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Production and characterisation of monoclonal antibodies to cell wall components of the flax rust fungus

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Abstract

Monoclonal antibodies have been raised against an haustorium-enriched sample prepared from flax leaves infected with the biotrophic flax rust pathogen *Melampsora lini*. The monoclonal antibodies were produced following conventional and co-immunisation procedures and the range of antibody specificities was compared. The preparation used as immunogen for the conventional protocol was a crude isolate of haustoria consisting of approx. 65% fungal haustoria, the other components being mainly mesophyll cells or cell wall and chloroplast fragments. Following hybridoma production, 40% of positive cell lines produced antibodies that reacted with haustoria and other fungal cells, but 60% bound to plant cells in the infected leaves. For the co-immunisation protocol, the preparation used for immunisation consisted of the crude isolate of haustoria mixed with serum raised against an haustorium-depleted leaf homogenate. In two fusions, 92-94% of the antibodies reacted with fungal cells, including 3 cell lines that localised specifically to the cell wall of haustoria. Only 6-8% of the antibodies produced via co-immunisation reacted with plant cells. The antigens targeted by the three haustorium-specific monoclonal antibodies are incorporated into the wall at early stages of haustorium development, remain in the wall throughout haustorium maturation, and are present in both compatible and incompatible interactions. The epitopes recognised by the monoclonal antibodies are oligosaccharide in nature and the antigens are highly resistant to extraction from the wall. These results highlight the value of the co-immunisation protocol for the production of monoclonal antibodies to specific components in an impure preparation and provide direct evidence for molecular differentiation within the wall of the haustorium of *M. lini*.

Introduction

Distinct races of the flax rust fungus *Melampsora lini* interact in a gene-for-gene manner with different cultivars of the host flax plant (Flor, 1956). On susceptible cultivars, virulent races of the biotrophic pathogen establish haustoria in living host cells. However, on resistant cultivars the formation of a young haustorium by avirulent races of the pathogen is associated with the initiation of a rapid hypersensitive response (known as hypersensitive cell death) by the infected host cells. It is considered that the host-haustorium interface of biotrophic fungi represents an important region of functional specialisation, one that is involved in recognition and signalling events that may control the induction or suppression of host cell death, as well as having a role in nutrient transfer events between the host and the pathogen (Manners and Gay, 1983; Mackie et al., 1991; Pain et al., 1992). It is thought that these events involve interactions between the invaginated host plasma membrane (known as the extrahaustorial membrane) and molecules on the surface of the haustorium (Albersheim and Anderson-Prouty, 1975; Pain et al., 1992).

As part of the characterisation of surface components at the haustorium-plant interface, a number of

studies of biotrophic fungal pathogens have identified molecular differentiation in haustorial complexes. Early ultrastructural studies of *Melampsora* and *Erysiphe* showed that the extrahaustorial membrane was different to other parts of the host cell plasma membrane; it differed in thickness, staining properties and patterns of intramembrane particles (Bracker, 1968; Littlefield and Bracker, 1972; Gil and Gay, 1977). More recently, the production of monoclonal antibodies (MAbs) to *Erysiphe pisi* haustorial complexes has revealed that the extrahaustorial membrane contains a 250 kD glycoprotein not detectable in other parts of the host plasma membrane (Roberts et al., 1993). The extrahaustorial membrane also has a decreased content of ATPase as compared to the rest of the host plasma membrane (Spencer-Phillips and Gay, 1981).

With respect to specialisation of fungal components within the haustorium itself, immunological studies of *Erysiphe pisi* have shown that the haustorial plasma membrane contains 62 kD and 45 kD glycoproteins that are not present in the plasma membrane of other fungal infection structures (Mackie et al., 1991, 1993). Early electron microscopy also showed an abrupt change in the stainability of the haustorial wall on either side of the neck ring in the penetration peg of flax rust haustoria (Littlefield and Bracker, 1972). This observation was interpreted as an indication of differences between the structure and composition of the wall of the haustorium and that of the subtending fungal cell, but to date no further details of this specialisation have been forthcoming.

The production of MAbs that target specific components of the haustorial complex of *E. pisi* has been made possible by the ability to isolate haustoria from infected leaf material (Gil and Gay, 1977; Mackie et al., 1991). It is also possible to isolate haustorial complexes from rust-infected plants to approximately 60-70% purity (Hahn and Mendgen, 1992), and in the present study MAbs have been raised against haustoria of *M. lini* with the aim to identify molecular components specific to the host-pathogen interface in this biotrophic association.

Materials and methods

Plant and fungal material

Two cultivars of flax (*Linum usitatissimum* L.), Hoshangabad (Hosh.) and Rust Differential No. 9 (RD9), were used. Hosh. possesses no known resistance genes, while RD9 contains the L^6 resistance gene. Three strains of flax rust fungus *M. lini* (Ehrenb.) Lev. were used: CH_5 (Lawrence et al., 1981), $CH_5F_2(84)$, the progeny strain of CH_5, and 271 (a strain unrelated to CH_5). All strains contain the avirulence gene corresponding to the L^6 resistance gene and give a compatible reaction on Hosh. but are incompatible with RD9. The spores and flax seeds were kindly provided by Dr G. J. Lawrence, Division of Plant Industry, CSIRO, Canberra, Australia. For plant inoculation, fresh urediospores were dusted over three to five week old plants and allowed to germinate overnight at 20 °C in a humid chamber (Lawrence et al., 1981). The plants were then left in a greenhouse until they were ready for harvest at various stages post-inoculation. Maize plants (*Zea mays* L.) infected with maize rust (*Puccinia sorghi*) and wheat plants (*Triticum aestivum*) infected with leaf rust (*P. recondita*) were provided by Dr A. Pryor, Division of Plant Industry, CSIRO, Canberra, Australia.

Haustorium isolation

Haustoria were isolated from flax plants (Hosh.) 6 days after inoculation with CH_5 *M. lini* spores following the method of Hahn and Mendgen [1992], with some modifications. All steps were performed at 4 °C. For each 10 g of infected leaf material 110 ml of cold homogenisation buffer [0.3 M sorbitol, 20 mM 3-morpholinopropanesulfonic acid (MOPS), 0.1% bovine serum albumin (BSA), 0.2% β-mercaptoethanol, pH 7.2] was added, and the material was homogenised in a Waring blender. The leaf homogenate was filtered through 20 μm nylon mesh. The filtrate was centrifuged for 5 min at 7000 $\times$ g, and the pellet washed once and then resuspended in 3 ml of suspension buffer (0.3 M sorbitol, 10 mM MOPS, 0.2% BSA, 1 mM $CaCl_2$, 1 mM $MnCl_2$, pH 7.2). 350 μl of the filtered homogenate was loaded onto a column (1 ml bed volume) of Concanavalin A (ConA) coupled to 6% agarose macrobeads. After 15 min, unbound material (primarily consisting of cell wall fragments and chloroplasts) was eluted from the column in 8 ml of suspension buffer, washed with phosphate buffered saline (PBS: 150 mM NaCl, 20 mM sodium phosphate buffer) and retained for MAb production following a co-immunisation protocol. Haustoria bound to the column were released in 3 ml of suspension buffer by vigorous resuspension with a pipette. The beads were allowed to settle and the supernatant, which contained the haustoria, collected. The procedure was repeat-

ed until all the leaf homogenate had been loaded onto the column. The haustorium-enriched sample was then centrifuged at 15 000 × g for 5 min and the supernatant carefully discarded to leave a soft pellet of haustoria and contaminating material which was brought into suspension with approx. 2.6×10^6 haustoria/ml.

Monoclonal antibody production

Monoclonal antibodies were produced by either conventional (Galfre and Milstein, 1981; Harlow and Lane, 1988) or co-immunisation (Barclay and Smith, 1986) procedures. For the production of hybridomas by conventional methods, the fusion used a mouse immunised with haustoria isolated from infected flax leaves. The mouse was given three intraperitoneal injections of approx. 10.4×10^5 haustoria in 400 μl PBS at intervals of 2 to 3 weeks, before a final injection 3 days prior to fusion. The spleen cells from the immunised mouse were fused with Sp_20 myeloma cells as described by Galfre and Milstein (1981).

The production of hybridomas by co-immunisation was as described by Barclay and Smith (1986), with minor modifications. Six week old female Balb/c mice were given three intraperitoneal injections, two weeks apart, of 500 μl of leaf homogenate (the material that did not bind to the ConA column and containing approx. 1 mg protein). The mice were bled at three and five weeks after the first immunisation and a polyclonal serum was prepared. The serum was tested in an indirect immunofluorescence assay on sections of butyl-methylmethacrylate (BMM)-embedded (Merck Lab, Darmstadt, Germany) infected leaf tissue which had been harvested 5 days post-inoculation. Balb/c mice of the same age and sex as above were then given intraperitoneal injections of haustorium-enriched samples, which had been incubated with the polyclonal antiserum in a 1:1 (v/v) ratio for 30 min. Three injections, each containing approx. 6.5×10^5 haustoria in 250 μl PBS, were made at two week intervals. A final injection of sample that had not been incubated with the polyclonal antiserum was made into the tail vein, and 200 μl of polyclonal antiserum was injected intraperitoneally. Four days later the spleen was removed and fused with Sp_20 myeloma cells. The tissue culture supernatants from growing hybridoma cells were screened for MAb production by immunofluorescence labelling on sections of BMM-embedded infected plants that had been harvested 5 days post-inoculation. Selected cell lines were cloned by limiting dilution and re-screened 10-14 days later. The immunoglobulin class and subclass of the selected MAbs were determined using a mouse Monoclonal Antibody Typing Kit (Amersham plc, Aylesbury, Bucks, U.K.).

Immunofluorescence labelling of infected leaf material and haustorium-enriched sample

Infected flax leaf tissue was fixed and embedded in BMM resin (Gubler, 1989; Baskin et al., 1992) as described in Kobayashi et al. (1994) and Murdoch and Hardham (1998). In brief, small leaf segments were fixed for 3 h in a fixative solution consisting of 4% paraformaldehyde, 0.2% glutaraldehyde and 0.05% Triton X-100 in 50 mM Pipes buffer (pH 7.0) containing 2 mM $MgSO_4$ and 5 mM EGTA. The samples were then dehydrated in a graded ethanol series and embedded in BMM resin by polymerisation under ultraviolet light and nitrogen gas.

Sections (10 μm in thickness) of BMM-embedded leaves or 10 μl aliquots of the haustorium-enriched sample were airdried onto gelatin-coated multiwell microscope slides (Flow Laboratories, USA). The resin was removed from the BMM sections by soaking the slide in acetone for 30 min, and samples were rehydrated in distilled water and PBS. The samples were incubated in 10 μl of blocking solution (1% BSA and 0.02% NaN_3 in PBS) for 10 min to 1 h, and then in primary MAb for 2 h at room temperature. The hybridoma supernatants were applied undiluted to sections when screening of antibody-producing hybridoma cell lines, but were diluted 1:3200 in blocking solution for all other immunofluorescence labelling assays. Non-immune mouse immunoglobulin IgM (10 μg/ml) was used as a control. After washing three times in PBS, sections were labelled with FITC-conjugated sheep anti-mouse antibody (SAM: Silenus Lab Pty Ltd, Dandenong, Australia), diluted 1:30 in blocking solution, for 2 h at room temperature under dark conditions. The samples were then rinsed in PBS followed by a final brief rinse in distilled water before mounting in mowiol mounting medium (Osborn and Weber, 1982) containing 0.1% paraphenylenediamine to prevent fading of the FITC. Samples were examined on a Zeiss Axioplan microscope using standard filters for fluorescein.

Processing isolated haustoria for ultrastructural analysis

Haustoria isolated from infected leaves were fixed in 2% glutaraldehyde in 50 mM Pipes buffer, pH 6.9 for

30 min. After fixation, the cells were pelleted at 7 000 × g for 2 min, washed in 50 mM Pipes buffer, and resuspended in 50 μl of 50 mM Pipes buffer. An equal volume of 2% agarose was then added, the suspension was mixed well and allowed to solidify on ice. The agarose-embedded cells were postfixed for 1 h in 1% osmium tetroxide in 25 mM phosphate buffer, pH 6.9, washed in 50 mM Pipes buffer, dehydrated in a graded series of acetone solutions and embedded in Spurr's epoxy resin. Ultrathin sections were stained in 2% uranyl acetate for 10 min and lead citrate for 5 min and viewed with either an Hitachi H600 or JEOL 2000 EX transmission electron microscope (TEM).

Processing infected leaf material for immunogold labelling

Infected leaf tissue was prepared and fixed in 2% glutaraldehyde in 50 mM Pipes buffer, pH 6.9, as described above, and rinsed in 50 mM Pipes buffer, pH 6.9. Some of the tissue was postfixed for 2 h in 1% osmium tetroxide in 25 mM phosphate buffer. All the steps following fixation were performed at 4 °C. The tissue was washed in 25 mM phosphate buffer, pH 6.9, dehydrated in a graded series of ethanol solutions and embedded in LR White resin (The London Resin Co., Hampshire, U.K.) with polymerisation at 60 °C for 24 h. Ultrathin sections of LR White-embedded tissue were collected on formvar-coated gold slot grids and were immunolabelled by immersing grids in 10 μl drops of reagent in the following sequence: (1) blocking solution, 10 min; (2) undiluted hybridoma supernatant or non-immune mouse immunoglobulin IgM (10 μg/ml), overnight at 4 °C; (3) five changes of PBS containing 0.2% Tween 20, 5 min each; (4) sheep anti-mouse antibody conjugated with 10 nm colloidal gold diluted 1:75 in blocking solution (Amersham Int., Buckinghamshire, U.K.), 1 h at room temperature; (5) three changes in PBS containing 0.2% Tween 20, 5 min each; (6) two changes in distilled water, 2 min each. Sections were then stained with 2% uranyl acetate for 4 min and lead citrate for 1 min and viewed with either an Hitachi H600 or JEOL 2000 EX TEM.

Antigen modification by periodate oxidation and pronase digestion

Procedures for antigen modification were based on methods described by Jones et al. (1990), with modifications. The analysis was conducted on sections (10 μm) of infected leaf tissue (harvested 5 days post-inoculation) embedded in BMM resin, or on haustoria isolated from infected leaves. For periodate oxidation, the sections were dried onto gelatin-coated multiwell microscope slides and washed with 50 mM sodium acetate buffer (pH 4.5). Sodium metaperiodate (20 mM $NaIO_4$ in 50 mM sodium acetate buffer pH 4.5) was then added to treated wells, while control wells received buffer alone. Following incubation for either 1 h or 4 h in darkness at 4 °C, sections were washed three times with acetate buffer and once with PBS before blocking and labelling for immunofluorescence microscopy as described above. Some treated sections or cells were labelled with anti-β-tubulin 1:50 (Amersham Int., Buckinghamshire, U.K.) as an additional control.

For pronase digestion, the sections or isolated haustorial complexes were mounted on multiwell microscope slides coated with Vectabond Reagent (Vector Laboratories Inc., Burlingame, CA, U.S.A.) and washed with PBS. Pronase (1 mg/ml diluted in PBS) was added to treated wells, while control wells received buffer alone. Following incubation for either 1 h or 4 h at room temperature, sections were washed four times with PBS and then blocked and labelled for immunofluorescence microscopy as described above. As for the periodate oxidation treatment, some treated sections or cells were labelled with anti-β-tubulin 1:50.

Preparation of samples for SDS-PAGE

Several methods were used to solubilise antigens from infected leaf material and haustorium-enriched cell suspensions. For all treatments, the Bio-Rad Protein Assay Dye was used to quantitate the total amount of protein present in each sample. The haustorial complexes were dried onto slides after each solubilisation treatment and immunofluorescently labelled, as described above. The solubilisation procedures are detailed in full in Murdoch and Hardham (1998) and included the following. (i) Addition of double strength (2X) sample buffer (0.125 M Tris-HCl pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, and 0.0025% (w/v) bromophenol blue), with or without 10% (v/v) 2-mercaptoethanol, to infected leaf tissue or haustorium-enriched sample that had been ground to a fine powder in liquid nitrogen, with or without prior treatment with protein extraction buffer pH 7.4 (John et al., 1989). (ii) Progressive extraction of finely ground leaf material with sodium bisulfite buffer (Murphy and Hood, 1993). (iii) Homogenisation of a suspension of haustorial complexes in non-reducing sample buffer (Mackie

et al., 1993). (iv) Enzymatic degradation of fungal wall material with chitinase and lyticase (Marcilla et al., 1991). (v) Treatment of haustorial complexes with 6 M guanidine, 8 M urea or 1 M KCl. (vi) Detergent lysis of haustorial complexes with 0.5% (v/v) Triton X-100 (Estrada-Garcia et al., 1990).

SDS-PAGE and western blotting

Solubilised proteins were separated by SDS-PAGE using a 4% (stacking) gel and either 7.5% or 12% (separating) acrylamide gels (Laemmli, 1970). Each lane on the gel was loaded with 10 μg of total protein. Separated proteins were transferred onto PVDF membrane (Millipore Corp., Bedford, MA, U.S.A.) following the protocol of Towbin et al. (1979).

For western blotting with MAbs, the membrane was washed twice with TBST (Tris-buffered saline pH 7.4 containing 0.05% Tween-20), 5 min each, and blocked for 1 h in 5% low fat milk in TBST. The membrane was washed three times, 5 min each, in TBST and then cut into strips. One strip was stained for total protein with Coomassie staining solution (50% methanol, 10% acetic acid and 0.1% Coomassie brilliant blue R-250) and the remaining strips were incubated overnight at 4 °C with MAb hybridoma supernatant (undiluted). A control strip was incubated in TBST. After washing the strips three times in TBST, they were then incubated for 1 h in secondary antibody (alkaline phosphatase-conjugated sheep anti-mouse diluted 1:10,000 in TBST) (Silenus Lab, Hawthorn, Vic., Aust.). Finally, the strips were washed twice in TBST, once in TBS, and once in substrate buffer (0.1 M Tris pH 9.5, 0.1 M NaCl and 50 mM $MgCl_2.6H_2O$). The strips were developed in 25 ml substrate buffer containing 25 μl of nitroblue tetrazolium solution (NBT, 150 mg/ml in 70% dimethyl formamide) and 25 μl of 5-bromo-4-chloro-indolyl phosphate solution (BCIP, 75mg/ml in 100% dimethyl formamide). After development, the membrane was washed for 10 min in distilled water then allowed to air dry.

Results

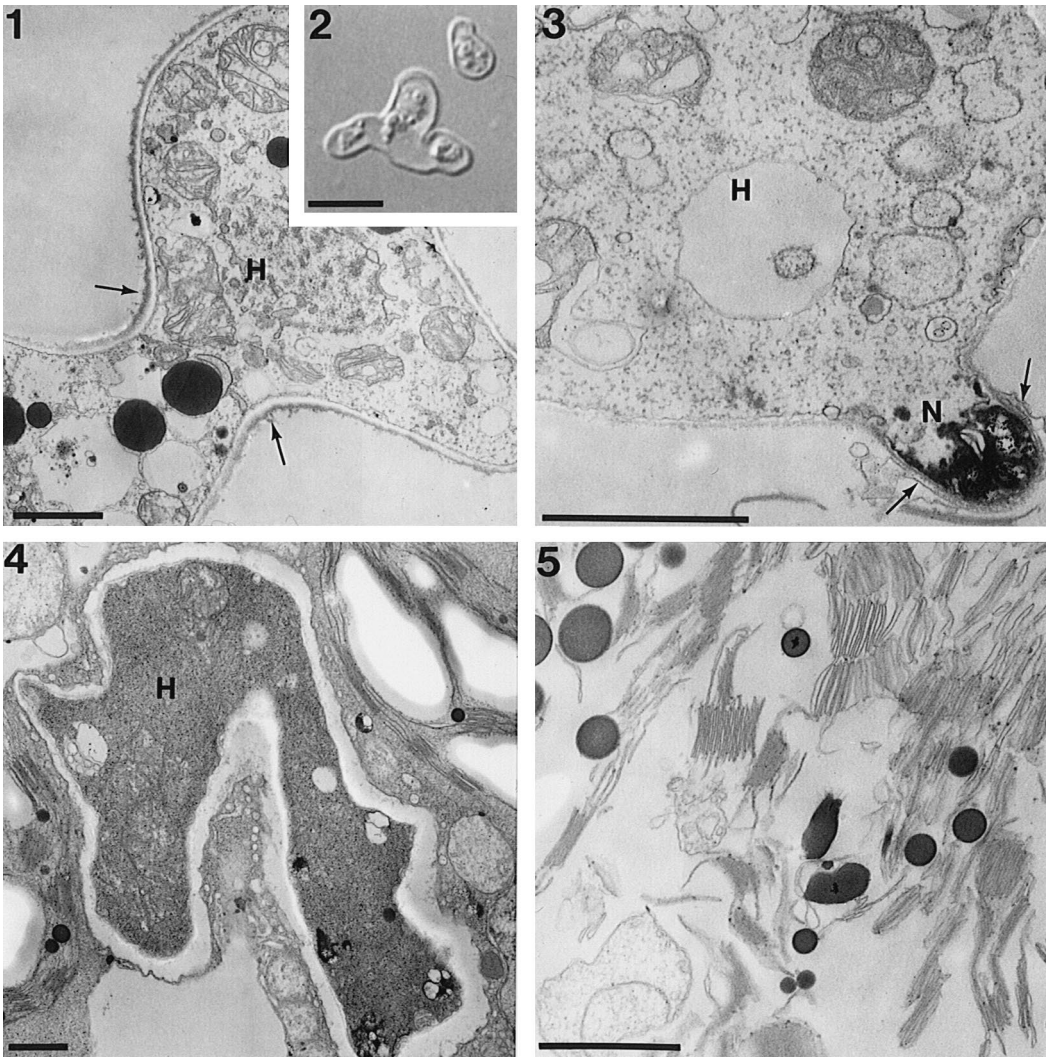
Ultrastructure of haustoria isolated from infected leaves

Isolated haustoria (Figure 1) maintained the irregular lobed shape that is characteristic of mature haustoria *in planta* (Figure 2). The haustorial wall was well preserved and, in most cases, had an electron-dense fibrillar layer attached to its surface (Figure 1). The extrahaustorial membrane was not present in the region surrounding the haustorial body, but could be found still connected at the haustorial neck (Figure 3). This neck region contained a plug of electron dense material at the point where the haustorium had been broken away from the haustorial mother cell (Figure 3). The fungal plasma membrane was intact and the haustoria possessed a normal complement of organelles, including mitochondria, nuclei, endomembranes and ribosomes (Figures 1, 3). Some single mesophyll cells containing haustoria could be found in the suspension (Figure 4), although the major contaminants of the haustorium-enriched suspension were cell wall fragments and broken thylakoid membranes from chloroplasts of the host plant (Figure 5).

Monoclonal antibody production

Haustorium-enriched preparations were used for the production of MAbs. Tissue culture supernatants from hybridoma cells were screened by immunofluorescence microscopy on 10 μm BMM sections of infected flax leaf tissue adhered to multiwell slides. For the initial fusion, mice were immunised with a preparation that contained approximately 2.6×10^6 haustoria/ml. Of the 554 hybridoma cell lines that were screened, 50 gave a positive reaction with the sections of infected flax leaves (Table 1). Forty percent of positive cell lines produced antibodies that reacted with fungal cells, 42% produced antibodies that reacted with plant cells and 18% produced antibodies that reacted with components of both plant and fungal cells. No cell lines produced antibodies that reacted specifically with the fungal haustoria.

For the second and third fusions, a co-immunisation protocol was followed. Mice were immunised with the haustorium-enriched preparation mixed with a polyclonal serum obtained from mice that had been immunised with flax leaf material that did not bind to the ConA column. In the second fusion, 92% of positive cell lines produced antibodies directed towards fungal cell components and only 8% of positive cell lines produced antibodies directed towards plant cells. No cell lines produced antibodies that reacted with both plant and fungal cells. In the third fusion, 93% of positive cell lines made antibodies that reacted with fungal cells, 6% produced antibodies that reacted with plant cells and three cell lines (1%) produced antibodies that specifically reacted with fungal haustoria (Table 1).



Figures 1–5. Ultrastructural features of an haustorium-enriched suspension prepared from flax leaves infected with a virulent race of *M. lini* 6 days after inoculation. (1) Mature lobed haustorium (H) showing electron dense material (arrows) on the outer surface of the haustorial cell wall. Bar = 1 μm. (2) Differential interference contrast (DIC) image of isolated haustoria. Bar = 10 μm. (3) The neck (N) of an haustorium (H) with some remnants of the extrahaustorial membrane still attached (arrows). The tip of the neck is blocked with an electron-dense plug at the point where it was ruptured from the haustorial mother cell. Bar = 1 μm. (4) An haustorium (H) within an intact mesophyll cell. Bar = 1 μm. (5) Broken thylakoid membranes derived from the chloroplasts of the host plant contaminated the haustorium-enriched suspension. Bar = 1 μm.

These three hybridoma cell lines, together with one fungal-specific cell line, were cloned for further study. The three haustorium-specific cell lines, termed ML1, ML2 and ML3, and the fungal-specific cell line, termed ML4, produced IgM immunoglobulins.

Localisation of antigens recognised by ML1, ML2, ML3 and ML4

Immunofluorescence labelling of BMM sections of flax leaves (cultivar Hosh.) infected for 24 h with *M. lini* (strain CH_5) showed that the MAbs ML1, ML2 and ML3 bound only to haustoria (Figure 6). The pattern and intensity of binding observed for the three MAbs was very similar, suggesting that the distribution of antigens recognised by these MAbs was

Table 1. Reactivity of hybridomas produced by conventional and co-immunisation protocols

	Conventional immunisation: Fusion 1[1]	Co-immunisation:	
		Fusion 2[2]	Fusion 3[2]
Number of cell lines screened	554	776	1098
Number of positive clones	50	398	280
Number of fungal-specific cell lines	20 (40%)[3]	366 (92%)	261 (93%)
Number of haustorium-specific cell lines	0	0	3 (1%)
Number of plant- and fungal-specific cell lines	9 (18%)	0	0
Number of plant-specific cell lines	21 (42%)	32 (8%)	19 (6%)

[1] Hybridomas were produced following the methods of Harlow and Lane (1988).
[2] Hybridomas were produced following the methods of Barclay and Smith (1986).
[3] Percent of positive clones.

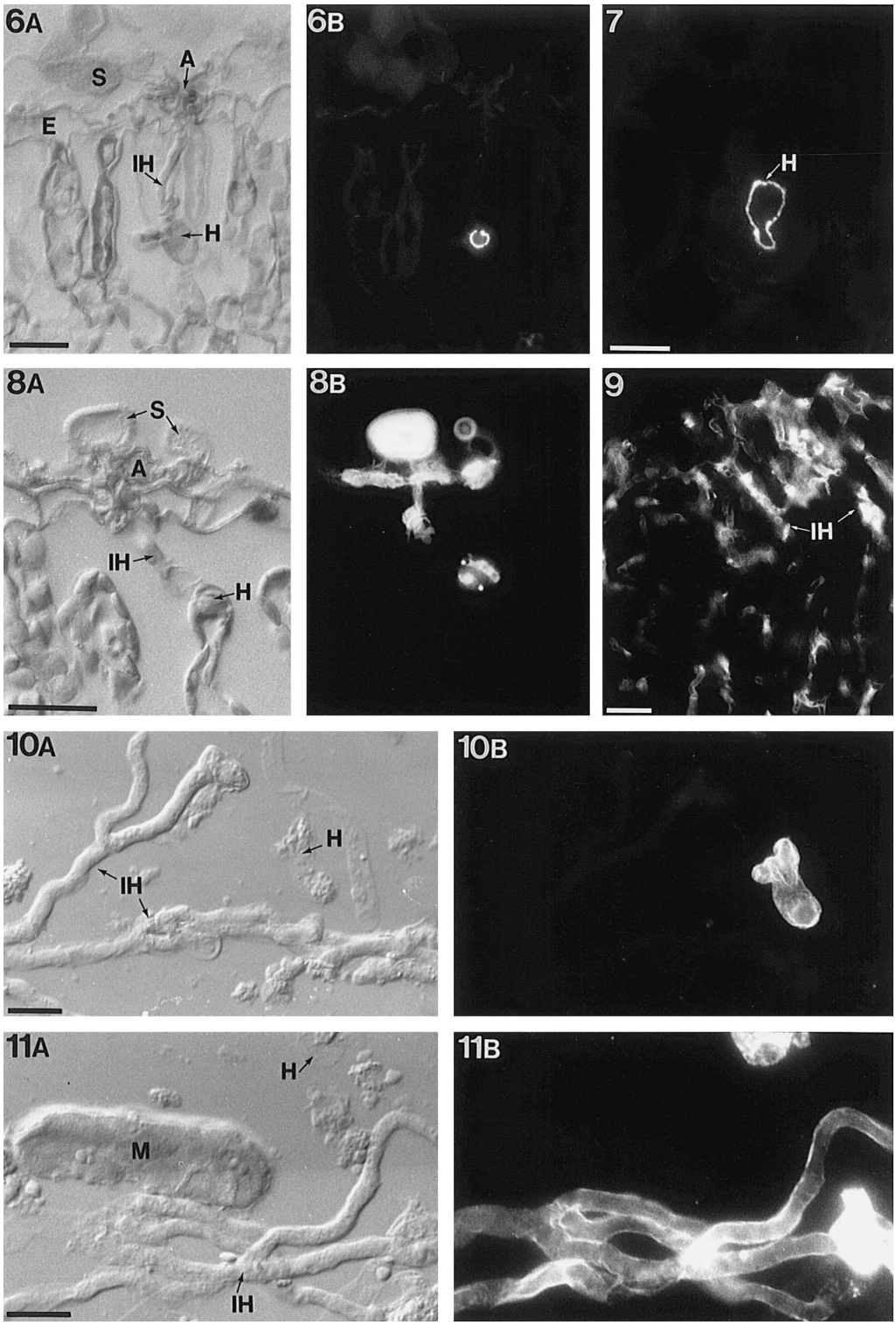
also similar. Labelling was localised to the periphery of the haustorial body and there was no detectable labelling of the haustorial cytoplasm. Labelling of the haustorial wall stopped at the neck of the haustorium (Figure 6B). The MAbs did not label fungal spores, appressoria, infection hyphae, or plant epidermal and mesophyll cells. At a later stage of infection (5 days post-inoculation), immunofluorescence labelling revealed that ML1, ML2 and ML3 all recognised the well-developed, lobed haustoria present at this time, with the same binding pattern and intensity to that observed at 24 h (Figure 7). MAb ML4 recognised all fungal infection structures (spores, appressoria, infection hyphae, haustoria) present at both 24 h and 5 days post-inoculation (Figures 8, 9).

Immunofluorescence microscopy was also performed on haustorial complexes that had been isolated from infected leaves and dried onto slides. Binding of ML1, ML2 and ML3 to the isolated haustorial complexes was intense and clearly localised to the haustorial body and lobes (Figure 10). Fungal-specific MAb ML4 bound to the haustorial complexes together with any fungal spores and infection hyphae that were present in the preparation (Figure 11). For all immunofluorescence analyses, sections or cells were completely unlabelled by non-immune mouse immunoglobulin IgM, indicating that neither mouse immunoglobulins nor the SAM-FITC conjugate bound non-specifically to the flax or fungal cell surfaces. No appreciable levels of autofluorescence were observed in the sectioned tissue or the isolated cells.

Immunogold labelling of infected flax leaves postfixed in osmium and embedded in LR White resin was performed in order to determine ultrastructural details of MAb binding. The level of membrane contrast in tissue that had not been postfixed in osmium tetroxide was low and it was difficult to identify the precise region of MAb-binding. Osmium treatment greatly improved contrast without affecting antigenicity; the level of labelling was found to be the same in osmicated and unosmicated tissue (data not shown). Immunogold labelling with the haustorium-specific MAbs, ML1, ML2 and ML3, was observed on the haustorial wall, with the density of binding being relatively constant over the entire wall area (Figure 12). All three MAbs showed similar binding patterns and each displayed equally intense labelling of the haustorial wall. The fungal-specific MAb ML4 also bound to wall material (Figure 13). It was localised to the wall region of all fungal infection structures, with the same level of binding being observed over the haustorial and hyphal walls. The sections were not labelled by non-immune mouse immunoglobulin IgM, suggesting that there was no non-specific binding of the antibodies to tissue components.

Immunofluorescence labelling of haustoria formed in an incompatible interaction

To investigate whether the antigens recognised by MAbs ML1, ML2 and ML3 were present in haustoria that partially develop in an incompatible interaction, a resistant cultivar of flax (RD9) was infected with the same strain of fungus (CH_5) as was used for the study of haustoria formed in a compatible infection. Immunofluorescence labelling was performed on BMM sections of infected leaf tissue that had been fixed 24 h post inoculation. It was found that haustoria within cells which had a healthy microscopical appearance were labelled by the haustorium-specific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Figures 6–11. Immunofluorescence and DIC images of *M. lini* haustoria labelled with the haustorium-specific MAb ML1 (Figs. 6, 7, 10) and the fungal-specific MAb ML4 (Figs. 8, 9, 11). The micrographs show transverse sections of flax leaves infected for 24 h (Figs. 6, 8) and 5 days (Figs. 7, 9) and haustoria and hyphae isolated from infected leaves (Figs. 10, 11). (6A) DIC image of a transverse section of an infected leaf showing fungal spores (S) on the upper epidermis (E) of the leaf, an appressorium (A), infection hypha (IH) and haustorium (H). Bar = 25 μm. (6B) Immunofluorescence labelling with MAb ML1 of the same section as in Figure 6A. Fluorescence, indicating MAb-binding, occurred only around the haustorial body. (7) The haustorium-specific MAb bound to the periphery of the mature haustorium (H). Bar = 10 μm. (8A) DIC image of a section of an infected leaf showing fungal spores (S), an appressorium (A) and germ tubes on the leaf surface and an infection hypha (IH) and haustorium (H) within the leaf. Bar = 25 μm. (8B) Immunofluorescence labelling with MAb ML4 of the same section as in (8A). The MAb recognised all fungal infection structures present in the section, including spores, germ tubes, the infection hypha and the haustorial body (H). (9) At 5 days post-inoculation, the fungal-specific MAb localised to the periphery of the numerous infection hyphae and haustoria that had formed within the leaf by this stage. Bar = 25 μm. (10A) DIC image showing an haustorium (H) and infection hyphae (IH). Bar = 10 μm. (10B) Immunofluorescence labelling with MAb ML1 of the same structures as in Figure 10A. Only the haustorial body was labelled by the MAb. (11A) DIC image showing a single mesophyll cell (M), infection hyphae (IH), and an haustorium (H). Bar = 10 μm. (11B) Immunofluorescence labelling of the same structures as in Figure 11A with MAb ML4. The MAb bound strongly to the infection hyphae and the haustorial body but did not recognise the plant mesophyll cell.

MAbs in a similar fashion to that described for the compatible interaction. Labelling of haustoria within autofluorescent (necrotic) cells could also be detected, although the intensity of binding appeared to be somewhat reduced, possibly an effect of the autofluorescence of the host cell (Figure 14). No labelling could be observed in highly necrotic cells (those with a dense build-up of phenolic compounds in the cytoplasm). In addition, the fungal-specific MAb ML4 was found to label all fungal infection structures formed by the pathogen on a resisitant host (Figure 15). The intensity and appearance of labelling of the spores, hyphae and haustoria, where present, by this MAb were similar to those observations detailed for the compatible reaction.

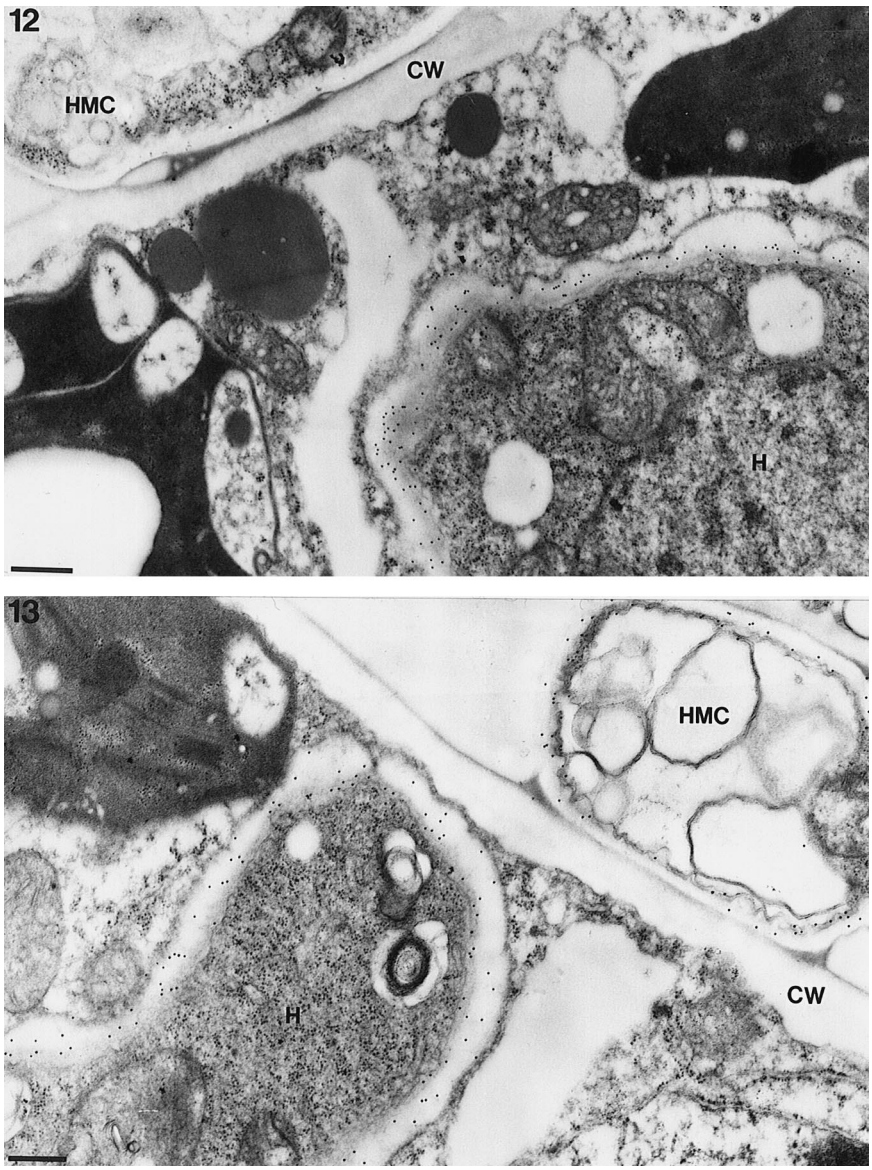
Specificity of MAb binding in the flax/flax rust system and other rust systems

To determine if the antigens targeted by the ML1-ML4 MAbs were present in strains of *M. lini* other than CH_5, flax cultivar Hosh. was infected with either a strain related to CH_5 [CH_5F_2(84)] or an unrelated strain (271). Immunofluorescence labelling of sections of the infected leaf tissue revealed that all four MAbs cross-reacted with these flax/flax rust systems and showed a similar affinity for the haustoria and/or fungal infection structures, at 24 h and 5 days post-inoculation, to that described for the interaction between Hosh. and CH_5. However, neither the haustorium-specific MAbs, ML1, ML2 and ML3, nor the fungal-specific MAb, ML4, showed any cross-reaction with infection structures formed by wheat or maize rust fungi, indicating that the antigens may not be present in other rust pathosystems (data not shown).

Epitope characterisation

To determine the biochemical nature of the epitopes recognised by the MAbs, periodate oxidation and pronase digestion of antigens was performed, and the treated and control samples of sectioned infected leaves were analysed by immunofluorescence microscopy (Figures 16–21). The samples were treated for 1 and 4 h. The haustorium- and fungal-specific MAbs responded in a similar fashion to the treatments. Binding of the MAbs was periodate sensitive, with labelling of haustoria and/or fungal structures being significantly reduced after 1 h of periodate treatment and completely eliminated after 4 h treatment (Figures 17, 20). Pronase treatment did not appear to effect MAb labelling of haustoria and/or fungal structures (Figures 18, 21) and the intensity of labelling of the pronase-treated cells was similar to that of untreated cells (Figures 16, 19). Treated sections were also labelled with an anti-β-tubulin MAb as an additional control. Labelling of microtubules by the anti-β-tubulin MAb was unaffected by periodate treatment but was pronase sensitive (data not shown), confirming that the pronase enzyme used was active. Similar results were found when periodate oxidation or pronase digestion was performed on an unfixed haustorium-enriched suspension that had been dried onto multiwell slides, indicating that the crosslinking action of glutaraldehye on proteins in the fixed and embedded leaves had not affected the action of pronase. These results suggest that all four MAbs recognise carbohydrate epitopes on antigens present in the haustorial or fungal walls.

ML4 was found to label the fungal wall of all infection structures formed by the pathogen, and thus it was thought that the MAb might bind to chitin. Potential inhibition of binding of ML4 by pre-incubation in

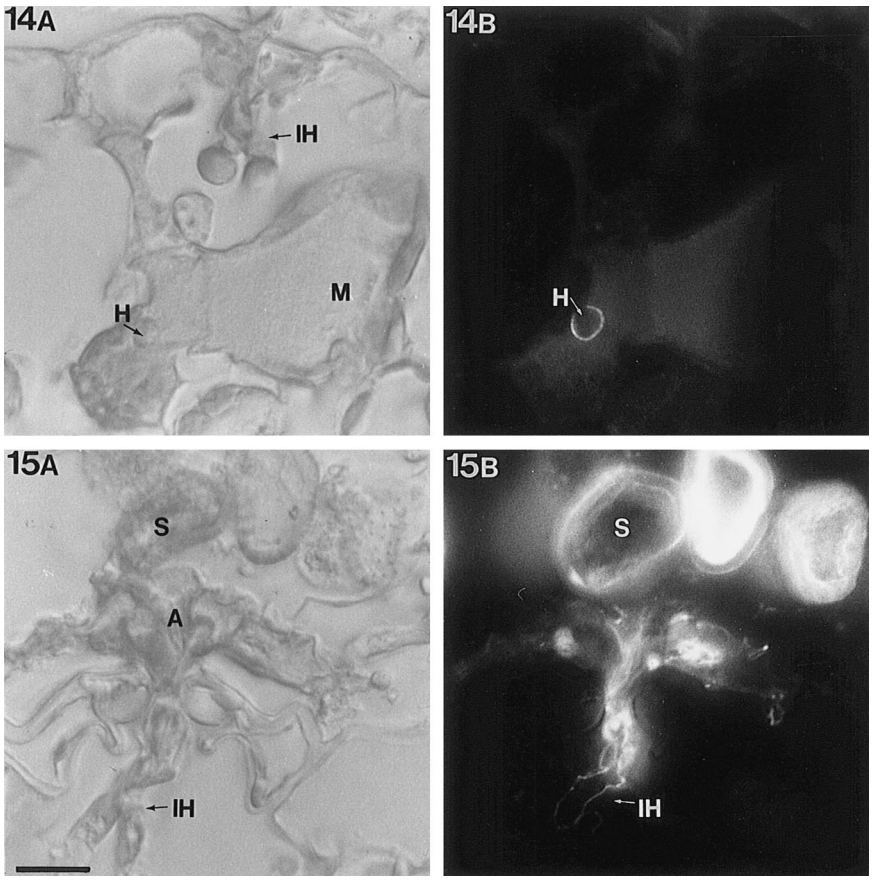


Figures 12–13. Immunogold labelling of flax leaf tissue infected with *M. lini* with haustorium-specific (ML1) and fungal-specific (ML4) MAbs. These micrographs show a compatible interaction at 60 h post-inoculation. Bar = 0.5 μm. (12) Ultrathin section of an haustorium (H) surrounded by the cytoplasm of a mesophyll cell and immunogold labelled with ML1. Dense labelling by the MAb is evident over the cell wall of the haustorium. The antibody did not bind to the cell walls of the host mesophyll cell (CW) or the haustorial mother cell (HMC). (13) Ultrathin section showing an haustorium (H) within a mesophyll cell and immunogold labelled with MAb ML4. The antibody bound specifically to the cell walls of the haustorium and of the haustorial mother cell (HMC), but did not label the cell wall (CW) of the host mesophyll cell.

pure chitin was tested in an immunofluorescence assay, however, the chitin was found to have no inhibitory effect on the binding of ML4 to the fungal wall (data not shown).

Immunoblot analysis

A number of problems were encountered in attempts to characterise the antigens recognised by the MAbs, ML1, ML2, ML3 and ML4, by western blotting. Haustorial complexes and infected leaves were solubilised using a range of approaches. Following separation by

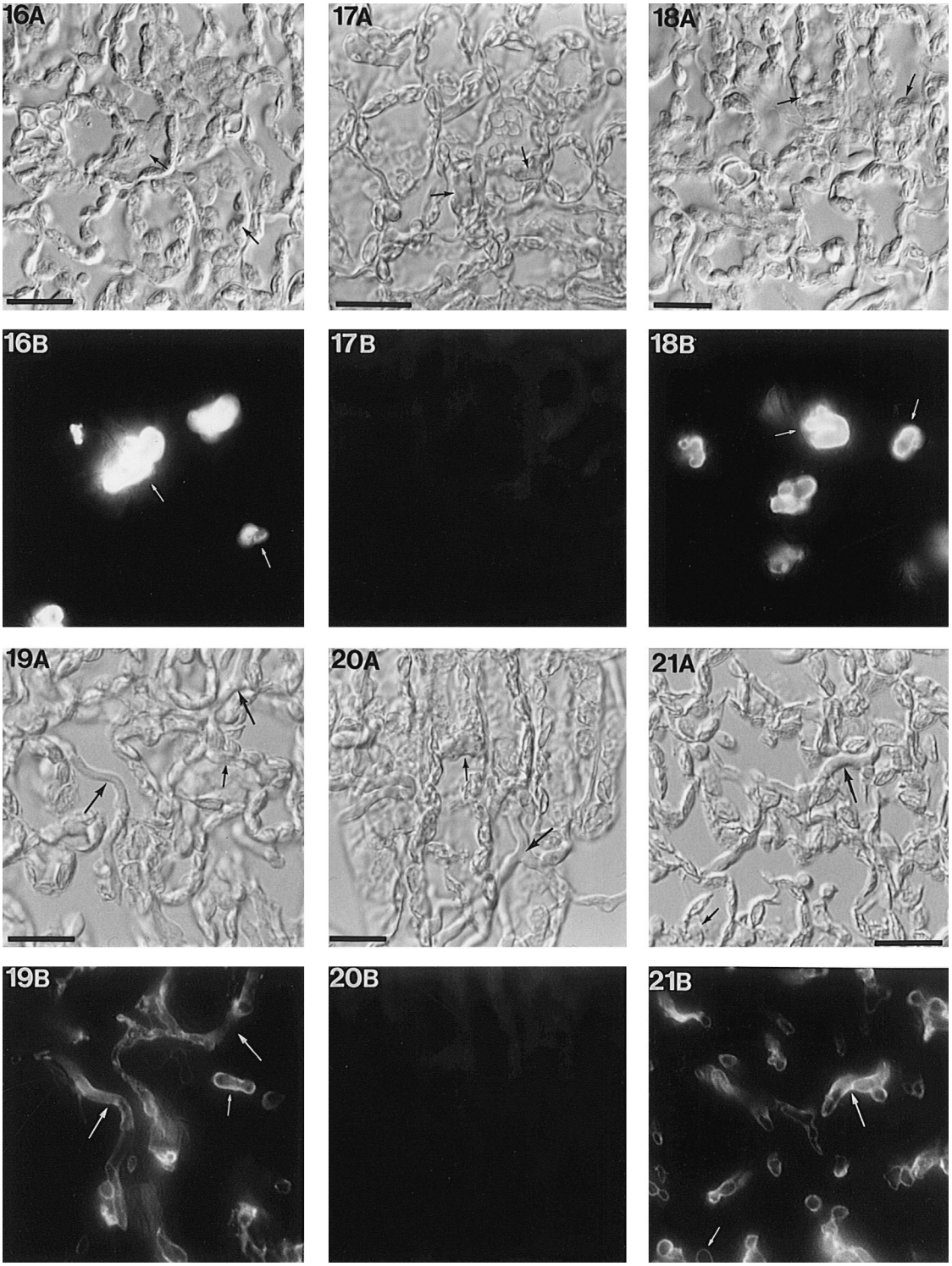


Figures 14–15. Immunofluorescence labelling with MAbs ML1 (Figure 14) and ML4 (Figure 15) of infection structures formed in an incompatible interaction between flax and *M. lini* in material fixed at 24 h post-inoculation. Bar = 10 μm. (14A) DIC image showing infection hyphae (IH) in the substomatal cavity of the leaf, and an haustorium (H) within a mesophyll (M) cell. (14B) Immunofluorescence labelling with ML1 of the same section as in Figure 14A. Only the periphery of the haustorial body (H) was labelled by the haustorium-specific MAb. The autofluorescence which is visible in the cytoplasm of the host mesophyll cell indicates that the cell was in the process of necrosis. (15A) DIC image showing fungal spores (S) and an appressorium (A) on the leaf surface and infection hyphae (IH) in the substomatal cavity. (15B) Immunofluorescence labelling with ML4 of the same section as in Figurel 15A. The spores (S), appresorium, and infection hyphae (IH) were all strongly labelled by the MAb.

SDS-PAGE, proteins were blotted onto PVDF membrane and probed with the MAbs. No specific labelling of protein bands in either the haustorium or infected leaf samples could be detected for any of the MAbs, regardless of the type of sample solubilisation procedure that had been used. To test the efficiency of antigen solubilisation from the flax rust haustoria, the haustorial cells were pelleted after each solubilisation treatment, washed with PBS, and then dried onto slides and immunofluorescently labelled with the MAbs. It was found that a wide range of solubilisation treatments including homogenisation of the sample with a protective protein extraction buffer, sonication of the sample in SDS sample buffer, detergent lysis, freeze-thaw treatment in the presence or absence of detergent, treatment of the sample with high salt solutions, such as 1 M KCl, or chaotrophic agents, such as 6 M guanidine and 8 M urea, and enzymatic digestion, with chitinase and lyticase, did not prevent labelling of the haustorial/fungal wall by the MAbs (data not shown), indicating that these treatments had not released the antigens from the wall.

Discussion

MAbs that are specific for infection structures formed by the dikaryotic uredospores of the flax rust fungus,


16A
17A
18A
16B
17B
18B
19A
20A
21A
19B
20B
21B

←

Figures 16–21. Effects of antigen modification treatments on binding of haustorium-specific (ML1; Figs. 16–18) and fungal-specific (ML4; Figs. 19–21) MAbs to fungal infection structures as assessed by immunofluorescence microscopy. All micrographs show transverse sections of infected leaf tissue fixed 5 days post-inoculation. DIC (16A, 17A, 18A, 19A, 20A, 21A) and immunofluorescence (16B, 17B, 18B, 19B, 20B, 21B) images of the same section are presented for each treatment. Bar = 15 μm. (16A-B) ML1: control. Haustoria (arrows) within the host mesophyll tissue were labelled by the MAb. (17A-B) ML1: periodate oxidation. Treatment of the infected tissue with periodate for 4 h significantly inhibited binding of ML1 to haustoria (arrows). (18A-B) ML1: pronase digestion. Treatment of the infected tissue with pronase for 4 h did not prevent labelling of haustoria (arrows) with ML1 (x 643). (19A-B) ML4: control. Infection hyphae (large arrows) and haustoria (small arrow) were specifically-labelled by the MAb (x 650). (20A-B) ML4: periodate oxidation. Treatment of infected tissue with periodate for 4 h inhibited binding of ML4 to infection hyphae (large arrow) and haustoria (small arrow) (x 583). (21A-B) ML4: pronase digestion. Treatment of the infected tissue for 4 h with pronase did not inhibit binding of ML4 to infection hyphae (large arrow) or haustoria (small arrow) (x 650).

M. lini, have been produced by inoculating mice with haustorium-enriched preparations from rust-infected flax leaves. Many of the MAbs (as represented by ML4) labelled components that were located in the walls of all fungal cells but which were absent from the walls of plant cells. Three MAbs (ML1, ML2 and ML3) showed even greater specificity by targeting antigens in the wall of fungal haustoria only. The four MAbs studied in detail reacted with carbohydrate epitopes on antigens that were present in several different strains of the flax rust fungus and which occurred in both compatible and incompatible interactions, although the MAbs did not recognise equivalent structures in wheat leaves infected with *Puccinia recondita* or in maize infected with *P. sorghi*. The antigens were not removed from the fungal cell walls by a wide range of extraction and solubilisation protocols used for the preparation of material for SDS-PAGE and immunoblotting. They thus appear to be integral wall components which may be covalently linked to other wall polymers.

Production of the MAbs to haustorial wall components was facilitated by the ability to isolate *M. lini* haustoria from infected flax leaves using a protocol based on that described for the isolation of *Uromyces viciae-fabae* and *P. graminis* haustoria from bean and wheat leaves (Hahn and Mendgen, 1992). As was the case for *U. viciae-fabae* and *P. graminis*, the structure and cytoplasmic organisation of the isolated haustoria were well preserved, with loss of cytoplasm through the broken haustorial neck being inhibited by the formation of a plug of electron-dense material. *M. lini* haustoria also had a layer of electron dense material over much of the outer surface of the wall, a feature observed previously on haustoria of other rust fungi and thought to represent remnants of the extrahaustorial matrix (Coffey and Allen, 1983; Heath, 1989; Hahn and Mendgen, 1992).

Hybridomas were first generated against the haustorium-enriched preparation by following conventional methods (Harlow and Lane, 1988). In this fusion, 40% of positive cell lines produced antibodies that reacted specifically with fungal cells present in infected leaves, but 60% recognised plant cell components. The two fusions conducted following the co-immunisation protocol, on the other hand, led to 92–94% of positive cell lines producing antibodies directed towards fungal infection structures, and only 6–8% that recognised plant cell components. The co-immunisation protocol thus more than doubled the production of antibodies to the fungal cells and dramatically reduced the production of antibodies to plant cell components.

Co-immunisation involves the initial preparation of a polyclonal antiserum against antigens that are not of interest, and the addition of this serum to the antigens of interest for the immunisation of another animal (Barclay and Smith, 1986). Hybridoma cells are then produced by conventional methods. The mechanisms by which this protocol restricts the immunological response of mice are not completely clear. Barclay and Smith (1986) propose that the co-injected polyclonal serum induces synthesis of anti-idiotypic antibodies which prevent the production of antibodies that have identical or crossreacting idiotypes to those found on antibodies in the polyclonal serum. Thus, antibodies that react with common antigens will not be produced if they have the same idiotype as those antibodies provided in the polyclonal serum. In this way the technique is thought to mask the immune response of the second mouse to antigens present in the first preparation, and results in an increased response to the antigens of interest.

The co-immunisation technique is particularly useful for MAb production if the cells of interest constitute only a small proportion of the immunogen or if they are in the presence of more immunodominant epitopes. This technique has been used to generate MAbs specific for conidia, infection hyphae or appressoria of *Colletotrichum lindemuthianum* which had been isolated from infected leaves of *Phaseolus vulgaris* L. (Pain et al., 1994; 1995). In the case of the

infection hyphae, although these structures constituted only about 3% of the infection structure-enriched suspension that was used for the immunisations, 18% of MAbs obtained were specific for the hyphae. Co-immunisation has also been used to produce MAbs specific to slime mold differentiation antigens (Barclay and Smith, 1986), human myeloma cells (Fisher et al., 1982) and plant arabinogalactan proteins (Pennell et al., 1991). In the present study, co-immunisation not only yielded a much greater percentage of antibodies that targeted fungal cells, but also resulted in the generation of three haustorium-specific antibodies.

The four MAbs that have been characterised in this study give evidence of two important features of the walls of *M. lini*: *M. lini* walls contain at least two molecules that are not present in the flax cell walls or in the walls of two other rust fungi (*Puccinia* species), and the haustorial wall of *M. lini* contains a molecule that does not occur in the walls of any other infection structure produced by this fungus.

The presence of a fungal wall component that does not occur in the plant cell wall is not surprising. Even in very general terms, basic differences are evident: plant cell walls are composed of microfibrils of cellulose embedded and crosslinked to other polysaccharides, including hemicelluloses and pectins, and structural proteins (Preston, 1974; Darvill et al., 1980; Kato, 1981) while the fungal cell walls are composed of microfibrils of chitin and polymers of β-glucans embedded in a matrix rich in mannoproteins and other glucans (Bartnicki-Garcia, 1968; Wessels and Sietsma, 1981; Gooday, 1994). In addition, it has long been recognised that the composition of fungal cell walls not only distinguishes fungi from other organisms but also is indicative of major taxonomic groupings within the fungal kingdom (Bartnicki-Garcia, 1968). The production of MAbs to fungal cells has led to evidence of various levels of taxonomic specificities in fungal cell wall components. Wall components that are genus-specific (Hardham et al., 1986, 1994; Pain et al., 1992), that react with defined groups of fungi such as arbuscular mycorrhizal fungi (Wright et al., 1996), that react across a class such as the Oomycetes (Estrada-Garcia et al., 1989; note, however, that reactions with organisms from other classes were not tested) or that react across class boundaries (Mitchell et al., 1997; Marshall et al., 1997) have been reported. The lack of binding of the *M. lini* MAbs to the walls of the two *Puccinia* species makes it unlikely that they are binding to common fungal wall constituents, although it is possible that they target a specific epitope on a more widespread molecule. Recognition of chitin was also eliminated by the fact that antibody binding was not inhibited by pre-incubation of the antibody with chitin or by treatment of the haustoria with chitinase.

The production of antibodies that react with components specifically localised within the wall of *M. lini* haustoria was one of the main aims of this project. Many authors have proposed that there must be molecular specialisations on both sides of the haustorium-plant interface, playing structural, metabolic and signalling roles in the interaction between host and pathogen (e.g. Albersheim and Anderson-Prouty, 1975; Manners and Gay, 1983; Mendgen et al., 1985; O'Connell et al., 1996). In support of this idea, immunocytochemical studies have now documented specialisation of proteins and glycoproteins in haustorial and plant extrahaustorial plasma membranes (Mackie et al., 1991, 1993; Roberts et al., 1993). MAbs have also revealed differences in the composition of the fungal cell wall at the level of cell type (Benhamou et al., 1990; Schuren et al., 1993; Pain et al., 1992, 1994). However, there has been no molecular information to substantiate the interpretations of structural and compositional wall differentiation made in early electron microscopy studies of flax rust haustoria (Littlefield and Bracker, 1972). The labelling of haustoria by MAbs ML1, ML2 and ML3 is the first to provide long-awaited new evidence.

For each of the three antibodies, loss of antibody binding after periodate treatment indicates that the antibodies recognise a carbohydrate epitope. It seems most likely that the insensitivity to pronase digestion means that the antigen is a polysaccharide. This evidence is not conclusive, however, because the protease might not have been able to penetrate the wall to gain access to a proteinaceous part of the molecule, or, even after cleavage of peptide chains, the oligosaccharide components containing the epitope might have remained attached to other wall polymers. Because glycoproteins are major constituents of the matrix of fungal cell walls (Gooday, 1994), considerable effort was made in the present study to extract and solubilise haustorial proteins and glycoproteins in order to characterise them by immunoblotting but unfortunately none was successful.

In this paper we have shown that co-immunisation can be used to raise MAbs to specific *M. lini* fungal cell components present in an impure suspension and that this technique causes a reduction in the number of antibodies that recognise structures which are not of interest. Immunofluorescent and immunogold

labelling with four of the MAbs produced in this study has revealed the existence of haustorial-specific and *M. lini*-specific wall components. Our results suggest that the ML1-ML4 antigens are an integral part of the walls of *M. lini* cells and are likely to be covalently crosslinked to other insoluble wall polymers. It also seems likely that the antigens are polysaccharides or glycoconjugates, however, the biochemical nature and function of these components remains to be determined. Future studies will attempt fractionation of wall components from haustorium-enriched preparations, including treatments that break covalent bonds. The presence of the ML1-ML4 antigens in the wall fractions may be assessed directly in enzyme-linked immunosorbant assays, or the fractions may be used in competitive assays in which the haustorium suspension coats the microtitre plate and inhibition of antibody binding by the various fractions is tested.

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