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Production and Characterization of Two Monoclonal Antibodies Specific for *Plasmopara halstedii*

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Sunflower downy mildew, caused by the fungus *Plasmopara halstedii*, is a potentially devastating disease. We produced two monoclonal antibodies (MAbs) (12C9 and 18E2) by immunizing mice with a partially purified extract of *P. halstedii* race 1. Both MAbs detected in enzyme-linked immunosorbent assay (ELISA) all races of *P. halstedii* present in France. No cross-reactions were observed with *Plasmopara viticola* or with other fungi commonly associated with sunflowers. Both MAbs recognized the same three fungal antigens with molecular masses of 68, 140, and 192 kDa. However, the epitopes on the fungal antigens were distinct and repetitive. Seed homogenates from infected plants were incubated in wells coated with MAb 18E2. This resulted in the trapping of *P. halstedii* antigens that were identified with biotinylated MAb 12C9. No reactions were seen with seed homogenates from healthy plants. Thus, our results suggest that these MAbs might be used to develop a sandwich ELISA detection system for *P. halstedii* in infected seeds.

Downy mildew caused by *Plasmopara halstedii* (Farlow) Berlese et De Tony is one of the economically most important diseases of sunflowers, *Helianthus annuus* L. The fungus, which is an obligate parasite of sunflowers, occurs in all areas where sunflowers are cultivated extensively except Australia, South Africa, and possibly parts of North Africa (21, 23). Systemic downy mildew infection alters the development of vegetative and generative parts of the plant, as well as its metabolism (26, 32). Inoculation of sunflower plants with *P. halstedii* at the two-leaf stage through apical buds greatly inhibits stem elongation. The yield of infected plants is usually less than 25% that of uninfected plants. There is no fungicide to control this disease after infection has occurred.

Little information is available on the epidemiology and biochemistry of *P. halstedii* or its relationship with its sunflower host. Seven physiological races of *P. halstedii* have been identified (10, 11, 24), and they are capable of attacking a wide range of sunflower genotypes. In France, in addition to the three races—1, A (equivalent to American race 4), and B (equivalent to American race 3)—classically encountered (19), two new races, designated C and D, have been detected recently (13). In the Red River Valley of North Dakota, Minnesota, and Manitoba, all but race 1 have been identified (24). Some sunflower varieties carry resistance genes (*Pl*) against *P. halstedii* races present in France and in the United States (15, 17, 18, 20, 28). Genetic variation in the pathogen appears limited, since no random amplification of polymorphic DNA variation was found among isolates from races 1, A, and B or between isolates of the same race, and very few (89% similarity) polymorphisms were identified among all races of *P. halstedii* present in France (22).

Downy mildew of sunflowers may result from oospores in the soil (6). Contamination of seeds by *P. halstedii* has also been implicated in the establishment of the disease (6). The only effective control technique for mildew-sensitive sunflower varieties is to treat seeds with metalaxyl (Apron 35SD) (1). However, metalaxyl-resistant isolates of *P. halstedii* have been described (1), and laboratory tests of the effectiveness of fungicide treatment of the seeds showed a decreased sensitivity of the fungus to the drug (13). In this context, there is a greater need for the development of efficient methods to detect *P. halstedii*. Our objective in this study was to develop a monoclonal antibody (MAb) that recognized all of the races of *P. halstedii* present in France.

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MATERIALS AND METHODS

Microorganisms and culture conditions. We used one isolate of each of the five races of *P. halstedii* present in France. Isolates were maintained by Groupe d'Etude des Variétés et des Semences (Angers, France) or Institut de Recherche Agronomique (Clermont-Ferrand, France). Races 1, C, and D were maintained on sunflower line HA89 (Peredovick variety), and races A and B were maintained on hybrid GH RHA266 (Pharaon variety), which contains the gene *Pl1* and is resistant to race 1. Artificial infections were made by immersing whole seedlings as described by Cohen and Sackston (3).

Preparation of crude fungal extracts. Zoosporangia and hyphae of *P. halstedii* were collected by scraping 150 contaminated cotyledons with a paintbrush in 50 ml of distilled water. The fungal suspension was sonicated for three periods of 5 min each using a Vibra Cell sonicator (Bioblock Scientific, Illkirch, France) (power, 24 W) and centrifuged at 12,000 $\times$ *g* for 10 min. The resulting supernatant, which corresponds to the crude fungal extract, was stored as 2-ml aliquots at −20°C until use.

For the preparation of MAbs, the crude extract of *P. halstedii* race 1 was partially purified by fractionated ammonium sulfate precipitation. Saturated ammonium sulfate was added to the crude extract to a final concentration of 0.65 M. The solution was incubated for 15 min at room temperature before being centrifuged (12,000 $\times$ *g*; 10 min). Ammonium sulfate was added to the resulting supernatant to a final concentration of 2 M. After incubation for 15 min and centrifugation as described above, the pellet was resuspended in distilled water and dialyzed for 24 h at 4°C against 200 volumes of distilled water.

Crude extracts of *Plasmopara viticola*, cultivated on vine leaves, and of other fungi (Table 1) potentially encountered on sunflower seeds as saprophytes or pathogens, cultivated on malt agar medium, were also prepared by sonication.

The total protein content of the extracts was determined by the method of Bradford (2), using bovine serum albumin (BSA) as a standard.

Germination of zoosporangia. Zoosporangia were isolated by scraping 10 contaminated cotyledons with a paintbrush in 50 ml of a 1% saccharose solution. Germination (release of zoospores and germ tube formation) followed incubation of the suspension of zoosporangia (about 5×10^6 per ml) at 16 to 18°C with gentle shaking. The first zoospores released under these conditions appeared 2 h after the beginning of the incubation, and most had initiated germ tube formation after 5 h (4).

TABLE 1. Specificities of MAbs 12C9 and 18E2 for *P. halstedii* as determined by ELISA

Species and race	OD[a]	
	MAb 12C9	MAb 18E2
P. halstedii race 1	0.97 ± 0.009	0.88 ± 0.004
P. halstedii race A	0.87 ± 0.003	0.84 ± 0.006
P. halstedii race B	0.76 ± 0.004	0.73 ± 0.008
P. halstedii race C	0.83 ± 0.002	0.83 ± 0.004
P. halstedii race D	0.83 ± 0.004	0.87 ± 0.002
P. viticola	<0.03	<0.03
A. tenuis	<0.03	<0.03
Aspergillus sp.	<0.03	<0.03
Botrytis sp.	<0.03	<0.03
Cladosporium sp.	<0.03	<0.03
Epicoccum sp.	<0.03	<0.03
Fusarium avenaceum	<0.03	<0.03
Fusarium poae	<0.03	<0.03
Fusarium graminearum	<0.03	<0.03
Fusarium tricinctum	<0.03	<0.03
Mucor sp.	<0.03	<0.03
Nigrospora sp.	<0.03	<0.03
Penicillium sp.	<0.03	<0.03
Phomopsis sp.	<0.03	<0.03
Pythium sp.	<0.03	<0.03
Trichothecium sp.	<0.03	<0.03

[a] Results indicate the optical density at 405 nm of a 1:4 dilution of the solutions obtained after color development and correspond to the mean values of triplicate experiments ± standard deviations.

Immunization of mice. MAbs were prepared from 8-week-old female BALB/c mice (Iffa-Credo, L'Arbresle, France) which received either three weekly subcutaneous injections or three intraperitoneal injections. The first injection consisted of 200 μl of a 1:1 mix of partially purified *P. halstedii* extract (450 μg of protein per ml) and complete Freund's adjuvant (Sigma Chemical Co., St. Louis, Mo.). Incomplete Freund's adjuvant was used for the two subsequent boosters. Twelve days after the last booster, blood samples were tested by immunoblotting. An additional injection of antigen was given intravenously 3 days before the mice were euthanized for removal of their spleens.

Hybridoma production. Murine plasmocytoma cells, X63/Ag 8.653, were grown in RPMI 1640 medium (Gibco Laboratories, Grand Island, N.Y.) containing 15% fetal calf serum, 2 mM glutamine, 1 mg of ampicillin/ml, and 0.1 mg of gentamicin/ml. Cell fusion and selection of hybrids were performed essentially as described by Dippold et al. (5) with minor modifications. Ten days after fusion, culture supernatants from wells with growing hybridomas were screened by enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence, and immunoblotting for the production of antibodies directed against *P. halstedii* race 1. Positive hybrids were subcloned twice by limiting dilution and stored in liquid nitrogen. MAbs were obtained either from hybridoma cultures or from ascites fluid. Isotypes were determined by ELISA with anti-isotype antibodies (Caltag Laboratories, Burlingame, Calif.).

Purification and labeling of MAbs. MAbs were purified by ion-exchange chromatography on a DEAE-Sepharose (Pharmacia-Biotech, St. Quentin, France) column in 25 mM Tris buffer (pH 8.8) containing 35 mM NaCl. Ascites fluids previously equilibrated in the same buffer were applied to the column at a flow rate of 2 ml/min. The MAbs were eluted using 25 mM Tris buffer (pH 8.8) containing 75 mM NaCl, and the eluate was collected in 4-ml fractions. Labeling of the purified MAbs was performed essentially as described by Guesdon et al. (8).

ELISA. Ninety-six-well flat-bottom microtiter plates were coated with crude fungal extract diluted in 50 mM carbonate buffer (pH 9.6) (25 μg of protein per ml; 100 μl per well). After a 1-h incubation at 37°C, followed by three washes in 0.15 M phosphate-buffered saline (PBS) (pH 7.2) containing 0.05% (vol/vol) Tween 20 (PBST), 200 μl of a 3% BSA solution in PBS were added to each well. The plates were incubated for 2 h at 37°C or overnight at 4°C, washed three times in PBST, and incubated for 1 h at 37°C with the pooled mouse immune sera diluted 1:400 or with undiluted culture supernatants (100 μl per well). After three additional washes in PBST, 100 μl of alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (IgG) γ chain antibodies (Caltag Laboratories) diluted 1:2,000 in PBS was added to each well. The plates were incubated for 1 h at 37°C and washed three times in PBST. *p*-Nitrophenyl phosphate (1 mg/ml in 1 M diethanolamine buffer [pH 9.8]) was used as a chromogen. The reaction was stopped after a 30-min incubation at room temperature by the addition of 3 N NaOH, and the absorbance at 405 nm was determined on a Titertek multiscan (Labsystem, Les Ulis, France). All tests were performed in triplicate. The controls were uncoated wells and the incubation of coated wells with PBS instead of the immune sera or culture supernatants.

Indirect immunofluorescence. Zoosporangia and hyphae obtained by scraping cotyledons in distilled water and zoospores and germ tubes initiated by germination of zoosporangia were pelleted by centrifugation for 10 min at 180 × *g*, and the pellets were resuspended in 200 μl of undiluted hybridoma culture supernatants. After incubation for 1 h at 37°C, followed by washing in PBS, the fungal elements were incubated for 1 h at 37°C in 200 μl of a 1:100 dilution in PBS of fluorescein isothiocyanate-conjugated goat anti-mouse IgG γ chain antibodies (Caltag Laboratories). After being washed, 20 μl of the fungal suspensions was dropped on glass slides, and the preparations were examined with a Nikon microscope equipped with epifluorescence. The specificity of the labeling was assessed by incubating the fungal elements in PBS instead of the culture supernatants.

Characterization of the antigens. Titration curves of the MAbs were established by ELISA on wells coated with crude extract of *P. halstedii* race 1. A concentration of the biotinylated MAb which gives about 50% binding (2 to 4 μg/ml for MAb 18E2 and 0.5 to 1 μg/ml for MAb 12C9) was used for competition experiments in combination with an equal concentration or a 2-, 10-, or 100-fold excess of the unbiotinylated MAb.

To demonstrate that the epitopes were repetitive, we used a procedure derived from that of Theolis and Breckenridge (29). Plates were coated with purified MAbs (20 μg/ml for MAb 12C9 or 10 μg/ml for MAb 18E2) and incubated successively with (i) crude extract of *P. halstedii* race 1, (ii) an appropriate dilution of the same biotinylated MAb corresponding to the minimum concentration that gives 100% binding (6 μg/ml for MAb 12C9 and 24 μg/ml for MAb 18E2), and (iii) streptavidin-labeled alkaline phosphatase polymer (Sigma) diluted 1:500 in distilled water. All incubation steps were carried out at 37°C for 1 h and followed by three washes in PBST. *p*-Nitrophenyl phosphate was used for color development as described above.

The antigen molecular mass was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Samples were dissolved in 62.5 mM Tris-HCl buffer (pH 6.8) containing 2% SDS, loaded on a 5 to 15% gradient polyacrylamide slab gel, and electrophoresed according to the method of Laemmli (12). After electrophoresis, the gels were stained with Coomassie brilliant blue or transferred electrophoretically to 0.45-μm-pore-size Immobilon membranes (Millipore Corp., Bedford, Mass.) as described by Towbin et al. (30). The blots were saturated overnight at 4°C in 10% nonfat dry milk in PBS, washed in PBS, and incubated for 1 h with a 1:50 dilution of the mouse immune sera in PBS or with undiluted culture supernatants. After being washed, the membranes were incubated for 1 h with alkaline phosphatase-conjugated goat anti-mouse IgG γ chain antibodies diluted 1:300 in PBS. Finally, bands were detected by using the nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate substrate system. The molecular masses of the antigens were calculated from the migration of molecular mass standards (Sigma): myosin, 205 kDa; β-galactosidase, 116 kDa; phosphorylase b, 97.4 kDa; BSA, 67 kDa; ovalbumin, 45 kDa; and carbonic anhydrase, 29 kDa.

Antigen sensitivity to proteolytic enzymes or chemical agents was also investigated by Western blotting and ELISA. The effects of 2-mercaptoethanol were determined by the addition of 5% 2-mercaptoethanol to the fungal extract prior to electrophoresis and Western blotting. Antigenic extract of *P. halstedii* race 1 immobilized on Immobilon sheets or microtiter plates was also incubated at 37°C for 1 h with either pronase E (2.5 mg/ml) or proteinase K (0.16 mg/ml) diluted in PBS (pH 7.6). After the plates or sheets were washed in PBS and incubated with nonfat dry milk, immunoreactivity with the MAbs was determined. Periodate oxidation was performed by incubating the immobilized antigens at room temperature in the dark for 1 h with a 20 mM periodate solution in 50 mM acetate buffer (pH 4.0) (33). After the antigens were washed in PBST, the reaction was stopped by the addition of 1% glycine and incubation for 30 min. The immobilized antigens were treated with the MAbs as described above. The control was treatment of the immobilized antigens with PBS or acetate buffer alone. ELISA results, which correspond to the means of triplicate determinations, are expressed as the binding (percent) relative to the control performed in the absence of any treatment of the antigens.

ELISA detection of the fungus in seeds from infected plants. Samples of seeds (2 g) collected from healthy or infected plants grown at nine different locations in France were homogenized in liquid nitrogen, sonicated in distilled water for three periods of 5 min each, and centrifuged at 12,000 × *g* for 10 min. ELISAs were performed by coating the plates with purified MAb 18E2 (10 μg/ml). After saturation with BSA, the centrifugation supernatant from seed homogenates was added to the coated wells (100 μl per well). The presence of the fungus was detected with biotinylated purified MAb 12C9 (8 μg/ml; 100 μl per well). The control was incubation with PBS instead of seed homogenates. The nonparametric Mann-Whitney test was used for statistical evaluation of comparisons between infected and noninfected seeds. A P value of ≤0.01 was considered statistically significant. To determine sensitivity, this ELISA was performed with various concentrations of the crude fungal extract instead of seed homogenates.

RESULTS

MAb isolation. The fusions between X63/Ag 8.653 myeloma cells and lymphocytes of BALB/c mice that had been immunized with the partially purified extract of *P. halstedii* race 1

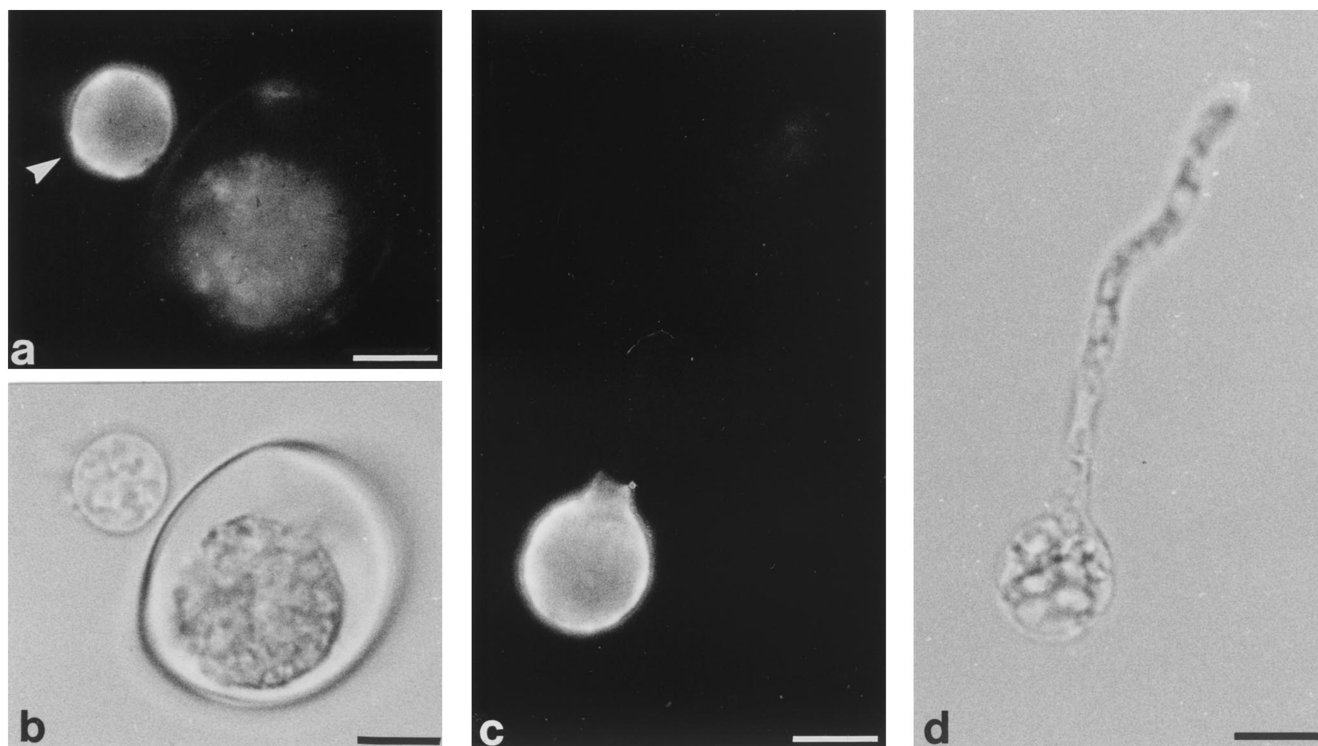

FIG. 1. Visualization by indirect immunofluorescence of the binding of MAb 12C9 to *P. halstedii* race 1 zoosporangia and zoospores (a) or germ tubes (c). Note the faint fluorescence of the granular contents of zoosporangia (a) and the intense staining of the surfaces of zoospores (a; arrowhead) and of mother cells of germ tubes (c). The surfaces of zoosporangia (b) and the hyphal walls of germ tubes (d) visualized on the same fields by phase-contrast microscopy were not labeled. Bars, 10 μm.

resulted in 4,000 hybridomas. Of these, 313 produced antibodies directed towards the fungus. However, only 16 remained positive after a second screening performed by Western blotting. These 16 included 12C9 and 18E2, which were IgG1 and IgG2b, respectively.

Immunofluorescence studies. Immunofluorescence studies performed on different morphological stages of the fungus revealed similar binding patterns for the MAbs. As soon as zoosporangia began differentiating, a faint fluorescence of their granular contents appeared with both MAbs, but their surfaces were not labeled (Fig. 1a and b). Extending the incubation time to 5 h resulted in production of germ tubes by almost all of the zoospores. When immunofluorescence was performed with these later developmental stages, the surfaces of the zoospores (Fig. 1a) and of the mother cells of the germ tubes (Fig. 1c) were intensely labeled. The hyphal part of the germ tubes did not stain (Fig. 1d), nor did hyphae (data not shown).

Specificity of MAbs 12C9 and 18E2 for *P. halstedii*. We coated the plates with crude extracts from the different races of *P. halstedii* present in France, *P. viticola*, a related fungus that causes downy mildew of grapevine, and other molds potentially pathogenic to sunflowers (e.g., *Alternaria tenuis* or *Phomopsis* sp.) or encountered on seeds of sunflowers. All races of the fungus tested were recognized by both MAbs (Table 1). The controls were negative, and no cross-reactions were observed with *P. viticola* or with the other fungi tested.

Characterization of the antigens recognized by MAbs 12C9 and 18E2. Titration curves (Fig. 2) demonstrated the saturability of the binding and permitted the determination of the minimal concentrations required for 50 and 100% binding. For example, when plates were coated with crude extract of *P. halstedii* race 1, a 0.5- to 1-μg/ml solution of biotinylated MAb 12C9 was required for about 50% binding, whereas the minimum concentration required to reach saturation was 6 μg/ml. These concentrations were four times greater for MAb 18E2, suggesting that the two MAbs differ in their affinity or their binding sites for the crude fungal extract.

Competition experiments suggested that distinct epitopes were detected by these MAbs on *P. halstedii* antigens (Table 2). Moreover, plates coated with an unbiotinylated MAb trapped

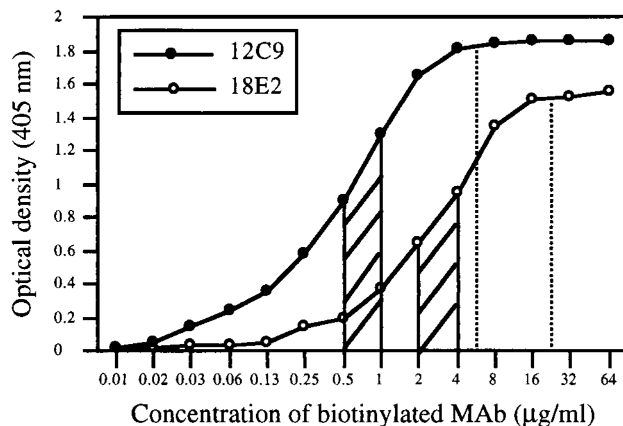

FIG. 2. Titration curve of biotinylated MAbs 12C9 and 18E2 by ELISA. Crude extract of *P. halstedii* race 1 was used for coating. The dotted lines correspond to the minimal concentrations of the MAbs which give 100% binding, and the hatched areas indicate the concentrations which give 50% binding.

TABLE 2. Competition experiments between MAbs 12C9 and 18E2[a]

Concn of unlabeled MAb	% Relative binding of biotinylated MAb			
	12C9		18E2	
	0.5 μg/ml	1 μg/ml	2 μg/ml	4 μg/ml
0	100	100	100	100
Equal concentrations	94	100	99	91
2-fold excess	94	100	97	87
10-fold excess	91	95	99	86
100-fold excess	90	93	78	84

[a] Competition experiments were performed on wells coated with crude extract of *P. halstedii* race 1 using a mixture of biotinylated MAb (at a concentration which gives about 50% binding) and an equal concentration or a 2- to 100-fold excess of the other unlabeled MAb. The results are the means of triplicate determinations and are expressed as the binding of the biotinylated MAb relative to that of the control performed in the absence of the unlabeled MAb. None of the values was significantly different from the others.

P. halstedii antigens that could be detected by the same, but biotinylated, MAb, suggesting the presence of repetitive epitopes for each MAb on the fungal antigens (data not shown).

From SDS-PAGE analysis of the crude extract of *P. halstedii* race 1, we identified about 15 polypeptides with molecular masses ranging from 20 to 250 kDa (Fig. 3, lane 1). Three proteins, with apparent molecular masses of 68, 140, and 192 kDa, were identified in Western blots by using MAbs 12C9 or 18E2 as the probes (Fig. 3, lanes 2 and 3). No bands were detected for either MAb when electrophoresis was performed under reducing conditions. Treatment of blots of the crude fungal extract with proteinase K or pronase E resulted in complete loss of immunoreactivity with MAb 12C9 or 18E2. Reduction of immunoreactivity with MAb 12C9 was also seen on blots treated with 20 mM periodate (Fig. 3, lane 5). Pretreatment of blots with acetate buffer alone (Fig. 3, lanes 6 and 8) or with PBS had no detectable effects. ELISA confirmed differences in the susceptibilities of the respective epitopes, since periodate oxidation resulted in a 43% reduction of the binding of MAb 12C9 while the binding of MAb 18E2 was only slightly modified (Table 3). Complete loss of binding was observed for both MAbs after treatment of the coated plates with proteolytic enzymes.

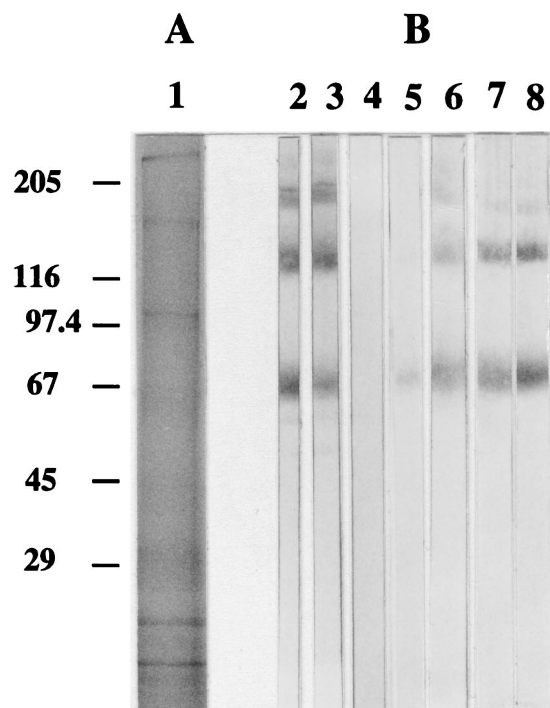

FIG. 3. Characterization of the antigens detected by MAbs 12C9 and 18E2. Lane 1, Coomassie blue staining of an SDS-PAGE gel of crude extract of *P. halstedii* race 1 (80 μg of protein per lane). Lanes 2 to 8, Western blot detection of the antigens recognized by MAb 12C9 (lanes 2, 5, and 6) or 18E2 (lanes 3, 7, and 8) without prior treatment of the crude extract (lanes 2 and 3) or after treatment with 20 mM periodate (lanes 5 and 7). Controls consisted of omission of the MAbs (lane 4) and incubation of the blots with acetate buffer (lanes 6 and 8) before the addition of the MAb. The molecular masses (in kilodaltons) of the protein standards are indicated on the left.

TABLE 3. Sensitivities of antigens detected by MAbs 12C9 and 18E2 to enzyme or chemical treatments as determined by ELISA

Treatment of crude fungal extract	% Relative binding[a]	
	MAb 12C9	MAb 18E2
None	100	100
PBS alone	98	97
Proteinase K	3	3
Pronase E	2	2
Acetate buffer alone	97	91
Periodate	57	81

[a] Results, which correspond to the means of triplicate determinations, are expressed as the binding relative to that of the control performed in the absence of any treatment of the antigens.

Detection of the fungus in seeds from infected plants. Since both MAbs recognized distinct and repetitive epitopes on the same antigens of *P. halstedii*, a sandwich ELISA system was developed to detect the fungus in infected seeds. *P. halstedii* antigens were trapped with MAb 18E2 and detected with biotinylated MAb 12C9 (Table 4). An optical density (OD) value higher than 0.250 was obtained with all lots of seeds from infected plants, but it did not exceed the control value (0.115) when seed homogenates from healthy plants were used. The difference between the results with infected and noninfected

TABLE 4. ELISA detection of fungus in seeds from healthy and infected plants

Seed homogenate	Geographic origin	OD value[a]
Control		0.12 ± 0.012
Infected plants		
Lot 1	Beaune (Cote d'Or)	0.46 ± 0.083
Lot 2	Beaune (Cote d'Or)	0.47 ± 0.066
Lot 3	Beaune (Cote d'Or)	0.56 ± 0.053
Lot 4	Beaune (Cote d'Or)	0.29 ± 0.052
Lot 5	Beaune (Cote d'Or)	1.16 ± 0.140
Lot 6	Chappes (Puy-de-Dome)	0.42 ± 0.080
Lot 7	Chappes (Puy-de-Dome)	0.48 ± 0.012
Lot 8	Chappes (Puy-de-Dome)	0.40 ± 0.034
Healthy plants		
Lot 9	Mazé (Maine-et-Loire)	0.093 ± 0.013
Lot 10	Corné (Maine-et-Loire)	0.12 ± 0.014
Lot 11	Brissac (Maine-et-Loire)	0.087 ± 0.025
Lot 12	St. Saturnin (Maine-et-Loire)	0.094 ± 0.015
Lot 13	Doué (Maine-et-Loire)	0.085 ± 0.027
Lot 14	Les Alleuds (Maine-et-Loire)	0.071 ± 0.005
Lot 15	Clermont-Ferrand (Puy-de-Dome)	0.077 ± 0.015

[a] Results (OD values at 405 nm) correspond to the mean values of triplicate experiments ± standard deviations.

seeds was highly significant ($P < 0.01$). The sensitivity of this assay, evaluated with the crude fungal extract, was estimated to be 1 ng/ml.

DISCUSSION

P. halstedii causes extensive damage to sunflowers, and the absence of efficient antifungal treatments has led to a search for new varieties of sunflower resistant to the fungus. Several races of *P. halstedii* have been identified (10, 11, 13, 19, 24), and some resistant sunflower hybrids have been produced (9, 16, 25), but the resistance is not durable. Moreover, metalaxyl is the only commercial antifungal agent available, and resistant strains of *P. halstedii* have been described (1).

An alternative control strategy is a screen to detect infected seeds and break the transmission of downy mildew by seeds by removing them. ELISAs have been developed for downy mildew of peas caused by *Peronospora viciae* f. sp. *pisi* (7), *Verticillium* wilt of potato (27), and blight of soybeans (31), as well as for downy mildew of sunflowers (14). These tests rely on rabbit polyclonal antisera specific for the causative agent of the disease. The target antigens in these tests have not been identified. The reproducibility of such ELISAs has been questioned, since the immunization of rabbits with crude or partially purified fungal extracts may result in qualitative or quantitative differences in the sera obtained. Such variation may be overcome by the use of MAbs.

In the present study, we produced two MAbs that recognized antigens found in all morphological stages of the fungus except the hyphae and the hyphal portion of the germ tubes. These MAbs were specific for *P. halstedii*. They recognized all races of the fungus that are present in France and did not cross-react with *P. viticola* or with other fungi commonly recovered from sunflower plants. All of the races tested have been reported in other parts of the world where sunflowers are grown commercially (19, 24). However, only a single isolate was available for each race of *P. halstedii*, and our results remain to be confirmed with other isolates. Both MAbs bound to the same three glycoproteins in the crude extract. The relationships among these glycoproteins remain to be defined. For example, they may be degradation products of the 192-kDa or a larger glycoprotein, different degrees of polymerization of the 68-kDa molecule, or three distinct glycoproteins sharing common epitopes. Both MAbs also bound distinct, repetitive epitopes. The reduction of immunoreactivity with MAb 12C9 after periodate treatment suggests that these epitopes are carbohydrates. The loss of recognition after reduction with 2-mercaptoethanol suggests that the epitopes are conformational.

We used the specificities of these antibodies for *P. halstedii* and their recognition of distinct and repetitive epitopes to develop a sandwich ELISA system for the detection of the fungus. Thus, we could detect the fungus in seeds from infected plants from two geographically distinct areas of France, and no reaction was observed with any lot of seeds from healthy plants. Although our study was conducted exclusively with isolates from France, these races have a global distribution. Therefore, our ELISA detection system can potentially be used in all countries where sunflowers are grown commercially. In conclusion, these MAbs may constitute valuable tools for the diagnosis of seed-borne downy mildew of sunflowers and help reduce the incidence of seed-borne disease transmission. As importing and exporting agencies in most countries require a phytosanitary certificate attesting the absence of downy mildew in the production area, this test could make this regulation easier to enforce by limiting movement of infected lots of seeds.

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