Localization of Melanin in Conidia of *Alternaria alternata* Using Phage Display Antibodies

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Melanins derived from 1,8-dihydroxynaphthalene (DHN) are important for the pathogenicity and survival of fungi causing disease in both plants and animals. However, precise information on their location within fungal cell walls is lacking. To obtain antibodies for the immunocytochemical localization of melanin, 83 phage antibodies binding to 1,8-DHN were selected from a naive semisynthetic single-chain Fv (scFv) phage display library. Sequence analysis of the heavy chain binding domains of 17 antibodies showed a high frequency of positively charged amino acids. One antibody, designated M1, was characterized in detail. M1 bound specifically to 1,8-DHN in competitive inhibition enzyme-linked immunosorbent assays, showing no cross-reaction with nine structurally related phenolic compounds. Epitope recognition required two hydroxyl groups in a 1,8 configuration. M1 also bound to naturally occurring melanin isolated from mycelia of *Alternaria alternata*, suggesting that epitopes remain accessible in polymerized melanin. Transmission electron microscopy-immunogold labeling, using M1 in the form of soluble scFv fragments, showed that melanin was located in the septa and outer (primary) walls of wild-type *A. alternata* conidia, but not those of an albino mutant, AKT88-1. The M1 antibody provides a new tool for detecting melanized pathogens in plant and animal tissues and for precisely mapping the distribution of the polymer within spores, appressoria, and hyphae.

Melanins are high-molecular-weight, brown-black pigments produced by animals, plants, fungi, and bacteria from phenolic or indolic precursors (Bell and Wheeler 1986; Butler and Day 1998). Some fungi (e.g., *Cryptococcus neoformans*) produce melanin by the oxidative cross-linking of DOPA (L-3,4-dihydroxyphenylalanine) present in their environment (Butler and Day 1998). However, melanin is synthesized from 1,8-dihydroxynaphthalene (DHN) in a wide range of economically important plant pathogens, including species of *Alternaria*, *Cochliobolus*, *Colletotrichum*, *Gaeumannomyces*, *Magnaporthe*, and *Verticillium* (Henson et al. 1999; Kubo and Furusawa 1991; Tanabe et al. 1995). The majority of dematiaceous fungi causing human mycoses, such as *Exophiala dermatitidis* and *Sporothrix schenckii*, also produce DHN-melanin (Dixon et al. 1991; Henson et al. 1999; Nosanchuk et al. 1998; Schnitzler et al. 1999). In all these fungi, DHN is synthesized via the polyketide pathway (Fig. 1), which involves the condensation and

cyclization of acetate to form 1,3,6,8-tetrahydroxynaphthalene (THN), followed by alternating reduction and dehydration reactions to form 1,8-DHN, which finally undergoes oxidative polymerization to form melanin (Kubo and Furusawa 1991). Little is known about the site of melanin biosynthesis in fungi, but 1,8-DHN is thought to be synthesized in the cytoplasm and then exported, without exocytosis, into the cell wall and extracellular matrix, where laccases probably catalyze the final polymerization step (Bell and Wheeler 1986; Butler and Day 1998).

Melanin enables fungal pathogens of plants and animals to survive adverse environmental conditions by protecting them against oxygen free radicals (Romero-Martinez et al. 2000), UV radiation (Kawamura et al. 1997), and wall-degrading enzymes produced by microbial antagonists (Butler et al. 2001). Melanins also contribute to the virulence of some human pathogens by protecting the fungal cells against host defense mechanisms (Romero-Martinez et al. 2000; Rosas et al. 2000; Schnitzler et al. 1999). In plant pathogens such as *Colletotrichum* and *Magnaporthe* spp. that produce melanized appressoria, melanin has been shown to be essential for host penetration (Howard and Ferrari 1989; Kubo and Furusawa 1991; Money and Howard 1996). The melanized appressorial cell wall acts as a semipermeable membrane, retaining solutes such as glycerol within the cell but allowing water to enter by osmosis. The exceptionally high turgor pressures generated in this way provide the mechanical force required for penetration of host epidermal cells (Money and Howard 1996).

A proper understanding of the role of melanin in pathogenicity requires knowledge of where the polymer is located in fungal cells. The ultrastructural localization of melanin has largely been based upon its electron-opacity: the polymer is inherently electron-opaque but becomes substantially more so after fixation with osmium and staining with lead salts. More information has been obtained by comparing the ultrastructure of wild-type cells with that of albino mutants blocked at various steps of the melanin biosynthesis pathway (Howard and Ferrari 1989; Kubo and Furusawa 1986; Tanabe et al. 1995) or cells treated with chemical inhibitors of the pathway (Wolkow et al. 1983; Woloshuk et al. 1983). However, such comparisons can be difficult to interpret because melanin is not the only electron-opaque component in fungal cell walls and is not always the most electron-opaque (Henson et al. 1999). Furthermore, shunt products accumulating after blockage of the melanin pathway can themselves form electron-opaque polymers (Kawamura et al. 1997). Procedures for the cytochemical localization of melanin by light and electron microscopy have been based on staining with Nile Blue and Azure A or the reduction of silver nitrate to metallic silver, but none of these

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methods are specific for melanin (Henson et al. 1999; McKeown et al. 1996).

Immunocytochemistry could provide more precise and less ambiguous information on the localization of melanin in fungal walls. However, although monoclonal antibodies and phage display peptides binding to DOPA-melanin have been reported (Nosanchuk et al. 1999; Rosas et al. 2000), no antibodies have yet been raised against DHN-melanin. The extraction of melanin from fungal walls involves harsh procedures, such as prolonged treatment with strong acids and alkali, which could modify the structure of the molecule (Bell and Wheeler 1986). Moreover, such procedures may not completely eliminate contamination from other wall components, such as proteins and polysaccharides (Butler and Day 1998). For these reasons, we have chosen in the present study to prepare antibodies against 1,8-DHN, the immediate precursor of melanin. DHN-binding antibodies were selected from a naive semisynthetic single-chain Fv (scFv) phage library, in which the V_H and V_L domains of human antibodies from an nonimmunized donor have been randomized to high diversity in vitro and displayed as scFv fragments on the surface of filamentous bacteriophage (Griffiths et al. 1994; Winter et al. 1994). Among other advantages, this approach allows the production of antibodies to toxic antigens, such as DHN, by completely avoiding animal immunization (Griffiths and Duncan 1998). Here, we describe the isolation of a set of phage antibodies (PhAbs) binding to 1,8-DHN and DHN-melanin, and the characterization of one these, designated M1, using competitive inhibition enzyme-linked immunosorbent assays (ELISAs). We also demonstrate the application of M1 antibodies for the localization of melanin in conidia of *Alternaria alternata* by transmission electron microscopy (TEM)-immunogold labeling.

RESULTS

Selection of PhAbs binding to 1,8-DHN.

PhAbs can be raised to small molecules such as 1,8-DHN without linking them to a carrier protein, as is necessary for animal immunization. However, to facilitate the coating of immunotubes and ELISA plates, 1,8-DHN was conjugated to bovine serum albumin (BSA) substituted with *p*-aminohippuric acid (PAHA). The conjugate was used as target antigen for panning the phage library; therefore, our selection strategy aimed to enrich for PhAbs binding to 1,8-DHN rather than the protein carrier. Thus, output phage from the first round of panning against the conjugate were subjected to a round of "subtractive" panning against unconjugated BSA, in which the nonbinding phage were selected, followed by three further rounds of panning against the conjugate. After the fifth round of panning, PhAbs derived from 168 randomly selected clones

were screened by ELISA for binding to plates coated with either the conjugate (1,8-DHN-BSA) or the carrier protein plus linker (PAHA-BSA). Eighty-three PhAbs were considered to be specific for 1,8-DHN on the basis that they showed strong reactivity against 1,8-DHN-BSA ($A_{405nm} \geq 0.4$) but very weak reactivity against PAHA-BSA ($A_{405nm} \leq 0.1$). Representative absorbance values for 17 PhAbs showing this differential binding pattern are given in Figure 2. A further 27 PhAbs showed similar differential binding but with less strong reactivity against 1,8-DHN ($A_{405nm} \geq 0.2$). Five PhAbs bound preferentially to PAHA-BSA, and 53 bound equally strongly to both antigens. The control PhAb THY, specific for human thyroglobulin, did not bind to either 1,8-DHN-BSA or PAHA-BSA in ELISAs (results not shown).

To assess phage clone diversity, the scFv inserts from 17 clones showing specificity for 1,8-DHN were sequenced. Analysis of the variable regions of the selected clones revealed a prevalent KAAKTRN motif at the V_H CDR3 binding domain (Table 1) in 9 of the 17 clones (M8, M16, M18, M20, M25, M34, M35, M38, and M41), with another, M29, containing a similar KSGKTR motif. Six of the nine clones containing the KAAKTRN motif were coded for by identical scFv genes, whereas one, M20, contained a different light chain component. Within the CDR3 domain of these clones, arginine, lysine, serine, and threonine were the most common amino acids (Fig. 3).

Characterization of PhAb M1 binding specificity.

Preliminary immunogold labeling experiments using antibodies produced by the 17 clones selected for sequencing showed that M1 labeled melanized fungal cell walls most strongly (see below). For this reason, only M1 was characterized further. Competitive inhibition ELISAs were used to test the ability of M1 to recognize unconjugated 1,8-DHN. In these experiments, serial dilutions of 1,8-DHN, ranging in concentration from 64 nM to 0.26 mM, were used to inhibit the binding of M1 phage to plates coated with the 1,8-DHN-BSA conjugate (Fig. 4). The concentration of 1,8-DHN giving 50% inhibition (IC_{50}) was estimated to be 0.51 μ M. The detection limit was 64 nM (= 10.25 ng/ml), and complete inhibition occurred at concentrations $>66 \mu$ M. In control experiments, binding of THY antibodies to thyroglobulin was not affected by 1,8-DHN at the highest concentration used (results not shown).

The specificity of M1 binding also was investigated with competitive inhibition ELISAs, using nine structurally related phenolic compounds (Fig. 5), at concentrations ranging from 0.13 μ M to 31 mM, to interfere with M1 binding. Catechol, phenol, naphthalene, 1,8-diaminonaphthalene, 1-naphthol, 2-naphthol, 1,5-DHN, 2,3-DHN, and 2,7-DHN all gave no significant inhibition of binding, even at the highest concentration

Fig. 1. Biosynthetic pathway leading to the formation of melanin from acetate, showing the site at which the pathway is blocked in the albino mutant AKT88-1 of *Alternaria alternata*. Adapted from Tanabe and associates (1995). 1,3,6,8-THN = 1,3,6,8-tetrahydroxynaphthalene; 1,3,8-THN = 1,3,8-trihydroxynaphthalene; 1,8-DHN = 1,8-dihydroxynaphthalene.

used, which was over 60,000 times higher than the IC_{50} value for 1,8-DHN. Competitive inhibition ELISAs also were used to test the ability of M1 to bind to DHN-melanin, purified from the mycelium of *A. alternata* (Fig. 6) and DOPA-melanin from the mollusk *Sepia officinalis* (data not shown). Suspensions of both types of melanin inhibited M1 binding in a concentration-dependent manner. *A. alternata* melanin had an IC_{50} of 33 $\mu\text{g/ml}$, whereas *S. officinalis* melanin had an IC_{50} of 100 $\mu\text{g/ml}$. Suspensions of *A. alternata* melanin completely blocked M1 binding at concentrations $>250 \mu\text{g/ml}$, but did not affect binding of the control antibody THY to thyroglobulin (results not shown). M1 also bound to *A. alternata* melanin ($A_{405\text{nm}} = 1.1$) in noncompetitive (direct) ELISAs, using plates coated with 280 μg of melanin per well.

Ultrastructure of melanized and albino conidia.

The mature, melanized conidia of wild-type *A. alternata* had a multicellular, muriform structure, with numerous transverse and longitudinal septa (Fig. 7A). The thick, deeply corrugated cell wall contained two distinct layers (Fig. 7A). These com-

prised a highly electron-opaque outer layer (400–550 nm thick) and a highly electron-lucent inner layer (250–350 nm thick). The outer layer (primary wall) is derived from the original wall of the developing spore, whereas the inner layer (secondary wall) completely surrounds individual cells within the spore and is deposited after the cells become delimited by septa (Campbell 1969). The septal partitions were highly electron-opaque and abutted directly onto the primary wall (Figs. 7A and 8A).

The structure of conidia produced by the albino mutant AKT88-1, which is deficient in the synthesis of THN from acetate (Fig. 1) (Tanabe et al. 1995), was markedly different from that of wild-type spores (Fig. 7B–D). All the albino conidia had a smooth surface, unlike the highly corrugated wild-type spores, and both primary (50–230 nm thick) and secondary (75–250 nm thick) cell walls were consistently thinner than those of the wild type (Fig. 7C and D). Furthermore, in many conidia (approximately 60 to 70% of the population) the constituent cells were loosely arranged and partially separated from each other (Fig. 7B and C) and the primary wall was ruptured and discontinuous (Fig. 7C and D).

Immunolocalization of melanin in fungal cell walls.

For immunogold labeling, antibodies were used in the form of soluble scFv fragments because their small size (4–5 nm) offered much higher spatial resolution and labeling efficiency than scFv displayed on phage particles (filaments 1–2 μm long). In wild-type conidia, primary walls and septa were strongly labeled by M1, but secondary walls were not labeled (Fig. 8A). Within the primary cell wall, labeling was most intense in the corrugated surface region (Fig. 8A). In contrast, M1 did not label the hyaline walls and septa of conidia of the albino mutant AKT88-1 (Fig. 8B) and the vegetative hyphae of the wild-type strain (Fig. 8C). The primary cell wall and septa of *A. alternata* conidia are believed to contain melanin on the basis that melanin is electron-opaque, and ultrastructural comparison of the conidia of melanin-deficient mutants with those of wild-type strains (Kawamura et al. 1999; Tanabe et al. 1995). Taken together with the data from competitive inhibition ELISAs described above, our results indicate that M1 specifically labeled melanin in wild-type conidia. The specificity of M1 binding was confirmed by the observation

Table 1. Predicted peptide sequences of CDR3 regions and V gene families of 1,8-dihydroxynaphthalene binding phage

Clone	CDR3 sequence	Heavy chain	Light chain
M1	GFGARRKL	V-III	λ V-I
M3	TRFRMSRWFR	V-III	λ V-III
M4	TPLVTM	V-III	λ V-III
M8	KAAKTRNR	V-I	κ V-III
M9	RTSRTIRT	V-I	λ V-III
M16	KAAKTRN	V-I	κ V-III
M18	KAAKTRNR	V-I	λ V-III
M20	KAAKTRN	V-I	λ V-IV
M21	HTGNHV	V-III	λ V-III
M24	RSSMPP	V-I	...
M25	KAAKTRN	V-I	κ V-III
M28	RTSRTIRT	V-I	λ V-III
M29	KSGKTRSL	V-I	κ V-III
M34	KAAKTRN	V-I	κ V-III
M35	KAAKTRN	V-I	κ V-III
M38	KAAKTRN	V-I	κ V-III
M41	KAAKTRN	V-I	κ V-III

^a ... indicates the absence of any light chain sequences in this clone.

Fig. 2. Differential binding in enzyme-linked immunosorbent assays of 17 phage-displayed antibodies. Plates were coated with either 1,8-dihydroxynaphthalene (DHN) conjugated to bovine serum albumin (BSA) via *p*-aminohippuric acid (PAHA) or PAHA-derivatized BSA alone. Antibodies were tested after the fifth round of selection from the phage display library. Absorbance (405 nm) was measured after incubation with substrate for 1 h at 25°C.

Fig. 3. Relative frequencies of amino acids in the CDR3 domains of phage binding 1,8-dihydroxynaphthalene. The histogram was constructed from the amino acid sequences presented in Table 1, using a representative clone from each clonal group.

that preincubation of the antibody with 1,8-DHN at 10 $\mu\text{g/ml}$ abolished labeling of the walls of wild-type conidia (Fig. 8D). The absence of labeling on cell walls when M1 was replaced with the control antibody THY (Fig. 8E) or extraction buffer (results not shown) demonstrates that neither scFv nor the antibody detection system bound to cell walls nonspecifically.

M1 consistently produced a small amount of gold labeling on the fungal cytoplasm (Fig. 8A–D), which was scattered randomly over all organelles and the cytosol but was absent from vacuoles. Synthesis of 1,8-DHN is likely to occur in the cytoplasm (Butler and Day 1998; Henson et al. 1999); therefore, a proportion of the cytoplasmic labeling observed with M1 could represent the intracellular location of melanin precursors. However, most of this labeling was probably not antigen specific, because it was also present on the cytoplasm of the

albino mutant (Fig. 8B) and was not eliminated by preincubation of M1 with 1,8-DHN (Fig. 8D). Fungal cytoplasm also was labeled to the same extent by THY antibodies (Fig. 8E) and five other antibodies from the Griffon.1 library, suggesting that scFvs from this library bind nonspecifically to cytoplasmic components. Blocking with a range of inert proteins or detergents and increasing the ionic strength or pH of the scFv preparations did not reduce this cytoplasmic labeling. Systematic deletion of the scFv coding region may reveal which portion is responsible for nonspecific binding, but the cationic polyhistidine tag probably is not involved because scFv lacking this hexapeptide also labeled the cytoplasm (results not shown).

DISCUSSION

In this article, we have described the selection of a phage antibody, M1, from a naive phage display library that specifically binds to 1,8-DHN. The phage-displayed antibody was used to develop a sensitive competitive inhibition ELISA for the detection of 1,8-DHN and DHN-melanin, while soluble scFv fragments of the antibody were successfully used to localize melanin in *A. alternata* conidia. Conventional monoclonal antibodies and phage display peptides binding to DOPA-melanin from the human pathogen, *Cryptococcus neoformans*, have been reported (Nosanchuk et al. 1999; Rosas et al. 2000). However, this is the first time antibodies of any type have been raised to DHN-melanin and used to immunolabel melanin in fungal cell walls. To our knowledge, this is also the first reported use of scFv fragments for immunogold labeling, although Fv fragments have been used for this purpose (Kleymann et al. 1995).

PhAb M1 showed remarkably high specificity for 1,8-DHN in competitive inhibition ELISAs, giving no cross-reaction with a range of structurally related compounds, including 1-naphthol and three forms of DHN with different patterns of hydroxylation. These results suggest that epitope recognition by M1 requires two hydroxyl groups located at the C-1 and C-8 positions. Competitive inhibition and direct ELISAs also demonstrated that M1 is reactive with fungal-derived melanin and that epitopes remain accessible to the antibody in the cross-linked DHN polymer. However, it should be noted that relatively harsh procedures were used to extract this melanin from *Alternaria* cell walls, so that its structure may differ from that of native melanin (Bell and Wheeler 1986). Although re-

Fig. 4. Inhibition curve showing the ability of the phage-displayed antibody M1 to recognize 1,8-dihydroxynaphthalene (DHN) in a competitive inhibition enzyme-linked immunosorbent assay. Plates were coated with 1,8-DHN conjugated to bovine serum albumin and absorbance was measured at 405 nm after incubation with substrate for 90 min at 25°C.

Fig. 5. Chemical structures of nine phenolic compounds used for competitive inhibition enzyme-linked immunosorbent experiments. DHN = dihydroxynaphthalene.

activity against DOPA-melanin was threefold lower in ELISAs, the binding by M1 suggests that this polymer shares some structural features with DHN-melanin. Both polymers contain aromatic rings and free hydroxyl groups, but DOPA-melanin differs in that it contains nitrogen and carboxyl groups (Henson et al. 1999).

The four most common amino acids in the CDR3 domains of 17 DHN-binding PhAbs, including M1, were either positively charged or neutral, with no representation of any negatively charged residues. Electrostatic interactions could therefore be important for scFv binding to 1,8-DHN which, like polymerized melanin, is negatively charged (Butler and Day 1998). Positively charged amino acids also were prevalent in peptides binding to DOPA-melanin, isolated from a decapeptide phage display library (Nosanchuk et al. 1999). However, unlike scFv binding to 1,8-DHN, these peptides also were rich in aromatic amino acids and contained the motif ++*H, where + represents a positively charged residue and * is either a positive or aromatic residue (Nosanchuk et al. 1999).

Previous comparisons of the ultrastructure of wild-type *A. alternata* conidia with that of two different albino mutants have provided indirect evidence that the electron-opaque layers in conidial walls and septa contain melanin (Kawamura et al. 1997; Tanabe et al. 1995). However, interpretation of these images is complicated by the fact that electron-opaque materials other than melanin were clearly present in walls and septa, as was observed here (Fig. 8B). In the present study, immunogold labeling has provided less ambiguous, high-resolution information on the distribution of melanin in *A. alternata*. Our results show that melanin is not deposited uniformly across conidial walls, being concentrated in septa and the outer region of primary cell walls but absent from secondary walls. Melanization of the primary wall begins at the start of spore formation (Campbell 1969), but appears to stop by the time secondary cell walls are laid down, suggesting that melanin biosynthesis is precisely regulated during conidiogenesis.

Melanin is not essential for the pathogenicity of the Japanese pear pathotype of *A. alternata*, because melanin-deficient mutants have the same capacity to produce necrotic lesions on

host tissue as the parent wild-type strain (Kawamura et al. 1999; Tanabe et al. 1995). However, the pigment does protect conidia from UV radiation and is likely to promote survival of the pathogen in the field (Kawamura et al. 1999). These authors also showed that melanin may have a role in conidial development, because targeted disruption of the *BRM2* gene, encoding 1,3,8-trihydroxynaphthalene reductase, reduced conidial size and septal number. In the present study, the unusual structure of albino mutant AKT88-1 suggests that melanization also affects cell wall thickness and surface ornamentation in *A. alternata* conidia. In addition, the partial disintegration of albino spores indicates that melanin may have an important structural role in this pathogen, perhaps contributing to the strength and cohesion of primary cell walls and septa. It remains possible that mutations unrelated to melanin biosynthesis are responsible for the observed abnormalities in conidial morphology, because AKT88-1 was obtained by chemical mutagenesis (Tanabe et al. 1995). However, the same phenotype was also reported in a spontaneous albino mutant in *A. brassicicola* (Campbell 1970).

In conclusion, the PhAb M1 provides a new tool for the specific and precise localization of DHN-melanin within fungal cell walls. Immunolabeling with M1 will be especially useful where it is necessary to distinguish melanin from other electron-opaque wall components or where melanin is not present in sufficient quantity to impart high electron-opacity (Henson et al. 1999) (e.g., during the early stages of melanization). In particular, immunolabeling with PhAb M1 could allow a detailed temporal and spatial analysis of melanin deposition during appressorial development in *M. grisea* and species of *Colletotrichum*. In these fungi, the crucial role played by melanin in generating the high turgor pressure required for host penetration is well established (Howard and Ferrari 1989; Money and Howard 1996). However, there remains uncertainty as to where the polymer is located within appressoria. Based on TEM observations of albino and wild-type appressoria, it has been concluded that melanin is concentrated in a very thin sheet just outside the plasma membrane (Kubo and Furusawa 1991) or is restricted to an inner layer of the appressorial cell wall (Howard and Ferrari 1989; Wolkow et al. 1983). On the other hand, the observation of flocculent electron-opaque granules in the extracellular matrix surrounding appressoria suggests that melanin deposition extends beyond the appressorial cell wall (Kubo and Furusawa 1986; O'Connell et al. 2000). Immunogold labeling with M1 should help to establish the precise location of melanin within appressoria of these fungi. Other applications of the antibody could include the immunodetection of DHN-melanin and melanized pathogens in plant tissues (e.g., melanized spores or appressoria on plant surfaces or hyphae of sap-staining fungi in wood) (Brisson et al. 1996).

MATERIALS AND METHODS

Microorganisms.

Two *Escherichia coli* strains were used in this study as hosts for the bacteriophage: TG1 suppressor strain for propagation of phage particles (Gibson 1984) and HB2151 nonsuppressor strain for expression of antibody fragments (Carter et al. 1985). Both strains were grown on tryptone yeast extract (TYE) medium containing 1% (wt/vol) glucose and antibiotic as required (Marks et al. 1991).

The phage display library used was a human semisynthetic $V_H + V_L$ scFv library (Griffin.1) containing 1.2×10^9 unique clones, which was kindly provided by the Center for Protein Engineering, MRC Center, Cambridge, UK. This library contains the same synthetic human V-genes as those described by

Fig. 6. Inhibition curve showing the ability of the phage-displayed antibody M1 to recognize dihydroxynaphthalene (DHN)-melanin, purified from mycelia of *Alternaria alternata*, in a competitive inhibition enzyme-linked immunosorbent assay. Plates were coated with 1,8-DHN conjugated to bovine serum albumin and absorbance was measured at 405 nm after incubation with substrate for 40 min at 25°C.

Griffiths and associates (1994) but is in an scFv rather than Fab format. The vector also is a phagemid rather than a phage, so the library is similar to that described by Nissim and associates (1994), but with diversity in the light chains as well as the heavy chains (Griffiths et al. 1994). A control phage antibody (THY) specific for human thyroglobulin also was provided by the Center for Protein Engineering.

Cultures of the Japanese pear pathotype of *A. alternata* were kindly provided by Takashi Tsuge (School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan). These included a melanized wild-type isolate (15A) and an albino mutant (AKT88-1), derived from 15A by nitrosoguanidine-mediated mutagenesis (Tanabe et al. 1995).

Isolation of DHN melanin from fungal cell walls.

Cultures of *A. alternata* isolate 15A were grown on potato dextrose broth (Sigma-Aldrich, Poole, Dorset, U.K.) under stationary conditions at $25 \pm 1^\circ\text{C}$ for 20 days. Melanin was extracted from the mycelia by a method adapted from Malama

and associates (1998). Briefly, mycelia were washed with de-ionized water, homogenized in an Atomix blender, and extracted with hot NaOH. After removal of the fungal residue by centrifugation, melanin was precipitated from the supernatant with concentrated HCl, washed with hexane, redissolved in NaOH and precipitated with acid, and finally washed extensively with deionized water and dried.

Synthesis of 1,8-DHN-BSA conjugate.

The target antigen, 1,8-DHN (Fluka, Gillingham, Dorset, UK), was conjugated to BSA (Sigma Product No. A-7638) via a diazotization reaction with PAHA-substituted BSA using the method of Jourdan and associates (1982), except that 5 mg of 1,8-DHN and 50 mg of substituted BSA were used for synthesis of the azo-conjugate. In this reaction, amino groups on the protein are likely to be linked via PAHA to the aromatic rings of 1,8-DHN at the C-2, C-4, C-5, or C-7 positions. The ratio of hapten to carrier protein in the conjugate was calculated from the absorbances of unconjugated 1,8-DHN, PAHA-BSA, and

Fig. 7. A, Transmission electron micrograph showing a longitudinal section through a conidium of *Alternaria alternata* wild-type isolate 15A. Note that the deeply corrugated primary wall (1) and septa (S) are highly electron-opaque, whereas the secondary wall (2) is highly electron-lucent. Section stained with uranyl acetate. Bar = 2 μm . B, Light micrograph showing the melanized conidium of wild-type *Alternaria alternata* and the hyaline conidium of albino mutant AKT88-1. Bar = 5 μm . C and D, Transmission electron micrographs showing a conidium of albino mutant AKT88-1. Note the loose arrangement of cells within the spore and the abnormally thin primary wall (arrowheads), which appears fragmented (arrows). Section stained with periodic acid-thiocarbohydrazide-silver proteinate. Bars = 1 μm .

the conjugate (measured at 250 nm in water), as described by Atzorn and Weiler (1983). On this basis, the molar ratio of 1,8-DHN to BSA in the conjugate was estimated to be 8:1.

Selection of 1,8-DHN-binding phage.

The Griffin.1 phage library was panned against antigens immobilized on Maxisorp immunotubes (Nunc, Roskilde, Denmark) as described by Marks and associates (1991), with the following modifications. Immunotubes were coated with 4 ml of either 1,8-DHN-BSA at 100 µg/ml or unconjugated BSA in 50 mM carbonate buffer (pH 9.5) at 100 µg/ml. Selection consisted of five rounds of panning. In the first of these, the li-

brary was panned against 1,8-DHN-BSA-coated tubes. Output phage then were subjected to one round of “subtractive” panning against BSA-coated tubes, in which nonbinding phage were selected and used for three subsequent rounds of panning against 1,8-DHN-BSA.

ELISA screening of PhAbs.

Out of the mixed population of output phage obtained from the final round of panning, 168 colonies were grown on individually to produce “monoclonal” phage (Marks et al. 1991). These were screened for binding to 1,8-DHN-BSA or PAHA-BSA by ELISA. Binding of the PhAb THY to plates coated

Fig. 8. Transmission electron micrographs showing immunogold labeling of melanin in conidia of *Alternaria alternata*. Sections were labeled with soluble single-chain Fv fragments of phage antibody (PhAb) M1, followed by mouse anti-*c-myc* antibodies and goat anti-mouse immunoglobulin G antibodies conjugated with 10 nm colloidal gold particles. Bars = 0.5 µm. **A**, Surface region of a wild-type conidium. Note M1 labels the electron-opaque primary wall (PW) and septum (S). The highly electron-lucent secondary wall (SW) is not labeled. **B**, Conidium of albino mutant AKT88-1, showing the absence of M1 labeling from all regions of the cell wall and S. Note that in this mutant, the surface of the cell wall is less corrugated than in wild-type conidia, and the abnormally thin PW retains some electron-opacity. **C**, The hyaline cell wall of the wild-type vegetative hypha (HW) is not labeled by PhAb M1, in contrast to the intensely labeled PW of the adjacent conidium. **D**, Following preincubation with 1,8-dihydroxynaphthalene at 10 µg/ml, M1 antibodies no longer label the PW of a wild-type conidium. **E**, Control PhAb THY, specific for human thyroglobulin, does not label the primary wall of a wild-type conidium.

with human thyroglobulin (SCIPAC Ltd, Sittingbourne, Kent, UK) provided a positive control. Microtiter plates (Maxisorp; Nunc) were coated overnight at 20°C with 50 µl per well of a solution of each antigen in carbonate buffer at 50 µg/ml. Plates then were processed for ELISA as described by Pope and associates (1996), except that binding phage were detected using rabbit anti-fd bacteriophage antibodies (Product No. B-7786; Sigma) followed by goat antirabbit immunoglobulin G (IgG) antibodies conjugated with alkaline phosphatase (Sigma). After development with *p*-nitrophenyl phosphate substrate for 1 h at 25°C, absorbance was read at 405 nm.

Characterization of PhAb specificity.

Competitive inhibition ELISAs were used to quantify PhAb binding in solution to the following: (i) DHN-melanin extracted from *A. alternata*; (ii) DOPA-melanin, extracted from ink glands of the cuttlefish *Sepia officinalis* (Sigma Product No. M2649), and (iii) nine phenolic compounds structurally related to 1,8-DHN (Fig. 5). These were 1,5-DHN (Aldrich, Gillingham, Dorset, UK), 2,3-DHN (L. Light & Co., Poyle, Bucks, UK), 2,7-DHN (Aldrich), catechol (BDH, Poole, Dorset, UK), 1-naphthol (BDH), 2-naphthol (Aldrich), 1,8-diaminonaphthalene (Aldrich), naphthalene (Aldrich), and phenol (BDH). Microtiter plates (Maxisorp) were coated overnight at 20°C with 50 µl per well of a solution of 1,8-DHN-BSA in carbonate buffer at 10 µg/ml, washed three times with phosphate-buffered saline (PBS) and blocked with 3% (wt/vol) fat-free milk powder (Marvel) in PBS (MPBS) for 2 h at 37°C. After incubating PhAbs (final concentration 10¹⁰ phage/ml) with serial dilutions of the competitor compounds in 3% MPBS for 1 h at 25°C, aliquots (50 µl per well) were added to the pre-blocked ELISA plates and incubated for 90 min at 25°C. Thereafter, plates were treated as described for PhAb screening, except that development in substrate was for 90–120 min. Absorbance values are expressed as a percentage of the maximum absorbance, obtained in the absence of competitor, and represent the mean of two replicate determinations.

To assess the binding of PhAbs to melanin directly, an aqueous suspension (70 µl) of *A. alternata* melanin at 4 mg/ml was placed in the wells of ELISA plates (Costar, Corning Life Sciences, New York) and heat-fixed to the plastic by drying at 85°C for 2 h (Nosanchuk et al. 1998). After blocking with 200 µl per well of 3% MPBS for 2 h at 37°C, plates were incubated with PhAbs and treated as described above for screening ELISAs, except that development in substrate was for 40 min at 25°C. Data are presented as means of four replicate determinations.

DNA sequencing.

To assess phage clone diversity, scFv inserts were amplified by the polymerase chain reaction (PCR) using the primers LMB3 (5' CAG GAA ACA GCT ATG AC 3') and fd SEQ1 (5' GAA TTT TCT GTA TGA GG 3'). Templates for sequencing consisted of either amplified DNA gel-purified with the Qiaquick kit (Qiagen, Crawley, West Sussex, U.K.) or plasmid DNA purified using a Quiagen miniprep kit. Templates were sequenced with the primers FOR_LinkSeq (5' GCC ACC TCC GCC TGA ACC) and PHEN-SEQ (5' CTA TGC GGC CCC ATT CA) using the ABI Prism dye terminator cycle sequencing reaction kit and an ABI Prism 377 automated sequencer. The sequences of V_H + V_L regions were aligned using CLUSTAL X software (Thompson et al. 1997) and compared with databases using FASTX.

Production of soluble antibody fragments.

Soluble scFvs were harvested from the periplasm of *E. coli* strain HB2151 infected with selected phage using methods de-

scribed by Pope and associates (1996), except that the extraction buffer was 50 mM Tris-HCl (pH 8) containing 1 mM EDTA and 20% wt/vol sucrose. After centrifugation of the periplasmic fraction (16,000 × *g* for 10 min at 4°C), the supernatant was passed through a 0.22-µm sterile membrane filter and stored at 4°C. The presence of scFv in the periplasmic preparations was confirmed by Western blotting. Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions on a 4 to 20% gradient gel, transferred to nitrocellulose, and probed using a mouse anti-*c-myc* monoclonal antibody (9E10; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) diluted 1:2000, followed by alkaline phosphatase-conjugated goat antimouse IgG antibodies as described by Hughes and associates (1999).

Preparation of conidia for electron microscopy.

Cultures of *A. alternata* were grown on V8 juice agar at 20 ± 1°C with illumination from black fluorescent lights (Philips TLD/36W; 12-h photoperiod). After 9 days, cultures were flooded with water containing 0.1% (vol/vol) Tween-20 and scraped with a glass rod to release conidia. After filtering the spore suspension through four layers of muslin, conidia were centrifuged (1,000 × *g* for 10 min) and the pellet resuspended in a mixture of 4% (wt/vol) *para*-formaldehyde and 0.5% (vol/vol) glutaraldehyde in 50 mM sodium phosphate buffer (pH 7.2). Postfixation with osmium tetroxide was omitted to avoid possible oxidation of epitopes within the melanin polymer. After fixation for 2 h, conidia were rinsed three times (10 min each) with deionized water and embedded in 4% (vol/vol) low-gelling-temperature agarose (Hughes et al. 1999). Samples then were dehydrated in an ethanol series, infiltrated with LR White acrylic resin for 3 days, and polymerized at 55°C for 24 h. Ultrathin sections were stained with either uranyl acetate or periodic acid-thiocarbohydrazide-silver proteinate to detect carbohydrates (O'Connell and Carzaniga 2001).

Immunogold labeling.

Ultrathin sections were mounted on Formvar-coated gold slot grids (1 by 2 mm). Grids were floated on drops of reagent in the following sequence: (i) blocked with PBS containing 5% (vol/vol) normal goat serum, 1% (wt/vol) BSA, and 0.1% (vol/vol) fish skin gelatin for 30 min; (ii) incubated in undiluted periplasmic preparation for 90 min; (iii) washed five times (3 min each) in PBS containing 1% BSA (PBSA); (iv) incubated in mouse monoclonal antibody 9E10 diluted 1:50 in PBSA for 60 min; (v) repetition of step (iii); (vi) incubated in goat antimouse IgG antibodies conjugated with 10 nm colloidal gold particles (British Biocell International, Cardiff, UK) diluted 1:50 in PBSA for 60 min; (vii) repetition of step (iii); (viii) washed five times (3 min each) in deionized water; and (ix) stained with 2% (vol/vol) aqueous uranyl acetate for 2 min and examined with a Hitachi H7000 TEM. Staining with lead citrate was omitted because it imparted too much contrast to the sections, making observation of the colloidal gold particles difficult. In control experiments, the periplasmic preparation was preincubated with 1,8-DHN at 10 µg/ml for 1 h before use, or replaced with either extraction buffer or a periplasmic preparation of the THY antibody.

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