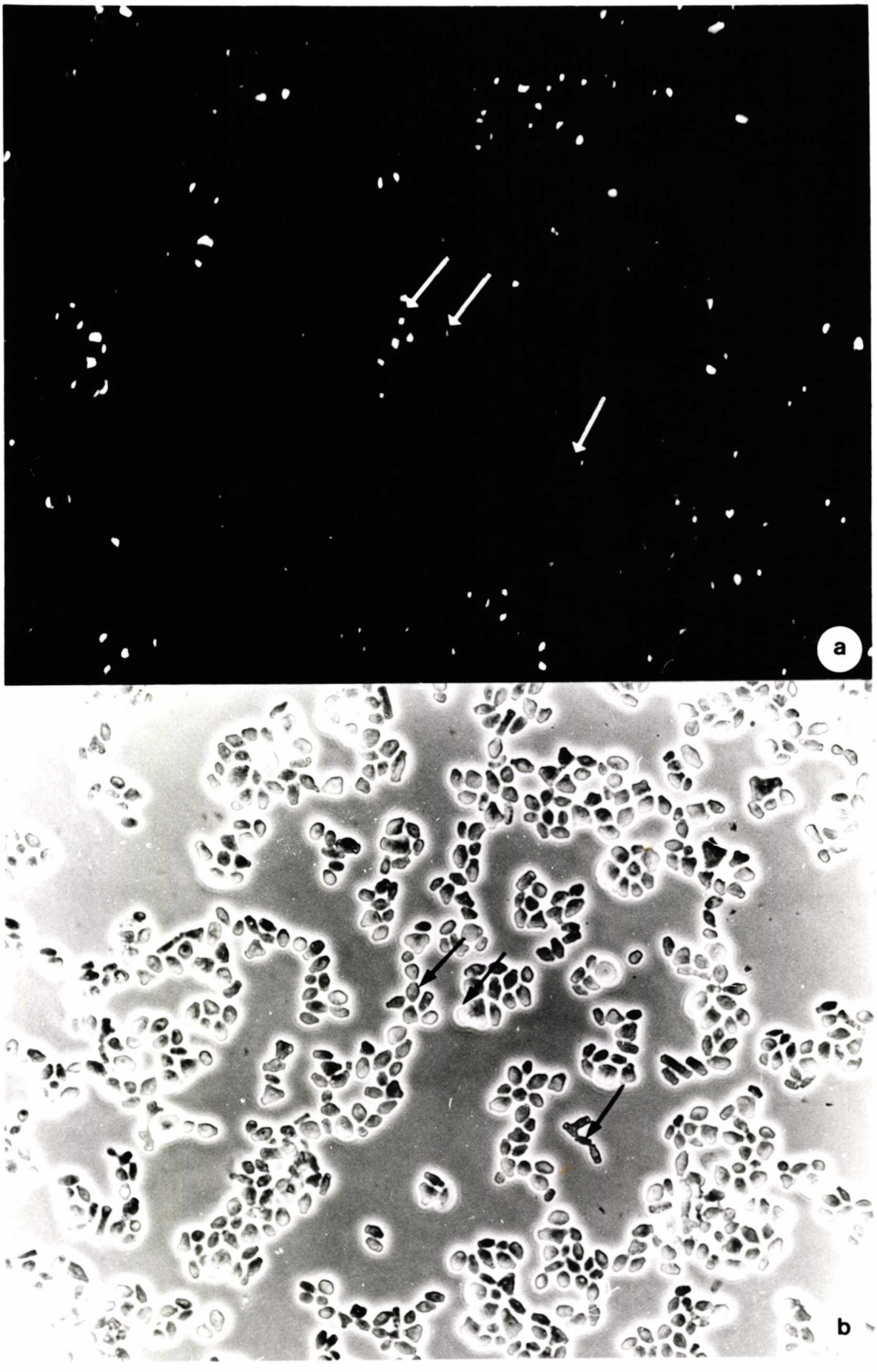

a
b

Fig. 6.2. The fluorescence patterns exhibited by conidia of *Neurospora crassa* following reaction with MAb S3B3.

(a) Fluorescence was seen at one end of some conidia, but not at all on others.

(b) Phase contrast of (a).

(c) Fluorescence was seen at one end of some conidia.

(d) Phase contrast of (c).

Scale bar represents 5μm and refers to all micrographs.

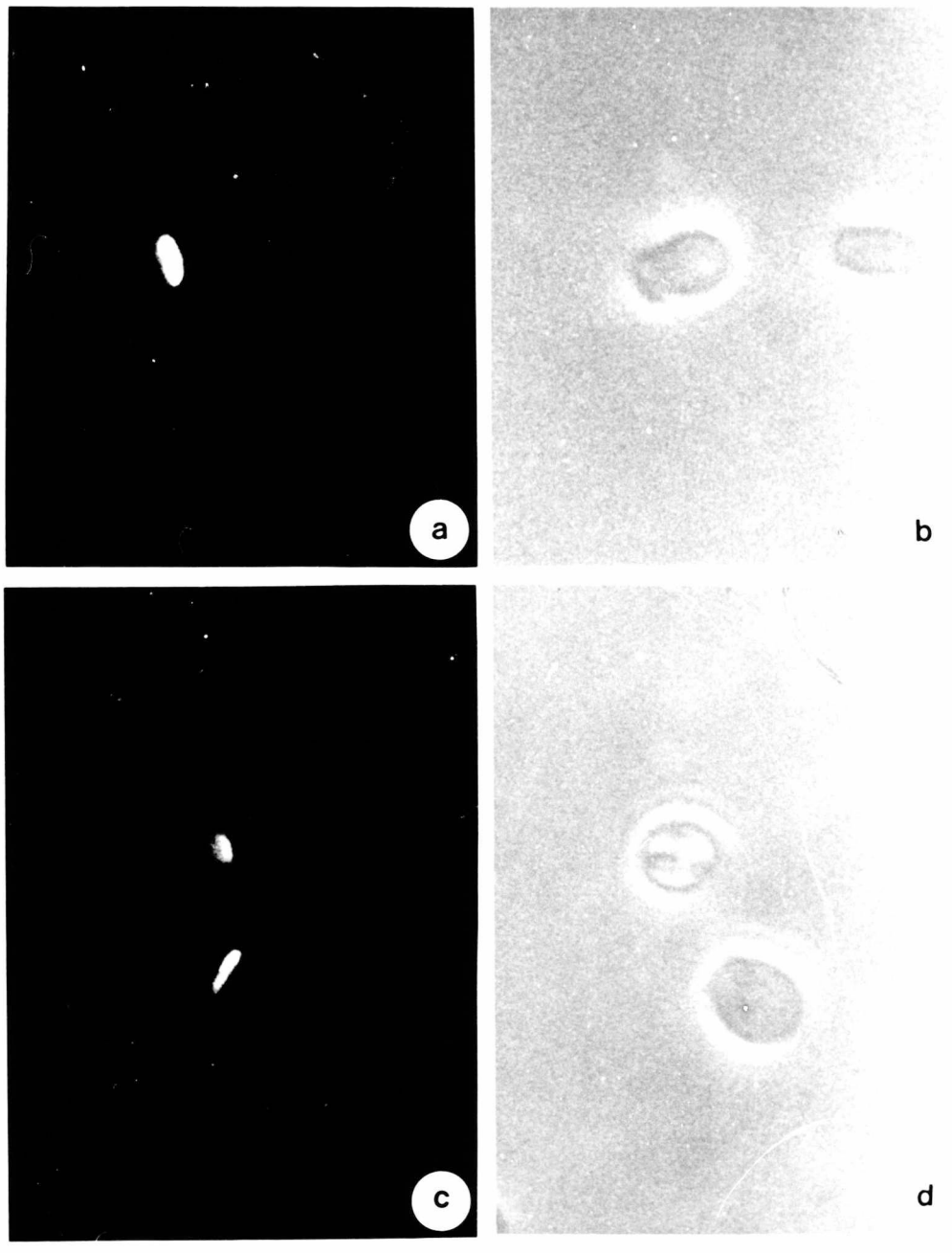
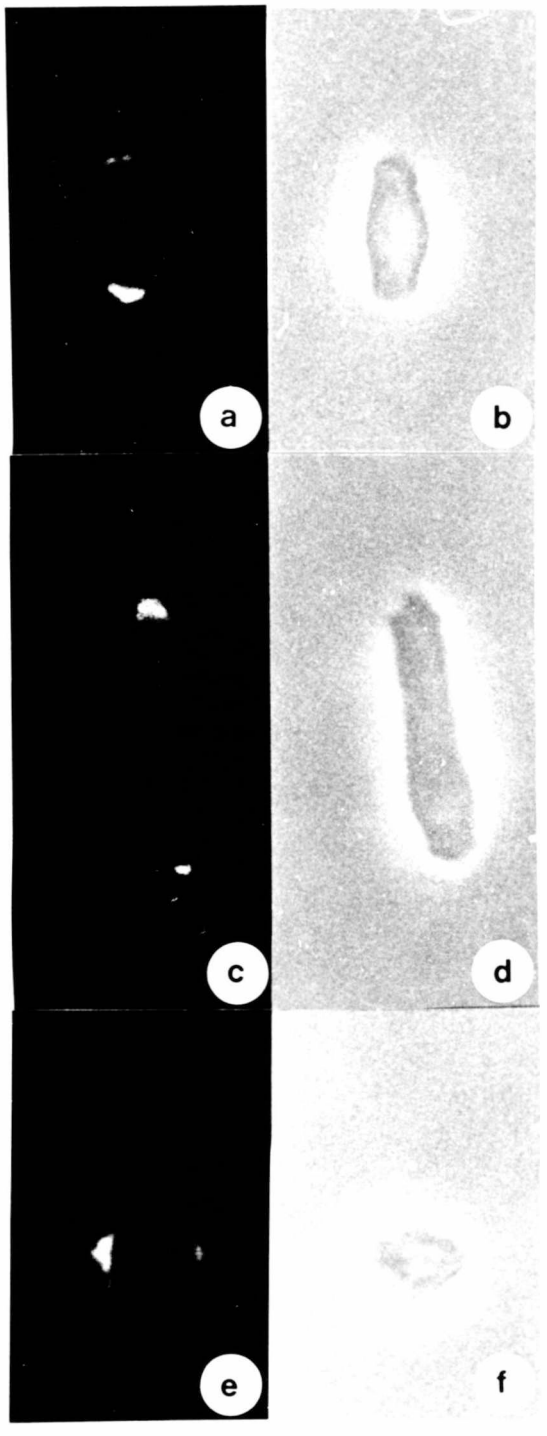

a
b
c
d

Fig. 6.3. The fluorescence patterns exhibited by conidia of *Neurospora crassa* following reaction with MAb S3B3.

(a), (c) and (e) Fluorescence was seen at both ends of some conidia.

(b), (d) and (f) Phase contrast of (a), (c), and (e), respectively.

Scale bar represents 5μm and refers to all micrographs.


a
b
c
d
e
f

Analysis of the distribution of the S3B3 epitope on *N. crassa* conidia reveals that in a population of conidia 32% of the conidia show fluorescence. These data are shown in Table 6.1, from which it can be seen that this percentage, 32%, refers to counts of conidial fluorescence which included conidia present in conidial chains, as well as individual discrete conidia. If only conidia which have become separated from chains are assessed, 18% show fluorescence.

Total conidia	Total fluorescing	Fluorescence		
		One end	Both ends	At 3 points
All conidia including those in chains.				
919	295	130	154	11
% of total	32	14	17	1
Discrete conidia only.				
311	55	21	32	2
% of total	18	7	10	0.6

Table 6.1. The fluorescence of *N. crassa* conidia with MAb S3B3.

In the population of conidia which do show fluorescence the ratio of conidia with fluorescence at one end to those with fluorescence at both ends (including those with fluorescence at three points around a conidium) is approximately as follows:

One end : Both ends

2 : 3

This ratio does not vary when discrete conidia only are assessed, unlike the overall percentage of conidia showing fluorescence.

On finding these differences in the distribution of the S3B3 epitope on *N. crassa* conidia it was decided to study the distribution of the S3B3 epitope in conidial chains. In a conidial chain the fluorescence occurs in the septal region (see Fig. 6.4); however, not all of the septal regions show fluorescence (see Fig. 6.4a, arrowed). The position of a conidium in the chain does not necessarily correlate with the presence or absence of the epitope as shown by fluorescence. For example, in Fig. 6.4a it can be seen that an older septal region does not show fluorescence and younger ones do, whereas in Fig. 6.5 the youngest septal regions do not fluoresce, but the older ones do; *N. crassa* conidia develop basipetally, *i.e.* the new conidium develops at the apex of the conidial chain.. The differential appearance of the S3B3 epitope in the septal region of conidial chains correlates with the differential appearance of the epitope in conidia separated both from the conidiophores and from other conidia. The fluorescence exhibited by the conidia does not disappear as a germ tube develops.

S3B3 also reacts with the septal region of vegetative hyphae (Fig. 6.6). As in the case of the conidial septal regions, not all of the septa exhibit fluorescence. The fluorescence appears as a halo around the septum.

6.3.2. With heterologous fungi.

S3B3 cross-reacts with the other *N. crassa* isolate, CMI 53420, and also the two other members of the genus, *Neurospora*, *N. sitophila* and *N. tetrasperma* (Fig. 6.7). The fluorescence pattern seen is the same as that seen with *N. crassa*.

S3B3 showed no reaction with *S. fimicola*, or *P. paucieta*. Nor did it show any reaction with any part of cells of the yeast, *Saccharomyces cerevisiae*. S3B3 did not react with the ends of the arthroconidia of *G. candidum*, which are also formed basipetally. In a mixture of *N. crassa* and *G. candidum* S3B3 differentiated germlings of *N. crassa* from conidia of *G. candidum* (Fig. 6.8). S3B3 did react with the ends of conidia from *P. chrysogenum* (Fig

Fig. 6.4. The fluorescence patterns exhibited in conidial chains following reaction with MAb S3B3.

(a) Fluorescence was localised in the septal region of the conidial chains, but fluorescence was not seen in all of the septal regions. The arrows indicate an older septal region which does not show fluorescence.

(b) Phase contrast of (a).

(c) A conidial chain in which all of the septal regions show fluorescence.

(d) Phase contrast of (c).

Scale bar represents 5μm and refers to all micrographs.

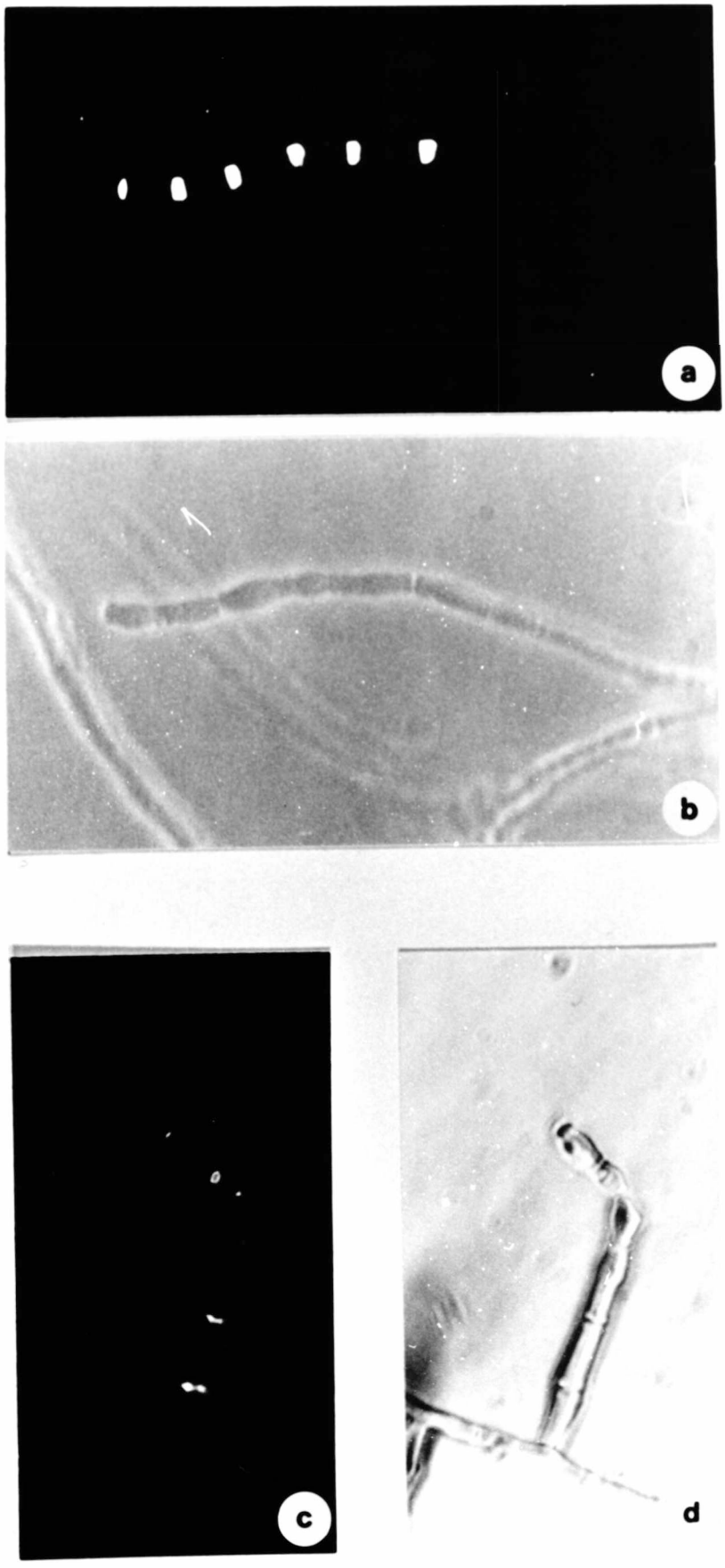

a
b
c
d

Fig. 6.5. The fluorescence patterns seen in conidial chains following reaction with MAb S3B3.

(a) Fluorescence was seen at the end of a conidium, corresponding to the septal region, but fluorescence was not seen in the region of other septa in the chain.

(b) Phase contrast of (a) showing position of septa.

(c) Fluorescence was seen in the septal region where the septum was seen to have to do so.

(d) Phase contrast of (c) showing orientation of septa.

Scale bar represents 5μm and refers to all micrographs.

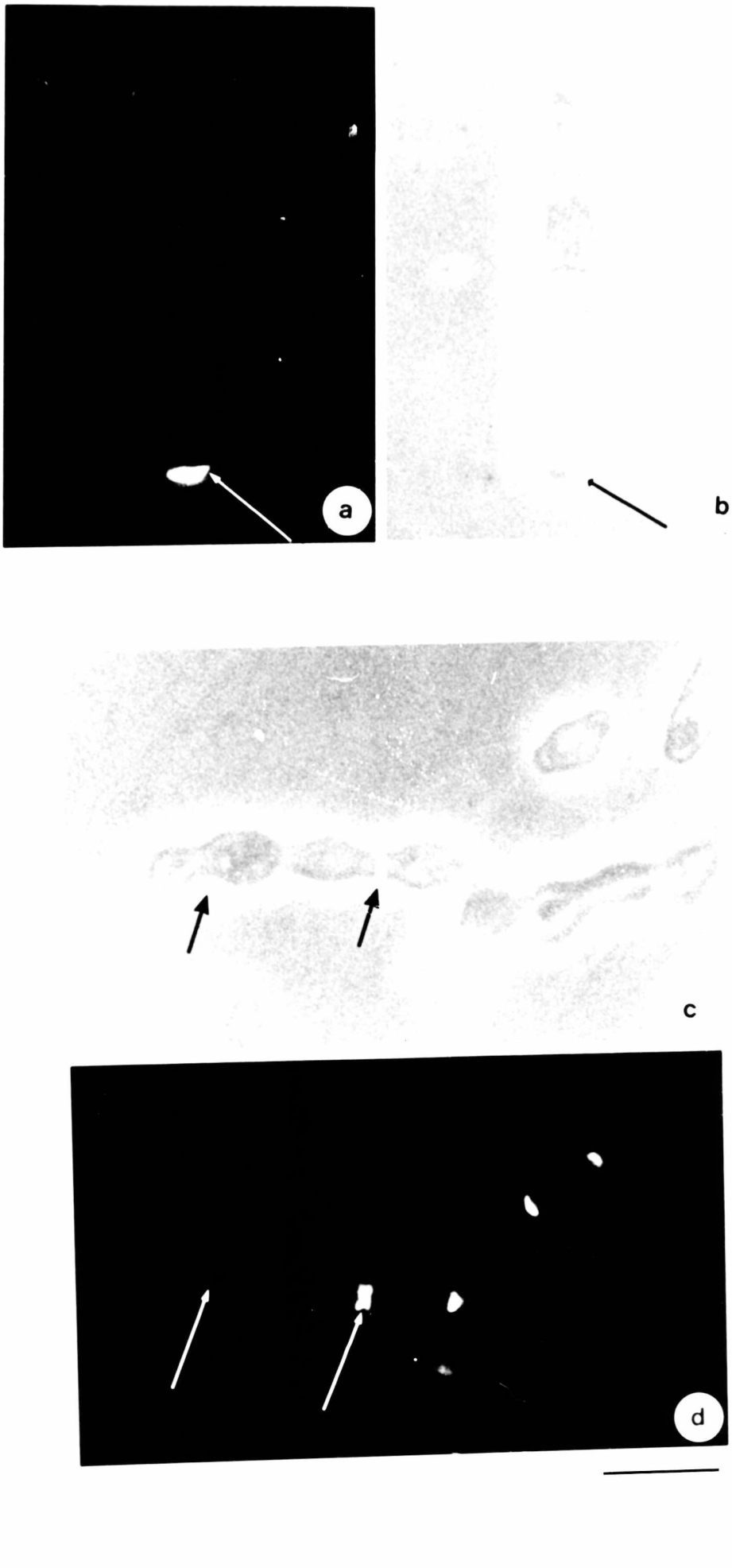

a
b
c
d

Fig. 6.6. The fluorescence pattern exhibited by hyphae of *Neurospora crassa* following reaction with MAb S3B3.

(a) Fluorescence was seen in the septal regions of the hyphae. The fluorescence was brightest at the ends of the septa indicating the presence of a higher level of the epitope at those points. Different levels of fluorescence were seen on different septa.

(b) Phase contrast of (a).

Scale bar represents 5μm.

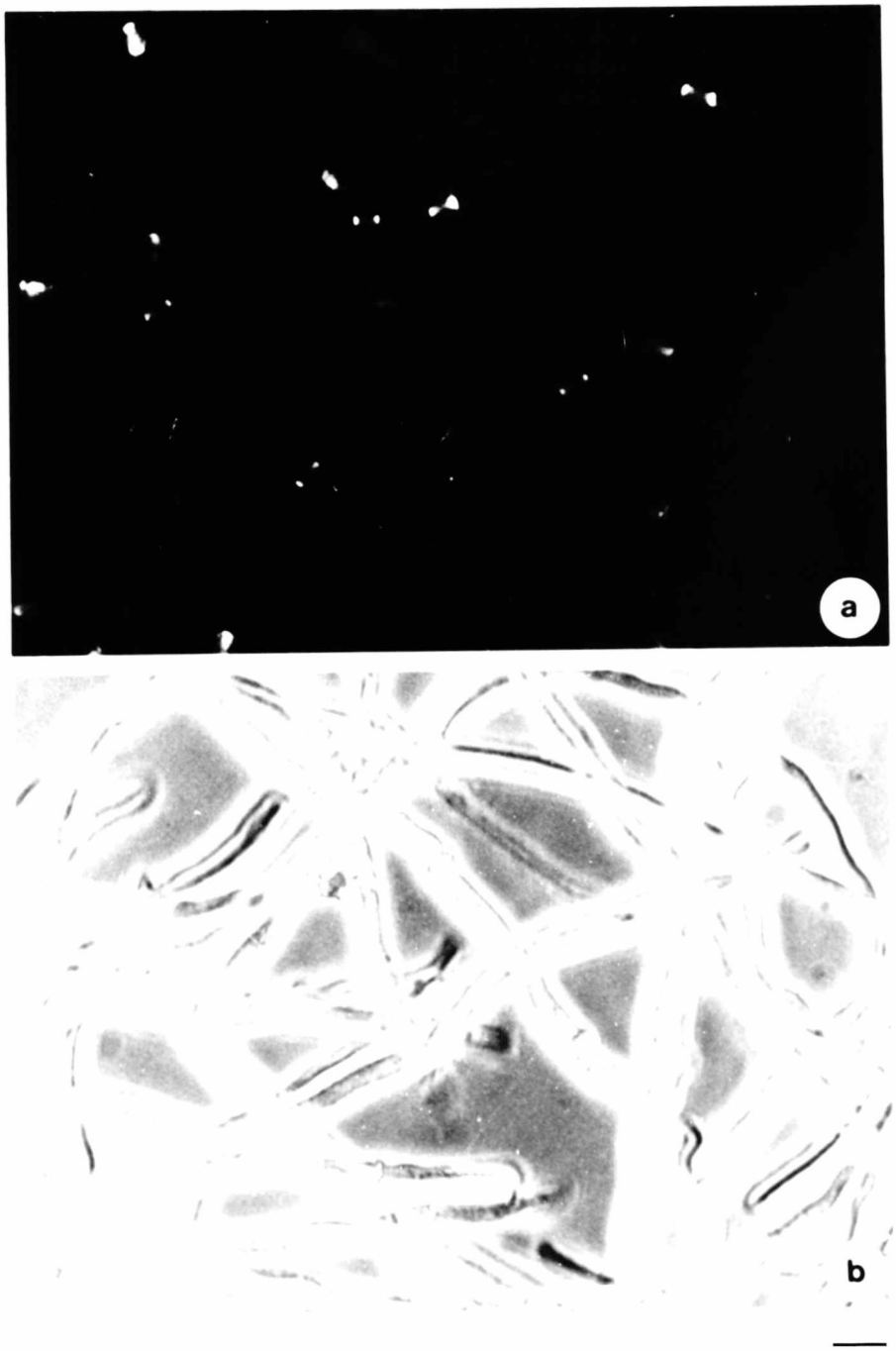

a
b

Fig. 6.7. The fluorescence patterns exhibited by *Neurospora tetrasperma* and *Neurospora sitophila* following reaction with MAb S3B3.

(a) Fluorescence was seen in the septal region of hyphae of *N. tetrasperma*

(b) Phase contrast of (a).

(c) The fluorescence pattern seen in a population of conidia from *N. sitophila* corresponded to that seen in conidia of *N. crassa* (see Fig. 6.1.), *i.e.* not all conidia showed fluorescence at their ends and of those that fluoresced some showed fluorescence at one end and some at both ends.

(d) Phase contrast of (d).

Scale bar represents 5μm and refers to both sets of micrographs.

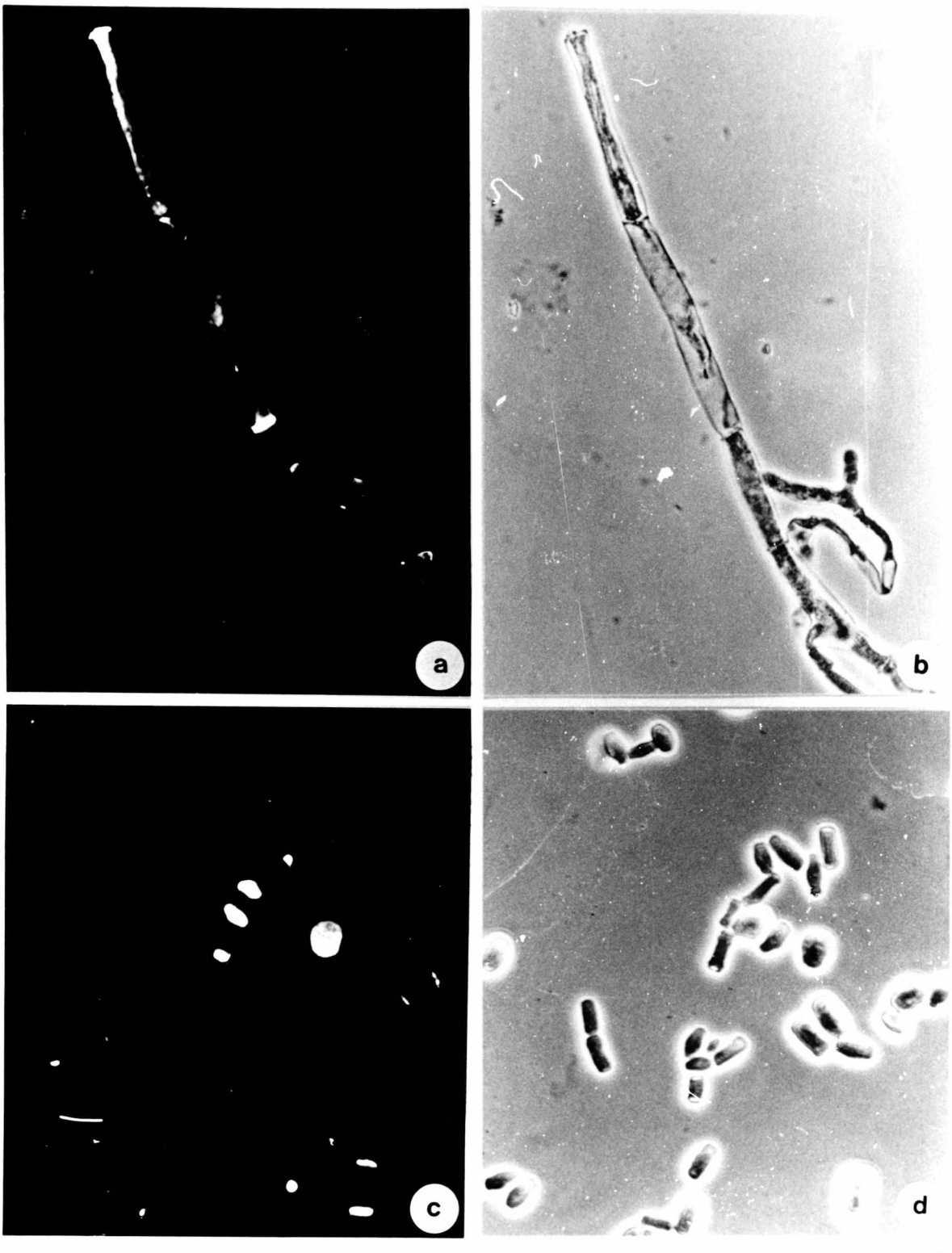

a
b
c
d

Fig. 6.8. The differentiation of germlings of *Neurospora crassa* and arthroconidia of *Geotrichum candidum* using MAb S3B3.

(a) The surface of the germlings of *N. crassa* showed weak fluorescence which enabled them to be seen in a mixture with arthroconidia of *G. candidum*. Some ends of conidia from *N. crassa* showed bright fluorescence and it was seen that the fluorescence was present on the conidia after germ tube emergence.

(b) Phase contrast of (a)

Scale bar represents 5μm.

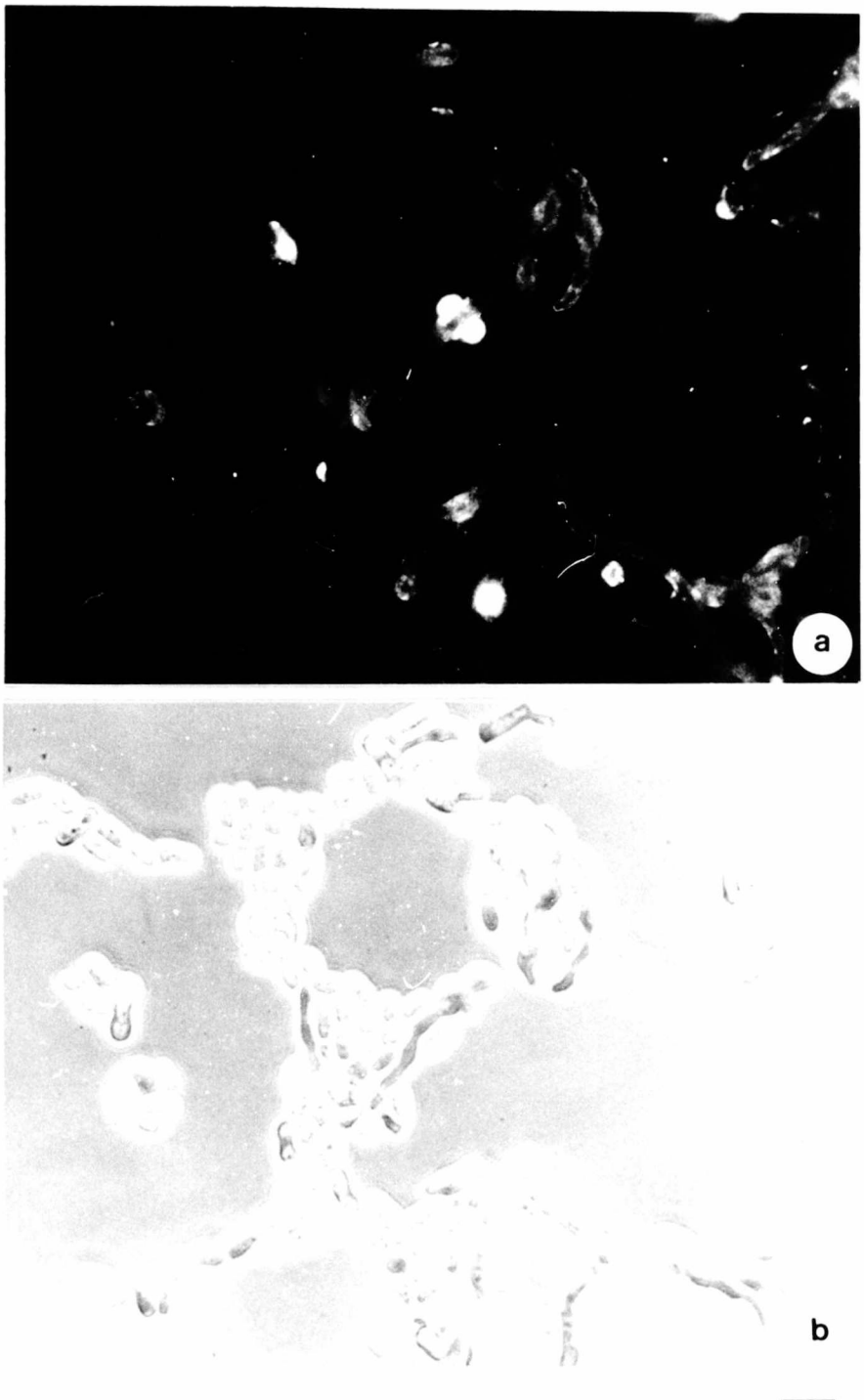

a
b

Fig. 6.9. The immunofluorescence reactions of *Penicillium chrysogenum*, *Aspergillus nidulans* and *Paxillus involutus* with the MAb S3B3.

(a) The fluorescence pattern exhibited by conidia of *P. chrysogenum* was similar to that exhibited by conidia of *Neurospora crassa*, *i.e.* fluorescence was seen at the ends of conidia, corresponding to the end adjacent to other conidia in a conidial chain, but not all conidia showed fluorescence.

(b) Phase contrast of (a).

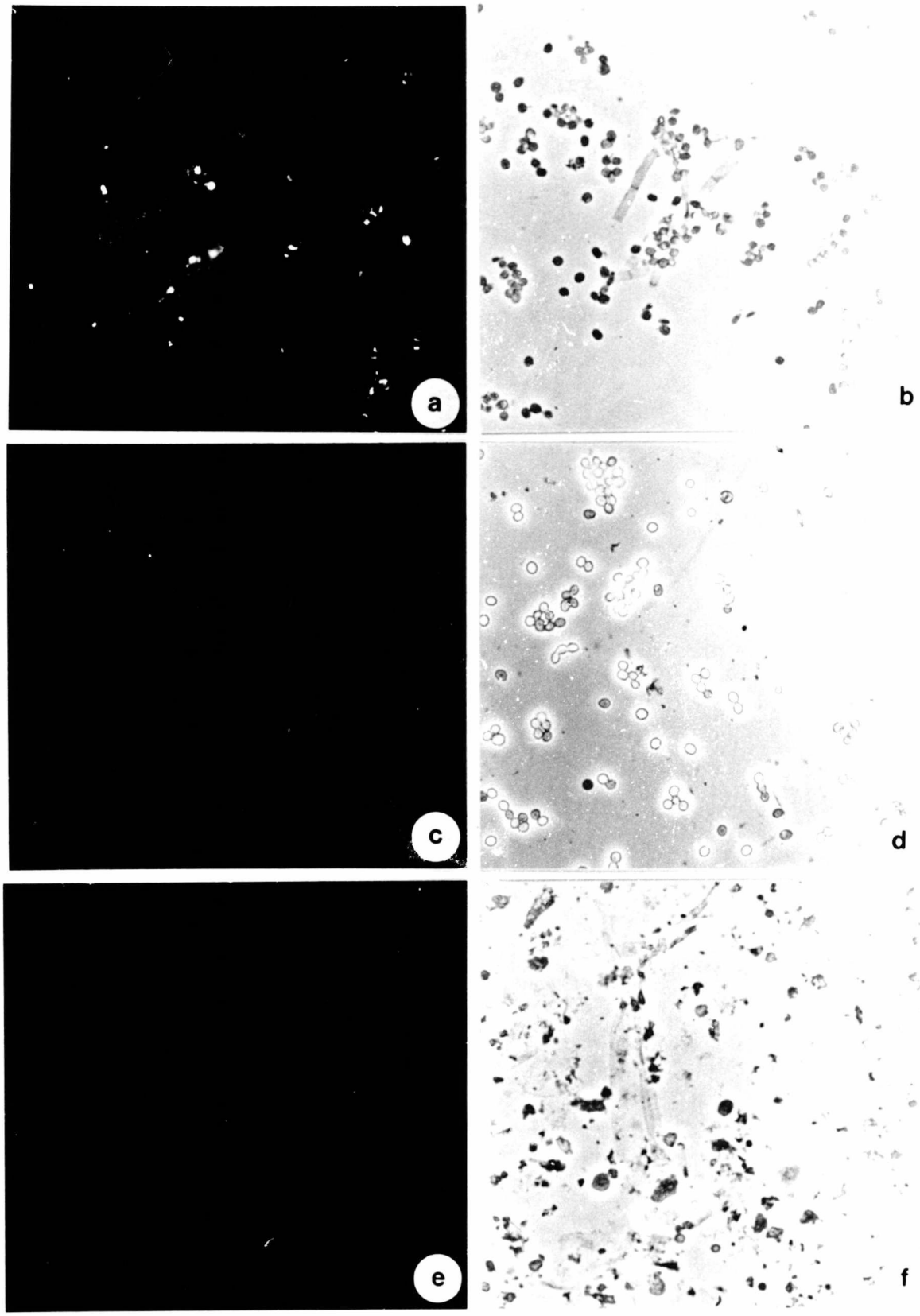
(c) Conidia of *A. nidulans* showed no reaction with S3B3.

(d) Phase contrast of (c).

(e) Hyphae and basidiospores from a fruiting body of *P. involutus* did not react with S3B3.

(f) Phase contrast of (e).

Scale bar represents 5μm and refers to all micrographs.


a
b
c
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e
f

6.9a), but it did not react with conidia from *A. nidulans* (Fig. 6.9c).

No reaction was seen with any member of the Basidiomycotina tested. These included *P. involutus* (Fig. 6.9e), *S. commune, Thelephora terrestris* and *Agaricus bisporus*. No fluorescence was seen on the spores of *M. hiemalis*.

6.4. The localisation of the S3B3 epitope in *N. crassa* using immunogold electron microscopy.

The S3B3 epitope is present in the walls of hyphae (Fig. 6.10a) and in the septal region (Fig. 6.10b) of hyphae. Labelling was also seen in the septal region of conidial chains (Fig. 6.11a). Labelling was seen on the ends of conidia, as might be expected from the fluorescence pattern shown, but the label was also seen on walls of conidia (Fig. 6.11b and c). The timing of the appearance of the epitope with regard to septal formation was not established.

6.5. The biochemical nature of the S3B3 epitope.

MAb S3B3 recognised *N. crassa* cell walls in an ELISA but it did not react with any of the cell wall fractions prepared using the Mahadeven & Tatum (1965) method, or any single fungal wall component tested. The MAb did not recognise any polypetide extracted in LSB, when tested by Western blotting. Unlike S4D1, S3B3 did not react with the LSB extracted wall material following slot-blotting (see Fig. 5.14).

6.6. Discussion.

MAb S3B3 recognises a component of *N. crassa* which is localised within the wall. Immunofluorescence study of *N. crassa* reveals that the epitope is only exposed at the fungal surface during and after the formation of septa.

Fig. 6.10. The localisation of the S3B3 epitope in the hyphal wall and septal region of *Neurospora crassa* using immunogold electron microscopy. .QE

(a) The epitope was found in the walls and septal region of hyphae.

Scale bar represents 0.5μm.

(b) A higher magnification micrograph of the septal region seen in (a).

Scale bar represents 0.5μm.

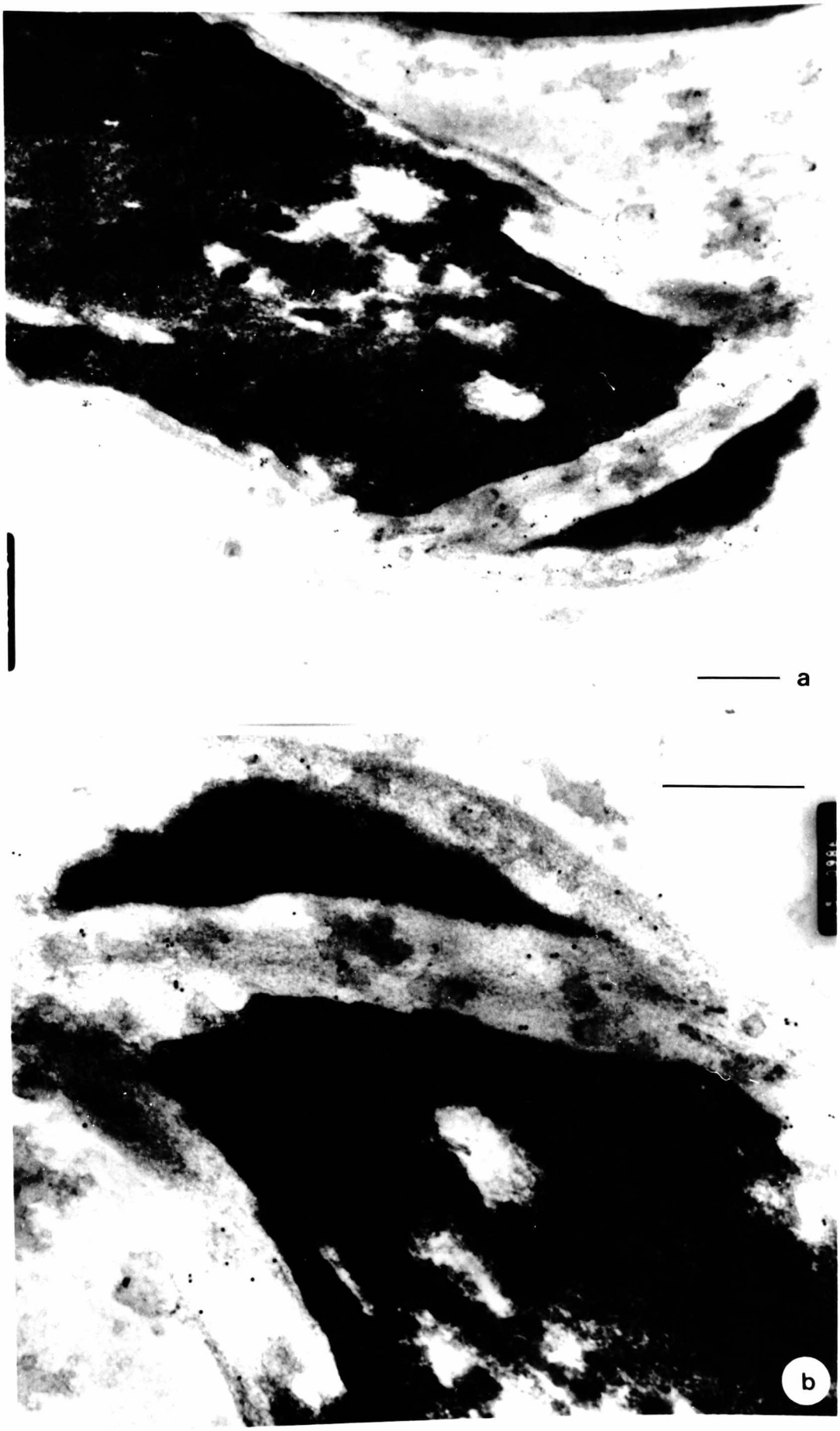
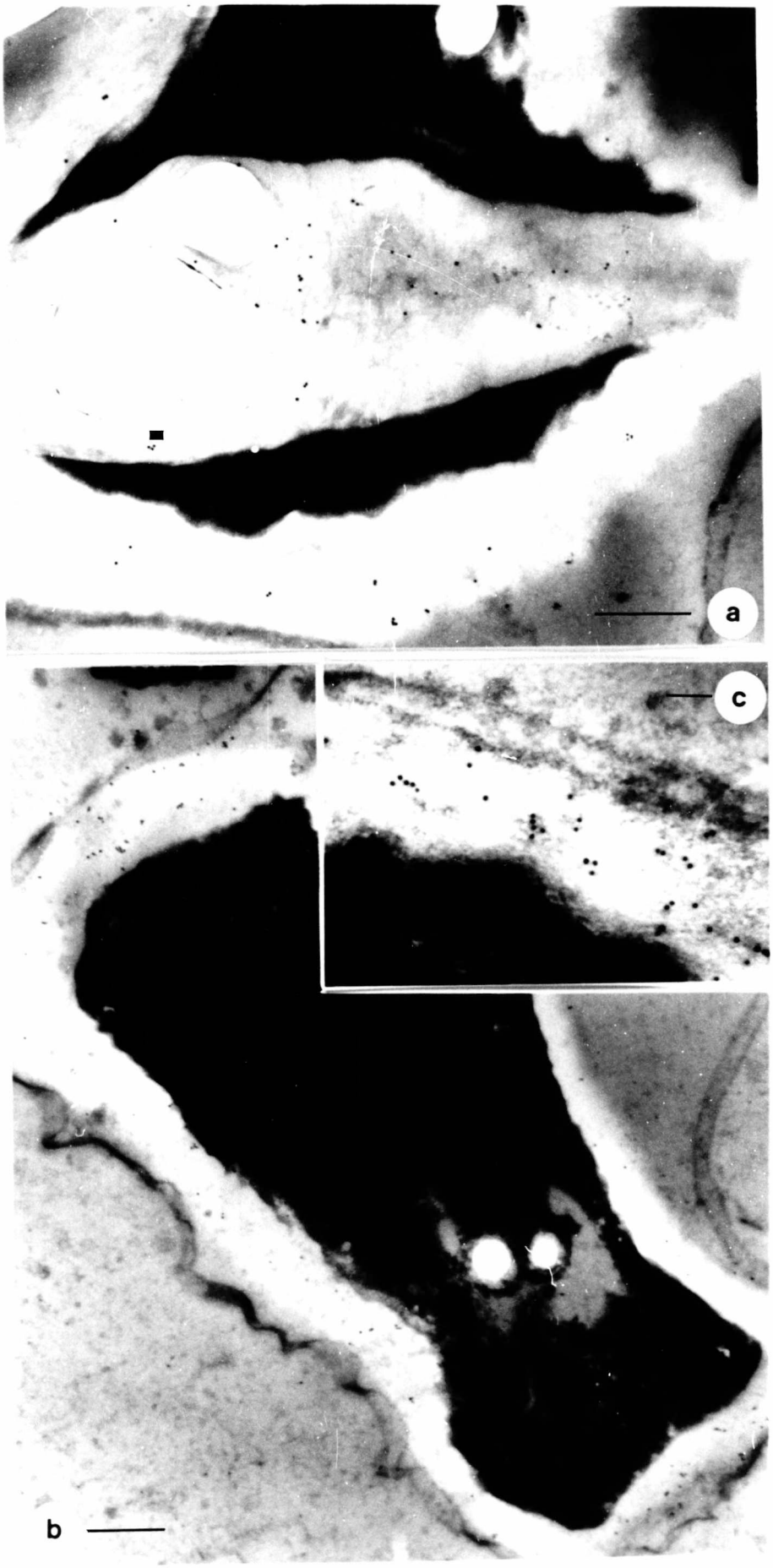

a
b

Fig. 6.11. The localisation of the S3B3 epitope in the conidial wall and septal region of *Neurospora crassa* using immunogold electron microscopy.

(a) The epitope was found in the septal region of conidial chains.
Scale bar represents 0.5μm.

(b) The epitope was present in the inner wall layers of conidia.
Scale bar represents 0.5μm.

(c) The gold label was associated with the inner conidial wall and not the two layer rodlet layer which can be seen clearly in this micrograph.
Scale bar represents 0.1μm.


a
c
b

Hunsley & Kay (1976) described an antiserum that only reacted with cell walls at the site of hyphal breakages, but this antiserum was raised against Fraction IV of *N. crassa* cell walls, the chitin fraction. They did not describe the reaction of the antiserum with septa. S3B3 does react with hyphal fragments at breaks, but did not react with chitin or N-acetyl-glucosamine, the chitin sub-unit. Hunsley & Gooday (1974) showed that chitin is incorporated in the septal region of *N. crassa*, and therefore a MAb which reacted with chitin would be expected to react with all septa.

The characteristic fluorescence pattern of S3B3 was the the most frequently seen pattern when hybridoma supernatants were screened and was the first pattern spotted. This suggests that the epitope may be an immunodominant component of cell walls. The nature of the epitope is not yet known. The MAb did not react with any of cell wall components tested, from *N. crassa* or any other fungus. The MAb did not react with any protein removed from the cell wall either. It might be that the epitope is present in one of the the four *N. crassa* fractions, or the proteins extracted, but its antigenicity is destroyed by the extraction treatments.

Chapter Seven

The monoclonal antibody S1E5

7.1. Introduction.

The characteristics of the MAb S1E5 are described in this chapter: its immunofluorescence reaction and immunogold labelling of *N. crassa*. This was the last MAb successfully derived from a mouse injected with *N. crassa* cell walls.

7.2. Antibody subclass.

MAb S1E5 was characterised as an IgM antibody using immunodiffusion.

7.3. The immunofluorescence reaction of MAb S1E5 with *N. crassa*.

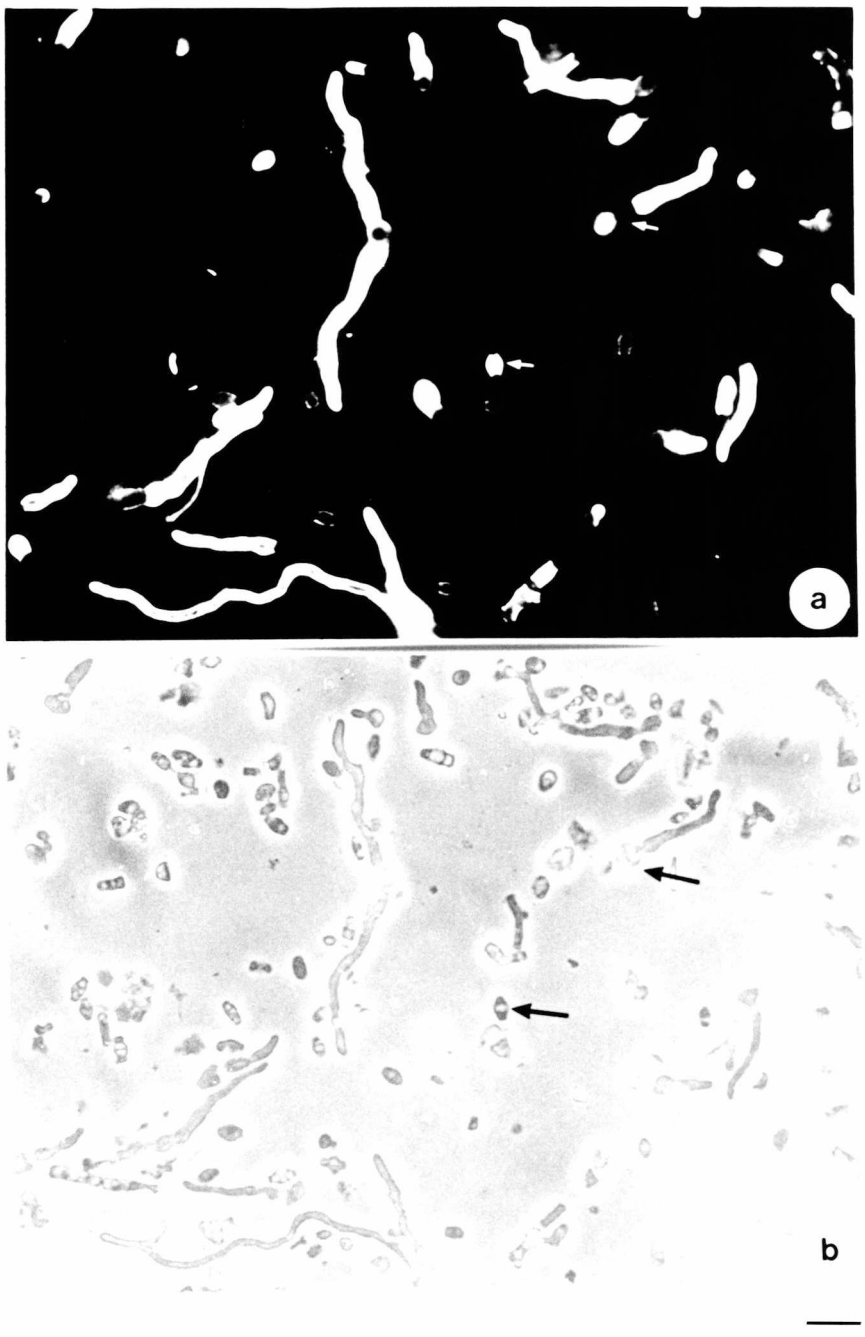
S1E5 reacts with an epitope present on the surface of *N. crassa* germ tubes, but which is not present on the surface of conidia. As a germ tube emerges from a conidium, the epitope is present (Fig 7.1). As the germ tube develops the epitope is present along the length of the germ tube, but is not seen on the conidium. The epitope is present on all germ tubes. Some conidia do, however, have the epitope present (Fig 7.1). The absence of the epitope at the surface of mature conidia suggests that the epitope is a hyphal wall component that is either lost as the conidia mature on the conidiophore or is covered by another wall component in the mature conidia. The conidia which do have the epitope present would therefore appear to be young conidia. The reaction of this MAb with heterologous fungi was not carried out and it will be informative to discover the cross-reactions of this MAb in

Fig. 7.1. The fluorescence patterns exhibited by germlings and conidia of *Neurospora crassa* following reaction with MAb S1E5.

(a) Fluorescence was exhibited by germ tubes as they emerged from the conidia and the fluorescence was seen along the whole length of germ tubes as they elongaed. Conidia from which germ tubes had emerged did not show fluorescence (arrowed), but some conidia did show fluorescence (also arrowed).

(b) Phase contrast of (a) with corresponding arrows.

Scale bar represents 5μm.


a
b

comparison to the other three MAbs described.

7.4. The localisation of the S1E5 epitope in conidia using immunogold electron microscopy.

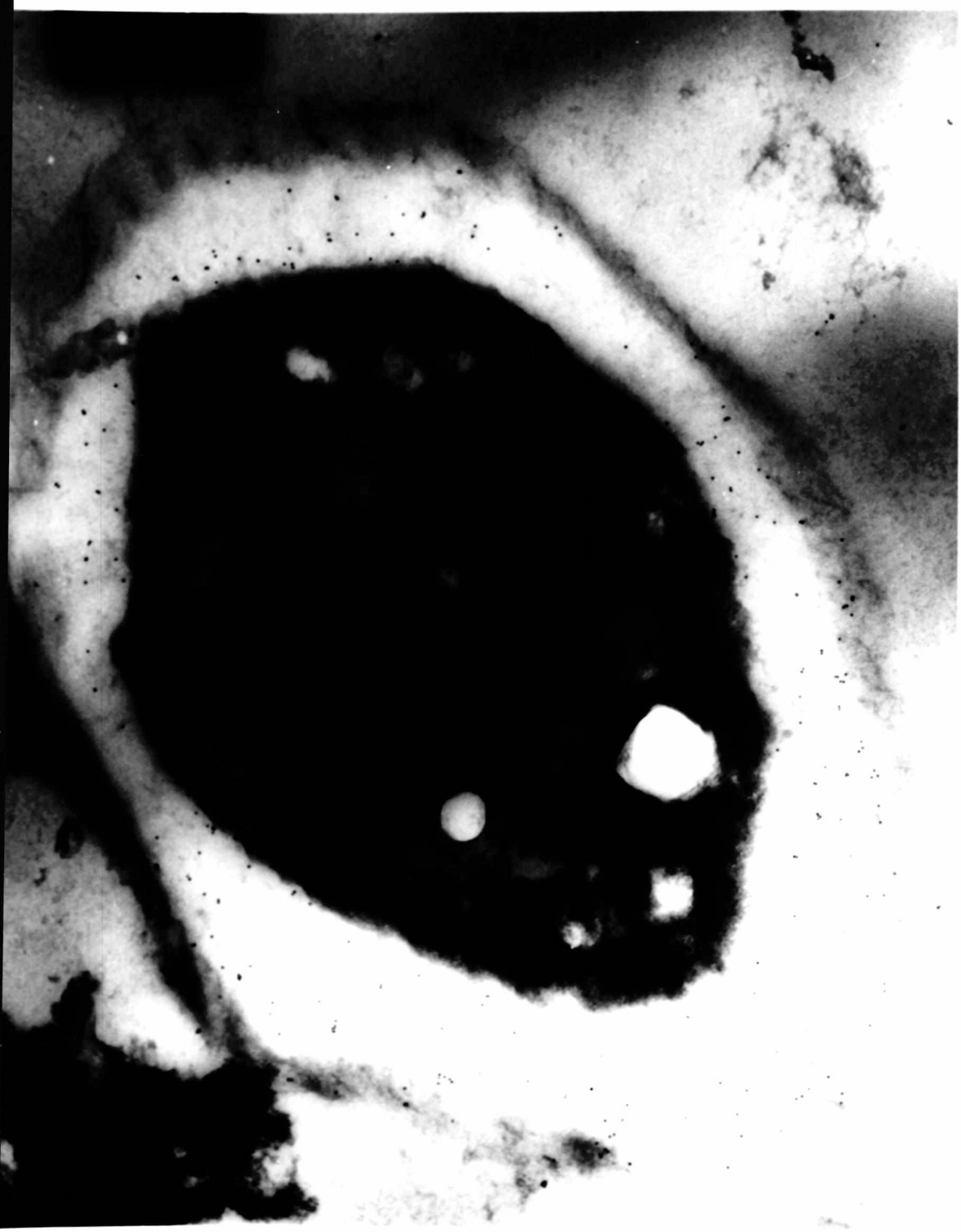
The fluorescence pattern exhibited by conidia showed that the epitope was not present at the surface of conidia, however, immunogold labelling revealed that the epitope is present in the cell wall of conidia (Fig. 7.2). The epitope is found in the inner wall layers, but is not present at the conidial surface.

7.5. Discussion.

The S1E5 epitope is present at the surface of *N. crassa* hyphae, but not conidia. The epitope, however, is present within the conidial wall, as revealed by immunogold labelling. Therefore, MAb S1E5 does not recognise an epitope specific to hyphae, since the epitope is present in the conidia. Two concepts follow from the analysis of this finding, one is that the presentation of an epitope in an antibody-antigen screening technique is crucial to the outcome of the screen; and the second is if this is so then this may well explain why some antibodies have been reported as having reacted with their antigens in some tests and not others (de Boer & Wierczorek, 1984). It also suggests that fungi indicated as having no reaction with a MAb by immunofluorescence screening might have an epitope present, but the MAb cannot gain access to the epitope because of other wall components hiding this epitope. All of the MAbs described in this thesis show differential patterns by immunofluorescence, and this will be discussed with reference to other work in Chapter 9.

Fig. 7.2. The localisation of the S1E5 epitope in the conidial wall of *Neurospora crassa* using immunogold electron microscopy.

The epitope was found throughout the inner wall layers of conidia, and only a few gold particles were seen associated with the outer surface of the wall. Scale bar represents 0.5μm.



Chapter Eight

The monoclonal antibody Pax-1

8.1. Introduction.

MAb Pax-1 was successfully derived from a mouse injected with *P. involutus* cell walls, not *N. crassa* cell walls as were the other three MAbs. In this chapter the immunofluorescence reactions of MAb Pax-1 with *P. involutus* and other fungi are described. The MAb was used in the immunogold labelling of *N. crassa* thin sections and this is also described.

8.2 Immunoglobulin class of MAb Pax-1.

MAb Pax-1 was characterised by immunodiffusion as an IgM class antibody.

8.3 The immunofluorescence reactions of Pax-1.

8.3.1. With its homologous fungus, *P. involutus*.

Pax-1 reacts with hyphal cell walls of *P. involutus*, ITE 16 (Fig. 8.1). It also reacts with the hyphal walls from *P. involutus*, ITE 32 (Fig. 8.2a) and the hyphae and basidiospores from a fruiting body of *P. involutus* (Fig. 8.2c). In a population of fragments, some show fluorescence along their whole length, some show fluorescence along only part of the fragment and others show no fluorescence at all. The strongest fluorescence seen is often associated with the ends of the fragments and this suggests that Pax-1 reacts with internal antigen. It is possible that Pax-1 reacts with an epitope similar in distribution to S1E5; but in

Fig. 8.1. The fluorescence pattern exhibited by cell wall fragments of *Paxillus involutus* (ITE 16) following reaction with MAb Pax-1.

(a) The fluorescence pattern seen on the fragments varied between fragments. Some fragments showed bright fluorescence along their whole length, whereas others had patchy fluorescence and others showed no fluorescence at all (arrowed).

(b) Phase contrast of (a).

Scale bar represents 5μm.

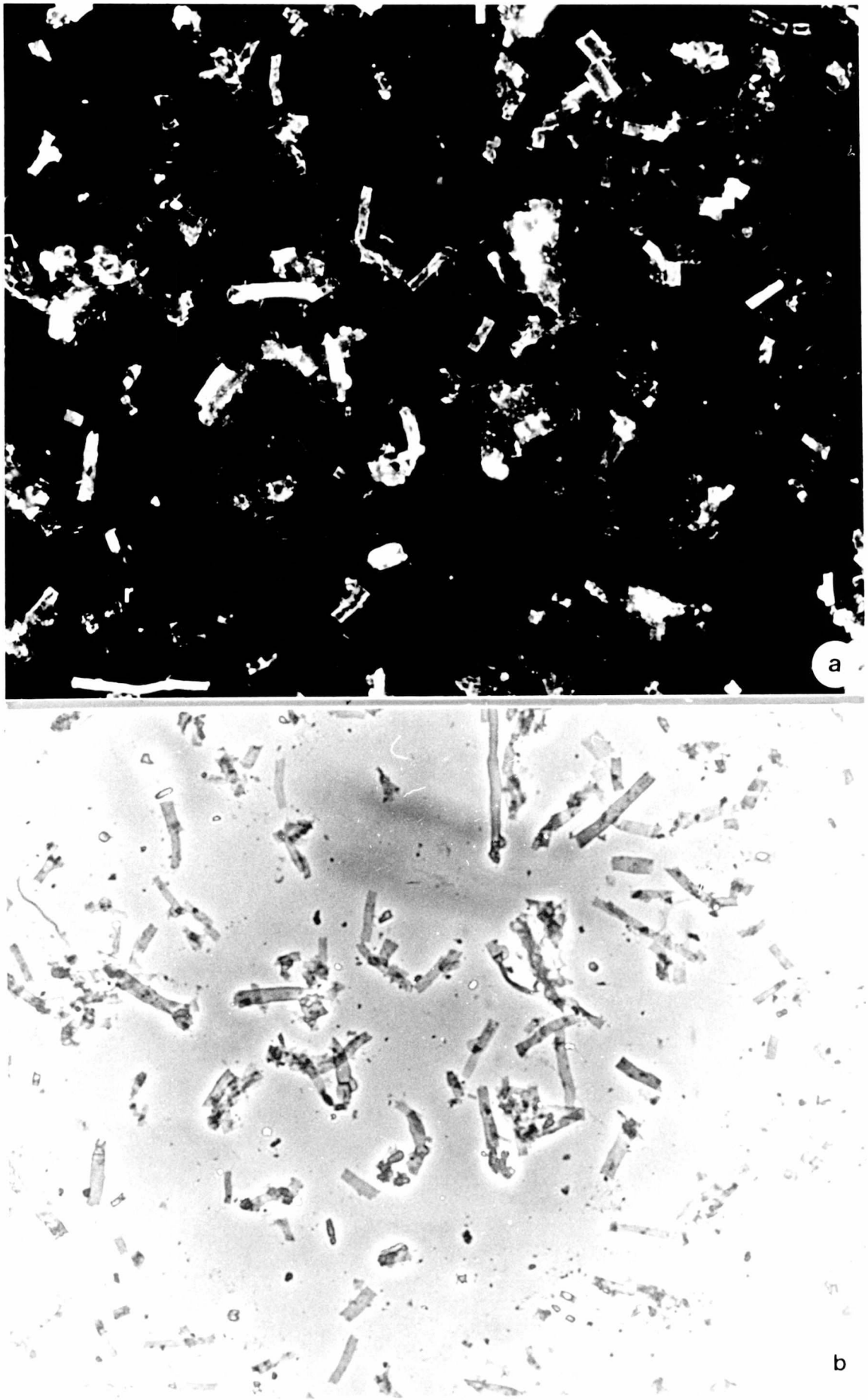

a
b

Fig. 8.2. The fluorescence patterns exhibited by other isolates of *Paxillus involutus* following reaction with MAb Pax-1.

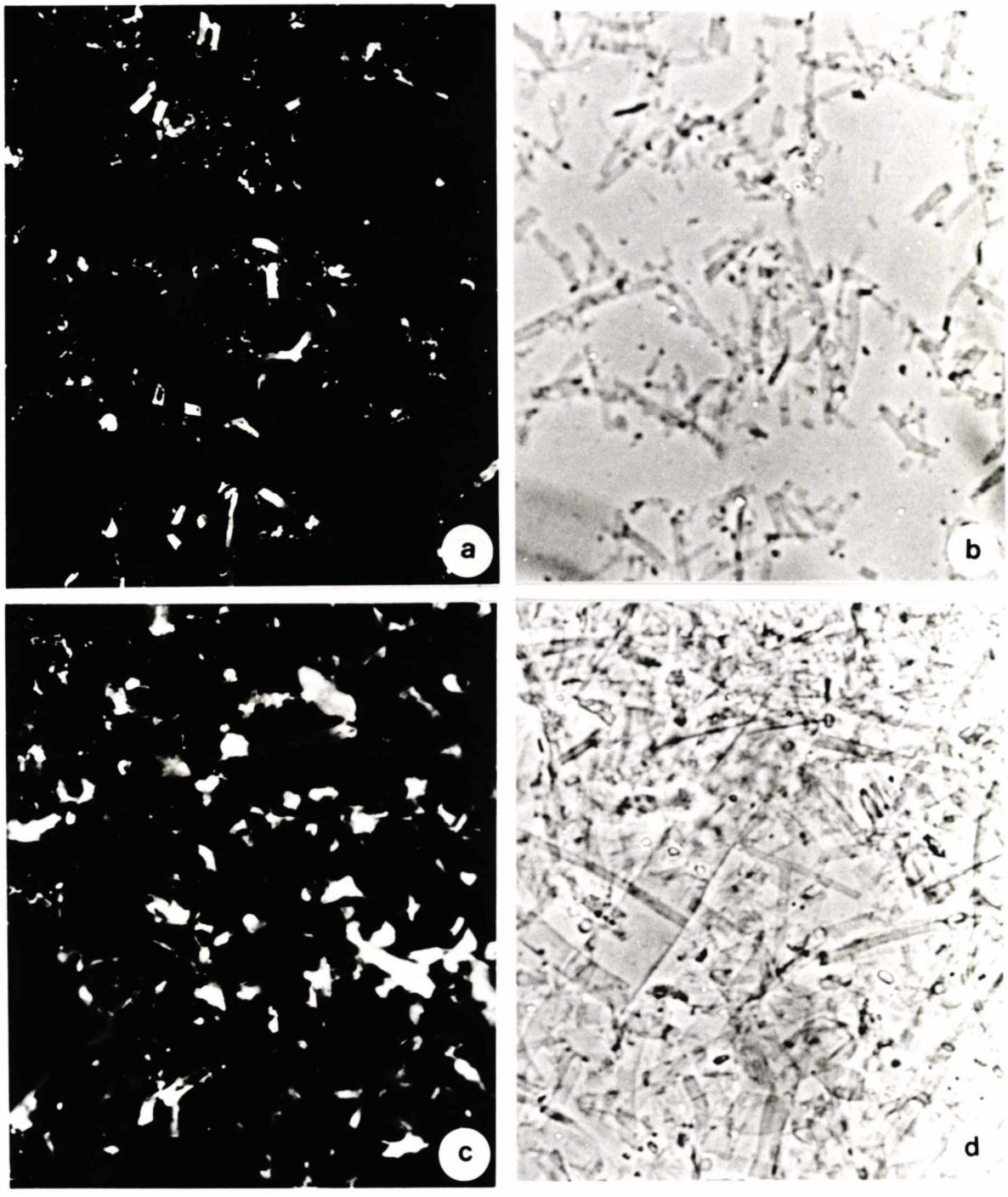
(a) The same pattern of fluorescence was seen on cell wall fragments of *P. involutus* (ITE 32) as on fragments from ITE 16.

(b) Phase contrast of (a).

(c) Hyphae from a fruiting body of *P. involutus* collected on the UKC campus showed a similar reaction to that seen in (a) and Fig. 8.1a.

(d) Phase contrast of (c).

Scale bar represents 5μm and refers to both sets of micrographs.


a
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c
d

P. involutus the epitope is distributed at the surface only at certain times during hyphal development *e.g.* in young hyphae. The pattern seen on mycelial strands did not, however, differ significantly from that on cell wall fragments.

8.3.2. With heterologous fungi.

Pax-1 reacted with all other species of the Basidiomycotina tested. These species were all members of the order Agaricales and were collected as fruiting bodies from the UKC campus (except for *A. bisporus*). The patterns of fluorescence exhibited by hyphae and spores extracted from these fungi were similar to that of *P. involutus*. The fluorescence patterns exhibited by hyphae of *Mycena* sp. and *Pluteus cervinus* were also similar (Fig. 8.3).

Pax-1 did not react with *Mucor hiemalis*, a member of the Zygomycotina. It did, however, cross-react with members of the Asco- and Deuteromycotina. Pax-1 showed the same pattern of fluorescence on *N. crassa* conidia as did S3B3 (Fig. 8.4c). The epitope recognised by S3B3, however, was never shown to be present in immunofluorescence screens of *P. involutus* using S3B3 (see 6.3.2), suggesting that these two MAbs are not identical antibodies. The Pax-1 reacted weakly with conidia of *Penicillium chrysogenum* in the same pattern as with *N. crassa* conidia, and as S3B3. Broken conidiophores and hyphae of *P. chrysogenum* reacted with the MAb (Fig. 8.4a), and the septa, especially, showed strong fluorescence. Pax-1 did not react with *A. nidulans* conidia or *G. candidum* arthrocondia, and only showed weak fluorescence with the germ tubes of *Colletotrichum gloeosporioides*.

Fig. 8.3. The fluorescence patterns exhibited by *Pluteus cervinus* and *Mycena galopus* following reaction with MAb Pax-1.

(a) Bright fluorescence was seen on some parts of the hyphae isolated from a fruiting body of *Pluteus cervinus*. No fluorescence was seen on the surface of basidiospores.

(b) Phase contrast of (a).

(c) The reaction of Pax-1 with the hyphal walls of *Mycena galopus* clearly differentiated the wall fragments from the cytoplasm in a crude preparation of the fruiting body.

(d) Phase contrast of (c).

Scale bar represents 5μm and refers to both sets of micrographs.

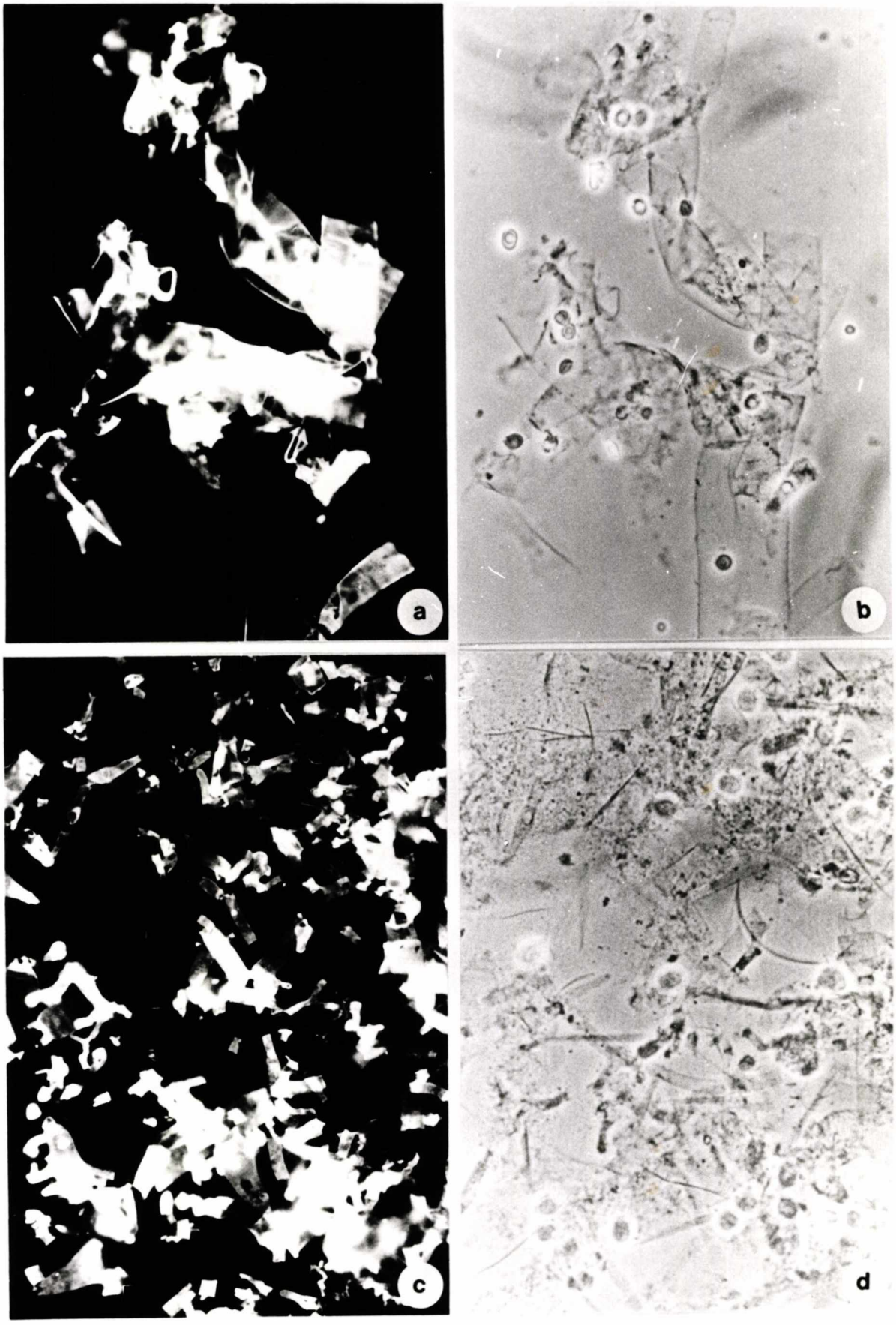
a
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Fig. 8.4. The fluorescence patterns exhibited by *Penicillium chrysogenum* and *Neurospora crassa* following reaction with MAb Pax-1.

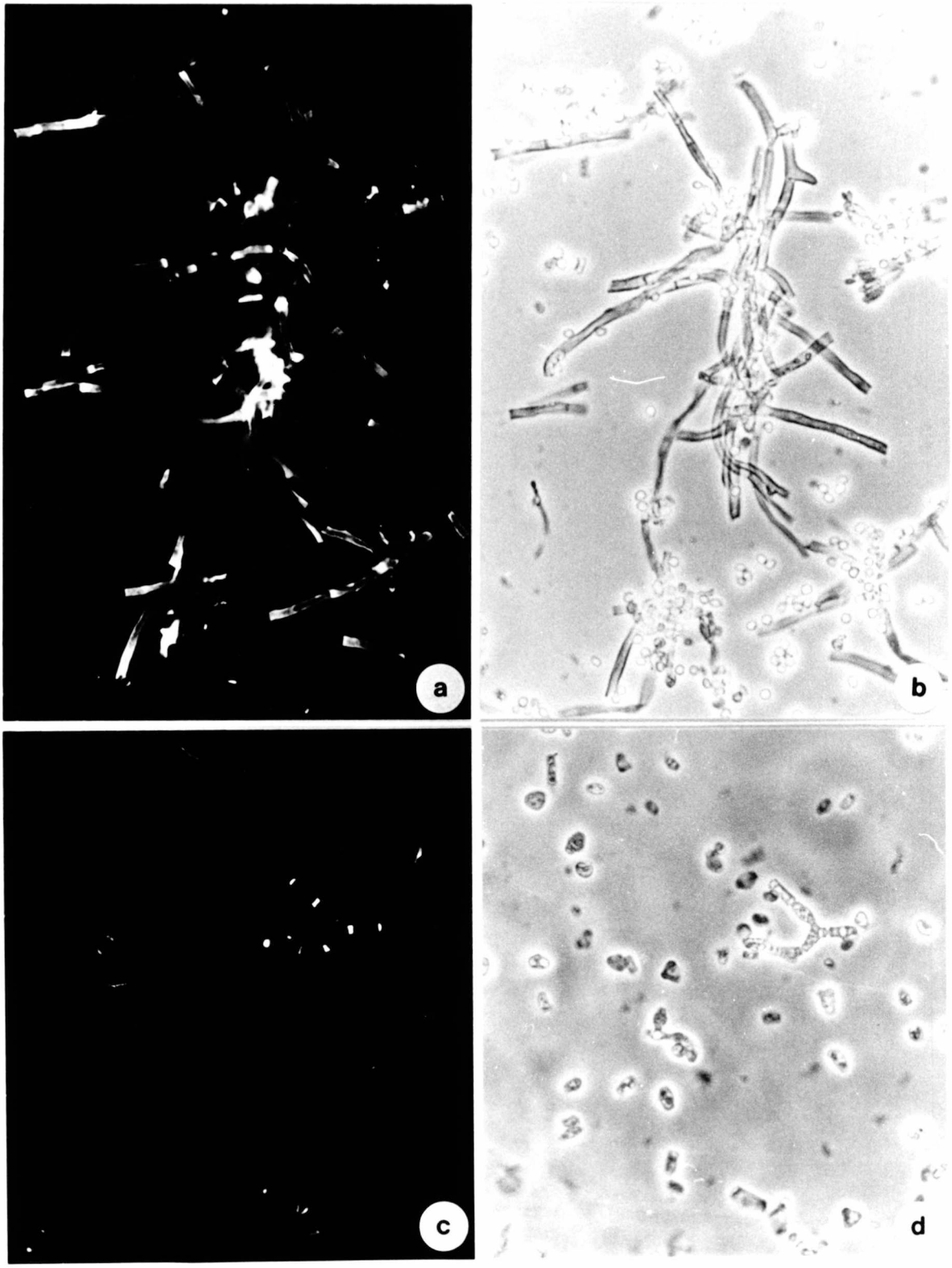
(a) Fluorescence was seen on hyphal fragments of *P. chrysogenum* and was brightest at the broken ends of fragments and on septa. Some fluorescence could be seen at the ends of conidia.

(b) Phase contrast of (a).

(c) A similar pattern of fluoresence was seen on conidia of *N. crassa* as was seen when conidia were reacted with MAb S3B3 (see Fig. 6.1), *i.e.* fluoresence at the ends of conidia, but not all conidia react.

(d) Phase contrast of (c).

Scale bar represents 5μm and refers to both sets of micrographs.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d

8.4. The localisation of the Pax-1 epitope in *N. crassa*.

The Pax-1 epitope is not only found in *P. involutus*, the fungus against which the MAb raised, but is also present in *N. crassa*. Immunogold labelling of *N. crassa* confirmed that the epitope is present in the septa of *N. crassa* as shown by immunofluorescence, but is also present in the rest of the wall (Fig. 8.5). The labelling pattern showed that the epitope lies adjacent to the cell membrane and not at the cell surface. This pattern was seen in all sections.

8.5. Discussion.

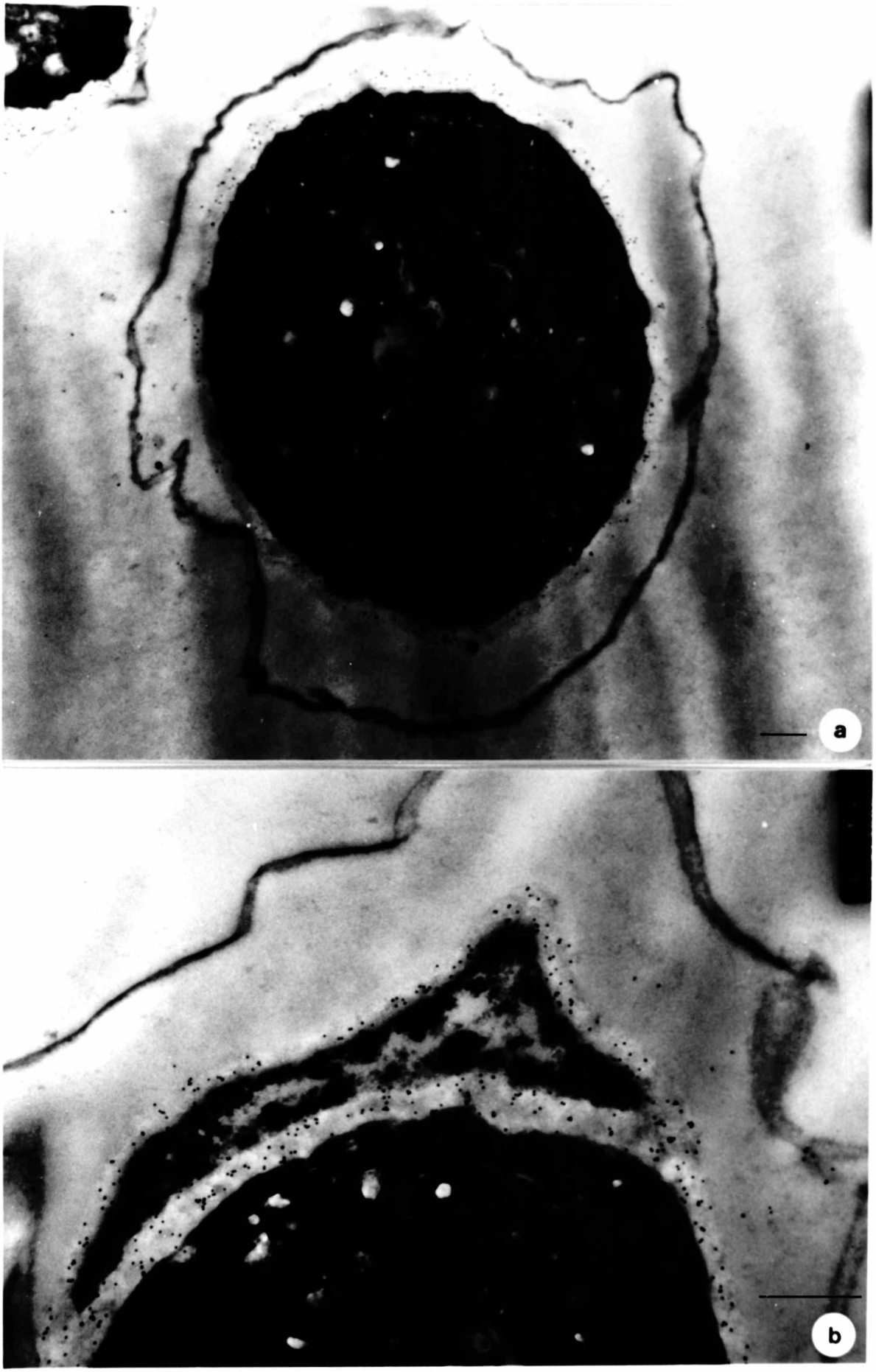
Pax-1 was derived from a mouse injected with cell walls of *P. involutus*, but it reacted with all fungi tested except a member of the Zygomycotina. Candidates for the epitope of such a widely reacting MAb raised against a member of the Basidiomycotina might be chitin or another type of glucan, other than α-1,3 glucan. The immunogold labelling pattern showed that the epitope lies next to the plasma membrane. Hunsley & Burnett (1970) suggested that chitin microfibrils lay next to the plasma membrane and the immunogold labelling of Pax-1 suggested it recognised such a layer. Pax-1, however, did not recognise chitin in an ELISA. Hunsley & Burnett (1970) were, therefore, wrong to suggest that the layer next to the plasma membrane consists of chitin only. The Pax-1 epitope is localised only in the layer next to the plasma membrane unlike the more diffuse distribution labelling patterns of the S4D1 and S1E5 epitopes.

The epitope might be a glucan, since glucans are the major components the walls of members of the Basidiomycotina (Barnicki-Garcia, 1968; Wessels & Sietsma, 1981). It may be that Pax-1 reacts with a β-linked glucan, although this would not agree with the suggested localisation of β-glucan in *N. crassa* (Hunsley & Burnett, 1970). Wessels *et al.* (1972) suggest that β-glucan forms an inner layer in the walls of *S. commune*. Wessels *et al.* (1981)

Fig. 8.5. The localisation of the Pax-1 epitope in the conidial wall of *Neurospora crassa* using immunogold electron microscopy.

(a) The gold labelling was localised in a discrete layer of the conidial wall adjacent to the plasma membrane. Scale bar represents 0.5μm.

(b) The labelling was present throughout the septal region of a conidial chain. Scale bar represents 0.5μm.


a
b

reported that the β-glucan in *S. commune* is linked to chitin in an insoluble complex. In the same paper they confirmed that such covalent linking between β-glucan and chitin did not exist in *N. crassa*, as suggested by the high level of β-glucan extracted by Mahadevan & Tatum (1965). It may be, however, that the glucan forms a complex with chitin in *N. crassa*, but the complex is soluble unlike that of *S. commune*. This may well be a function of the relatively fast growth rate of *N. crassa* compared with that of *S. commune*. In a fast growing fungal hypha the wall needs to have more plasticity to enable hyphal extension to occur. Wessels *et al.* (1983) suggest that the *S. commune* glucan-chitin complex accounts for the change from plasticity to rigidity of the wall during hyphal morphogenesis. The existence of such a complex next to the plasma membrane in *N. crassa* may well explain the localisation of the Pax-1 epitope in the so-called chitin layer.

Chapter Nine

Discussion

In this chapter discussion points which refer to all four MAbs will be considered. The immunological recognition of fungi will be considered with reference to the four areas outlined in Chapter 1, *i.e.* identification, quantification, classification and investigation.

None of the MAbs raised in this project were species-specific. In this work cell walls were used as the immunogens, and although other workers have raised species-specific MAbs using other complex antigens, there is no consensus on the best antigen to be used to produce such specific MAbs. For example, Hardham *et al.* (1985, 1986) produced isolate and species-specific MAbs, which recognise zoospores and cysts of *Phytophthora cinnamomi*, using formaldehyde fixed zoospores and cysts as the immunogen. In contrast, Wright *et al.* (1987)injected a mouse with a soluble fraction from *Glomus occultum* to produce a species-specific MAb. Dewey *et al.* (1989b) used a mycelial homogenate to raise MAbs against *Ophiostoma ulmi* which showed few cross-reactions with other fungi, but which cross-react with host tissue (elm). Dewey *et al.* (1989b) used surface washings of cultures to raise MAbs against *Humicola lanuginosa*, which were able to be used as species-specific probes as the cross-reacting fungi are not usually present in rice shipments. This was a principle used by several workers who successfully used polyclonal antisera for specific identification of one particular fungus (see Section 1.2.2.1).

Identification of a fungus is usually taken to mean the specific recognition of one particular fungus from all others. Identification, however, can occur at different levels as described for polyclonal antisera in Section 1.2.2. The MAb Pax-1 showed the most

widespread cross-reactivity of the three MAbs tested and might have uses as a general fungal probe. It could be used to detect fungal contaminants in foodstuffs as in the polyclonal example of Robertson *et al.* (1988); or it could be used to detect a fungal infection in plants, either by immunofluorescence or ELISA. The advantages of a MAb in this case would be the indefinite supply and the non-variable nature of the MAb which would allow strict comparison between all results.

All four of the MAbs recognise epitopes which have differential distribution at the surface of fungi. This suggests two problems in regard to the production of species-specific MAbs. The first of these is the different reactions of MAbs with their antigens in different tests. If only one test is used to determine the cross-reactivity of a MAb, anomalous results may be obtained. As an example, the presence of both the S1E5 and Pax-1 epitopes in the conidial wall layers of *N. crassa*, but not at the surface, indicates that other techniques must be used to confirm that the negative results from immunofluorescence screens mean that the epitope is not present.

The second problem, related to this, is that if an epitope does show differential localisation in a fungus during its life-cycle a MAb which recognises such an epitope may not recognise the fungus during all stages of its life-cycle. Within this second problem there may be several variations. One is illustrated in immunofluorescence studies by S4D1 especially. Using immunofluorescence it was shown that the S4D1 epitope is present at the surface of *N. crassa* conidia and hyphae and so would recognise the fungus in both of these stages. The epitope is not, however, present on the surface of *P. chrysogenum* conidia, but it is present on hyphae, and vice versa in *Amanita muscaria*. Immunofluorescence studies of a soil sample using S4D1 would, therefore, give different answers to the question "are these fungi there?" depending on the stage of the life-cycle present. An ELISA would overcome the problem in this case, if it were shown that the epitope was present in all of the life-cycle

stages, but was simply inaccessible to the MAb during the immunofluorescence technique. Another problem that might arise with epitopes that show differential localisation is if an epitope is present in one stage of the life-cycle but not another. The accessibility of the MAb to the epitope is not important in this case and an ELISA would have no advantage compared with immunofluorescence.

In other examples of MAbs, raised against non-medical fungi, the differential expression of an epitope has only be considered in detail by Hardham and her co-workers. The species-specific MAb to *G. occultum*, raised by Wright *et al.* (1987) recognises an antigen which is present in both spores and hyphae. Dewey *et al.* (1989a, b) presented data which showed the reaction of their MAbs with "mycelial antigens", and did not distinguish between reactions with different morphological forms. The isolate and species-specific MAbs raised by Hardham *et al.* (1986) recognised zoospores and cysts of *Phytophthora cinnamomi*, therefore, any identification of cultures requires induction of sporangia first. This point was mentioned in the paper, but no information as to the reaction of the MAbs with hyphae was given. The need to induce sporangia to enable use of these MAbs to identify *P. cinnamomi* may reduce the speed at which identification might occur and this may not be satisfactory if speed is important.

The differential expression of fungal epitopes is also an important factor to consider in the quantification of fungal biomass. Several workers have referred to non-uniformity of "indicator" molecules during the different stages of a fungal life-cycle (see Sections 1.4 and 1.5). Use of the MAb S4D1 to quantify *N. crassa* biomass indicated that there was a linear relationship between the S4D1 epitope and *N. crassa* biomass. Other workers have shown such a relationship using polyclonal antisera (see Section 5.7). It is possible, however, that because a MAb only recognises one epitope that a quantification assay using a MAb would be more sensitive to differential expression of the epitope than would be the case with

polyclonal antisera. In the case of a polyclonal antiserum, providing it was not raised against only one antigen, the change in one epitope would be masked by the reactions of the antisera with its other epitopes.

S4D1 reacts with α-1,3 glucan and the level of this glucan has been shown to vary with nutritional status in *A. nidulans* (Zonneveld, 1972). An experiment to test Zonneveld's finding using S4D1 in a quantitative ELISA was not carried out, but such an experiment would yield useful data on the factors needed to be considered in the quantification of fungi using MAbs, as well as giving useful developmental data on α-1,3 glucan in different fungi grown under different conditions.

Polyclonal antibodies are particularly useful for studying serological relationships between fungi and this information can be used as an aid to taxonomic classification. MAbs have a similar potential, but because they only react with one epitope it may be argued that the level of recognition is at the molecular rather than species level. Ferguson, Wycoff & Ayers (1988) suggested that the panel of MAbs, raised by Wycoff, Jellison & Ayers (1987), showed differentiation of glycoproteins rather than *Phytophthora* species. Ferguson *et al.* (1988) were able, however, to group different phytopathogenic fungi using cluster analysis of the ELISA results into taxonomic groups which agreed with conventional taxonomic classifications of the fungi tested. The MAbs in this panel showed quantitative differences in their reactions with different fungi. From this Ferguson *et al.* (1988) suggested that production of MAbs to every single species is not necessary and, that a panel of MAbs would overcome any concern over antigenic variation within a species.

The MAbs raised in this project can be used to show the distribution of four different wall components throughout the fungal kingdom. Whether a combination of the results from this small panel could be used in a similar manner to that of Ferguson *et al.* (1988) was not investigated. The MAbs could certainly be used to confirm, and enlarge upon, the taxonomy

data of Bartnicki-Garcia (1968) based on cell walls. Results from the MAbs also suggest that a panel of MAbs may be needed to ensure recognition of all morphological forms of a fungus.

The four MAbs recognise four different wall components and this can be used in investigations of the cell wall of *N. crassa* and other species. The epitope was characterised for MAb S4D1 only, and further work would be needed to establish the epitopes of the other three MAbs before they could as useful as S4D1 in cell wall studies. One of the problems of previous cell wall work has been a lack of co-ordination between composition and ultrastructure of fungal walls. Hunsley & Burnett (1970) used enzyme dissections to overcome this, and the MAbs, S4D1, S3B3, S1E5 and Pax-1, enable elaboration of their data as described below. Polyclonal antibodies may reveal more data with regard to immunological relationships between fungi, but the specificity of MAb for one epitope only is more useful in the investigation of a fungus and its component molecules.

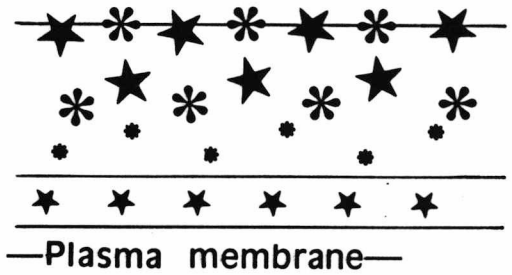
The four MAbs produced during this project were used to investigate not only the surface localisation of the four epitopes in different fungi, but also the localisation of the epitopes throughout the wall of *N. crassa*. The immunogold studies using the different MAbs on thin sections of *N. crassa* showed that the model of Hunsley & Burnett (1970) may be elaborated using such probes and techniques. Of the MAb epitopes, only the Pax-1 epitope conformed to a "layer-type" distribution pattern. This layer was adjacent to the plasma membrane, but was not chitin. As discussed in Section 8.5 this questions whether chitin is also present in this layer and, if so, would this be its only location in the wall. No anti-chitin MAb was raised in this project, but a second clone hybridoma cell line, produced at Essex University, recognises chitin in an ELISA (Hitchcock, pers. comm., 1989) and use of the antibody produced would solve this problem.

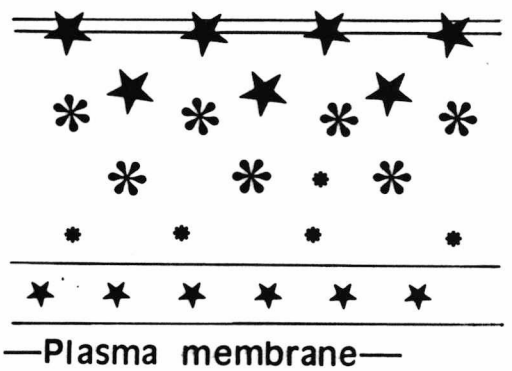
The distributions of the epitopes in the walls of *N. crassa* suggest that a layer model may not be the best way to represent the ultrastructure of fungal cell walls. Burnett (1979) did stress that the layers are not supposed to be discrete but grade into one another. Wessels & Sietsma (1981), however, suggested that published studies are in agreement with a model of the wall in which the various wall components are closely associated with one another to form essentially one layer, with some components accumulating at the outside to from apparently extra layers. The epitope distribution revealed in this work using immunogold electron microscopy support the views of Wessels & Sietsma (1981). Wessels (1988) suggested that this model applies to vegetative hyphae but not to the walls of specialised structures such as spores where genuine outer walls may be present. The electron microscopy studies in this project suggest that the wall model proposed by Wessels & Sietsma (1981) is also essentially correct for conidial walls of *N. crassa*, as well as the hyphae. A specialised outer layer is present, but throughout the rest of the wall the same close association between wall components occurs as in the hyphal walls.

A summary of the distribution of the MAb epitopes in the walls of *N. crassa* is shown schematically in Fig. 9.1.

It has been in medical mycology that most studies of the antigenic nature of cell wall antigens have been made (see Section 1.7). These studies also suggested that the antigenic nature of the wall varies between different stages of the fungus life-cycle. Brawner & Cutler (1987) also showed that the distribution of two *C. albicans* wall components varied between cells grown *in vivo* and *in vitro*. Immunogold studies using two MAbs showed that two mannan-like antigens were expressed in greater quantities on the surface of germ tubes than on mother cells *in vivo* and that the antigens were expressed more in the outer wall of cells grown *in vitro* than in the outer wall of cells grown *in vivo*.

Fig. 9.1. Wall epitope distribution in Neurospora crassa.





Key

- S4D1
- S3B3
- S1E5
- Pax-1

In Section 1.9 it was suggested that "immunological recognition of fungi" is usually taken to mean the "specific recognition of one species of fungus from another" and that this is only one aspect of the information immunological probes can reveal. The production of four MAbs which recognise fungi and their use during this project has shown that MAbs can also be used in other areas, for example quantification of fungal biomass, and investigation of the epitope distribution throughout different fungi and in one particular fungus. The MAbs produced in this project have also highlighted some areas for further research in the study of immunological recognition of fungi. Some of these are considered below.

The idea that one MAb alone would be sufficient to recognise one particular fungus from another would appear to be simplistic. It is possible to produce species-specific probes, but they may only recognise the fungus in one stage of its life-cycle. It may, therefore, be necessary to raise more than one species-specific MAb to recognise a fungus in all stages of its life-cycle. The only "true" species-specific fungal MAb raised thus far is that of Wright *et al.* (1987).

In order to raise such species-specific MAbs successfully either components specific to the fungus in question should be used as immunogens, or if a complex antigen is used methods such as those described by George & Converse (1988) should be used. George & Converse (1988) showed that enrichment of the desired B lymphocyte population prior to screening increased the number of hybridomas which secreted the desired antibody by up to 70%. By selective absorption of B lymphocytes with an antiserum raised against cauliflower cytoplasm, they were able to produce MAbs which recognised cauliflower mosaic virus, following immunisations of mice with infected plant tissue rather than purified virus.

For quantification it may be difficult to raise a species-specific MAb that also has a linear relationship with biomass throughout all stages of the fungal life-cycle, as discussed in 5.7. In studies of fungal population in any environment, it might be that a panel of MAbs is

required to both specifically identify and quantify fungi. A panel of MAbs would, for example, be necessary to follow one fungus in a mixed indigenous population of fungi. One MAb would be required to "track" the particular fungus and other/s would be necessary to indicate the presence of the other fungi in the population. If information on fungal biomass was also desired other MAbs might be needed to show this, as well as other MAbs or probes to indicate the viability of the biomass present.

This study also showed the potential of MAbs in the investigation of fungi. MAbs can be used to investigate specific fungal components, both within and between fungi, as well as in infections of any type.

In conclusion, immunological recognition of fungi involves more than simple identification. The aim to produce species-specific MAbs, although not achieved in this project, led to successful use of the MAbs in the investigation of fungi, as well as allowing a deeper insight into the problems the production of a species-specific MAb entails.

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Appendix I: Fungal Culture Media

Malt extract agar and broth (MEA, MEB)

Malt extract	30g l^{-1}
Mycological peptone	5g l^{-1}
Oxoid agar No. 3	12g^{-1} (omitted if broth)
pH5.4	

Modified Melin-Norkrans (MMN) agar or broth.

Glucose	10g l^{-1}
$CaCl_2$	0.05g l^{-1}
NaCl	0.025g l^{-1}
KH_2PO_4	0.5g l^{-1}
$(NH_4)_2HPO_4$	0.25g l_{-1}
$MgSO_4.7H_2O$	0.15g l^{-1}
$FeCl_3$ (1% w/v)	1.2ml l^{-1}
Thiamine HCl	100µg/ l^{-1}
Oxoid agar No. 3	10g l^{-1} (omitted for broth)

Neurospora culture agar or broth (NCA, NCB)

Yeast extract	5g l^{-1}
Proteose peptone	5g l^{-1}
Maltose	40g l^{-1}
Oxoid No.3 agar	15g l^{-1} (omitted for broth)

Neurospora crassa minimal medium (NCMM) agar or broth.

Mineral salts

Na_3citrate.$2H_2O$	2.5g l^{-1}
KH_2PO_4(anhydrous)	5.0 g l^{-1}
NH_4NO_3(anhydrous)	2.0 g l^{-1}
$MgSO_4.7H_2O$	0.2 g l^{-1}

The mineral salts were dissolved in 800ml distilled water and sterilised at 121°C, 15p.s.i. for 15 min. Following sterilisation the following were added aseptically:

Sucrose	20 g l^{-1} (sterilised at 121°C, 10p.s.i., 10 min, in 200ml dH_2O
Biotin	1ml l^{-1} (0.5% w/v in 50% ethanol)
Trace element solution	0.1ml l^{-1}

The trace element solution contained the following:

Citric acid.$1H_2O$	5.0 g l^{-1}
$ZnSO_4.7H_2O$	5.0 g l^{-1}
$Fe(NH_4)_2(SO_4)_2.6H_2O$	1.0 g l^{-1}
$CuSO_4.5H_2O$	0.25 g l^{-1}
$MnSO_4.1H_2O$	0.05 g l^{-1}
H_3BO_3(anhydrous)	0.05 g l^{-1}
$Na_2MoO_4.2H_2O$	0.05 g l^{-1}

The trace element solution was made up in 95ml sdH_2O and the volume adjusted to following addition of 1ml chloroform as a preservative. This solution was stored at 4°C.

Neurospora germination medium (Chang & Trevithick, 1972)

The mineral salts listed for NCMM were dissolved in 0.04M sodium succinate buffer, pH 5.2. The buffer contained 4.72g l^{-1} succinic acid and 1.6g l^{-1} sodium hydroxide. The biotin and trace element solutions were added as for NCCMM, but the 20g l^{-1} sucrose was supplemented with 27g l^{-1} galactose.

Yeast extract peptone digest (YEPD)

Peptone	10 g l^{-1}
Yeast extract	10 g l^{-1}
Glucose	20 g l^{-1}

Appendix II: Tissue culture media

Dulbecco's Modified Eagle Medium (Gibco, Cat no. 074-2100)

Component	$mg\ l^{-1}$
Inorganic salts	
$CaCl_2$ (anhyd.)	200.00
$Fe(NO_3.9H_2O$	0.10
KCl	400.00
$MgSO_4$ (anhyd.)	97.67
NaCl	6400.00
$NaH_2PO_4.H_2O$	125.00
Other components	
D-glucose	4500.00
Phenol red	15.00
Amino acids	
L-arginine.HCl	84.00
L-cystine.2HCl	62.57
L-glutamine	584.00
Glycine	30.00
L-histidine.$HCl.H_2O$	42.00
L-isoleucine	105.00
L-leucine	105.00
L-lysine.HCl	146.00
L-methionine	30.00
L-phenylalanine	66.00
L-serine	42.00
L-threonine	95.00
L-tryptophan	16.00
L-tyrosine	103.79
L-valine	94.00
Vitamins	
D-Ca pantothenate	4.00
Choline chloride	4.00
Folic acid	4.00
I-inisitol	7.20
Nicotinamide	4.00
Pyridoxal.HCl	4.00
Riboflavin	0.40
Thiamine.HCl	4.00

Modified Eagle Medium Non-essential Amino Acid solution, 100x (Gibco, Cat no. 043-1140).

Component	mg ml^{-1}
L-alanine	890.00
L-asparagine	1500.00
L-aspartic acid	1330.00
L-glutamic acid	1470.00
Glycine	750.00
L-proline	1150.00
L-serine	1050.00

Appendix III: Miscellaneous

Phosphate buffered saline, pH7 (PBS-7)

NaCl	8g l^{-1}
KCl	0.2g l^{-1}
$Na_2HPO_4.12H_2O$	2.27g l^{-1}
if $.7H_2O$	2.26g l^{-1}
if $.2H_2O$	1.435g l^{-1}
KH_2PO_4 (anhyd.)	0.114g l^{-1}

ELISA solutions

a. Second antibody diluent

NaCl	0.5M
Na_2HPO_4 (anhyd.)	0.01M
Triton X-100	0.5%
Bovine serum albumin	0.1%

Filter sterilised, stored at 4°C.

b. Substrates

i. ABTS, 2,2'azino-bis (3 ethylbenzthiazoline sulphonic acid)

220mg of ABTS was dissolved in 10ml H_2O, to give a 40mM solution. This was stored at -20°C in 250µl aliquots. To use in an ELISA this solution was diluted in citrate buffer (0.1M citric acid/0.1M trisodium citrate.$2H_2O$, pH4) at 1 in 50. 30µl of hydrogen peroxide (30% w/v), diluted 1 in 10, was added immediately prior to use.

ii. *o*-phenylenediamine

o-phenylenediamine was dissolved in 0.05M sodium citrate/0.15M sodium phosphate buffer (pH6) at 0.04mg ml^{-1} and 0.01% hydrogen peroxide added. The solution was prepared immediately before use.

Immunofluorescence solutions

i. Formaldehyde from paraformaldehyde

3.7g of paraformaldehyde was stirred into 80ml of buffer, either PBS-7 or PEM (see below). The supension was heated to 60°C and a few drops of concentrated sodium hydroxide solution added to dissolve the paraformaldehyde. When the solution was clear it was removed from the heat and cooled, before the pH was adjusted back to 7 by addition of sulphuric acid. The solution was made up to 100ml and filtered using Whatman No.1 paper to remove any large particles.

ii. PEM buffer, pH7

PIPES	15.12g l^{-1}
EGTA	1.90g l^{-1}
$MgSO_4$	1.23g l^{-1}

iii. Mowiol 4-88 (mountant)

2.4g of Mowiol was stirred into 6g of glycerol (AR), 6ml of ddH_2O was added and the mixture incubated for 2h at room temperature. 12ml of 0.2M Tris (pH8.5) was added and the mixture incubated in a water bath at 50°C until most of the Mowiol had dissolved. The mixture was then spun at 4500r.p.m. (2250*g*) for 15min and

the supernatant aliquoted into 1ml volumes and stored at -20°C.

iv. *p*-phenylenediamine

A 10mg ml^{-1} solution of *p*-phenylenediamine solution was made up in 0.2M Tris-HCl, pH8.5. It was used as an anti-fade agent in the mountant, Mowiol at 1mg ml^{-1}.

Sample extraction buffers for 1-D PAGE.

i. Laemmli sample buffer (LSB)

TrisHCl	0.0625M
SDS	2%
Glycerol	10%
Mercaptoethanol	5%
Bromophenol blue	0.01%

Three times LSB was obtained by using three times the concentration of SDS and mercaptoethanol given above.

ii. Urea sample buffer (USB), 10M

Tris	0.0625M
SDS	2%
Glycerol	10%
Mercaptoethanol	5%
Urea	60%
Bromophenol blue	0.001%

iii. Phosphate sample buffer (PSB)

Sodium phosphate	0.1M
SDS	1%
Mercaptoethanol	2%
Sucrose	10%
Bromophenol blue	0.001%

1-D polyacrylamide gel electrophoresis

Lower gel buffer

Tris	181.65g^{-1}, 1.5M, pH8.8
SDS	0.4%

Acrylamide, 30% w/v

Acrylamide	29.29g/100ml
Bisacrylamide	0.78g/100ml

Ratio of acrylamide to bisacrylamide 37.5 : 1

Ammonium persulphate, 10% w/v

Upper gel buffer

Tris	60.55g l^{-1}, 0.5M, pH6.8
SDS	0.4%

Agarose, 1% in 1/4 strength upper gel buffer

Running buffer

Tris	3g l^{-1}
Glycine	14.4g l^{-1}
SDS	0.1%

Main gel mix

	10%	12.5%	15%
Water	25ml	20ml	15ml
Lower gel buffer	15ml	15ml	15ml
Acrylamide	20ml	25ml	30ml

Degassed and 200µl ammonium persulphate and 30 TEMED added.

Stacking gel mix

Water	6.0ml
Acrylamide	1.5ml
Upper gel mix	2.5ml

Degassed and 45µl ammonium persulphate and 15 TEMED added.

(TEMED = N,N,N',N'-tetramethylethylenediamine)

Electroblot transfer buffer (blot electrode buffer, BEB)

Tris	3g l^{-1}
Glycine	14.4g l^{-1}
Methanol	200ml l^{-1}

Immunoblotting solutions

i. TBS

TrisHCl	1.21g l^{-1}
NaCl	8.18g l^{-1}

ii. TBS-Tween

TBS containing 0.1% (v/v) Tween 20

iii. HS-Tween

TrisHCl	1.21g l^{-1}
NaCl	58.4g l^{-1}
Tween 20	0.5% v/v

iv. Substrate solution

18mg of 4-chloro-1-naphthol was dissolved in 6ml of methanol. The volume was made up to 100ml by addition of TBS and 0.025ml 30% hydrogen peroxide added. The substrate was made up immediately prior to use.