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Production of monoclonal antibodies against surface antigens of spores from arbuscular mycorrhizal fungi by an improved immunization and screening procedure

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Abstract. Monoclonal antibodies (mAbs) were produced against surface antigens of chlamydospores of arbuscular mycorrhizal (AM) fungi by immunizing mice with crushed or complete spores. The intrasplenic approach proved to be superior to the intraperitoneal method of immunization with regard to the amount of antigen required for the immune response. The hybridoma technology was combined with an improved screening procedure, applying an immunogold-silver staining technique to semi-thin sections of spores. In this way, mAbs to surface antigens on the outer wall could be selected. Two mAbs were raised against *Glomus etunicatum* and *G. scintillans* spores. Cross-reactivities of the antibodies to other structures of the fungus, to other species of *Glomus* and to other soil-borne fungi were tested with indirect immunofluorescent labelling. The mAbs did not react with non-AM fungi. One mAb (A5B1) selectively recognized *G. etunicatum*, another (D12F11) exhibited limited interspecies cross-reactivities. One further mAb (H8F7), which reacted with spores of all AM fungi but not with other fungi, was shown to be specific for *Bacillus mycoides*. The implications are discussed.

Key words: *Glomus etunicatum* – *Glomus scintillans* – Intrasplenic immunization – Immunogold-silver staining

Introduction

Antibodies (Abs) as highly specific and sensitive molecular probes are gaining increasing attention in plant sciences (Hampton et al. 1990). The potential of serological methods for quantification and identification of chemical and biological structures has been especially recognized for plant-microbe interactions. Abs have been raised against plant pathogenic fungi and bacteria (Dewey et al. 1991). For research on arbuscular mycorrhizal (AM) fungi, serological tools have been utilized in studies of the host-symbiont dialogue (Sanders et al. 1992), as well as in ecological investigations in soil (Aldwell and Hall 1986; Friese and Allen 1991) and in taxonomic studies (Aldwell et al. 1985). However, a wide range of cross-reactivities was reported in most cases when polyclonal sera were employed. The strength of these cross-reactivities can be used in taxonomic studies for differentiation at the strain and/or species level, but the serological quantification of fungal matter in complex systems like soil is severely hampered by cross-reactivities. Not only is the supply of a polyclonal antiserum from laboratory animals limited but the Ab titer and Ab specificity also vary greatly between individuals and even between blood samples from one individual taken at different times. Once a defined polyclonal serum has been depleted, it is irreversibly lost.

The monoclonal antibody (mAb) technology, based on the work of Köhler and Milstein (1975), provides solutions to these problems. Here Ab-producing cells from the spleen of immunized animals are cloned and immortalized in cell culture. Morton and co-workers (Morton et al. 1987a, b; Wright et al. 1987; Wright and Morton 1989) first raised mAbs towards AM fungi and showed that mAbs directed against *Glomus occultum* had a high specificity for the respective isolate and no cross-reactivity to related fungi. When a hybridoma cell line secreting an antibody of defined specificity and sensitivity has been isolated, it can produce virtually unlimited amounts of this Ab. A further advantage of this technology is that highly specific mAbs can be generated without using purified immunogens. The multiple components of the mycorrhizal structures can be resolved by cloning mAb-producing hybridomas. Contaminant structures or structures that mask species-specific antigenic determinants can be eliminated by the screening process. The mAb potential in mycorrhizal research has been reviewed by Perotto et al. (1992).

The production of mAbs usually requires large amounts of antigen for immunization and screening of

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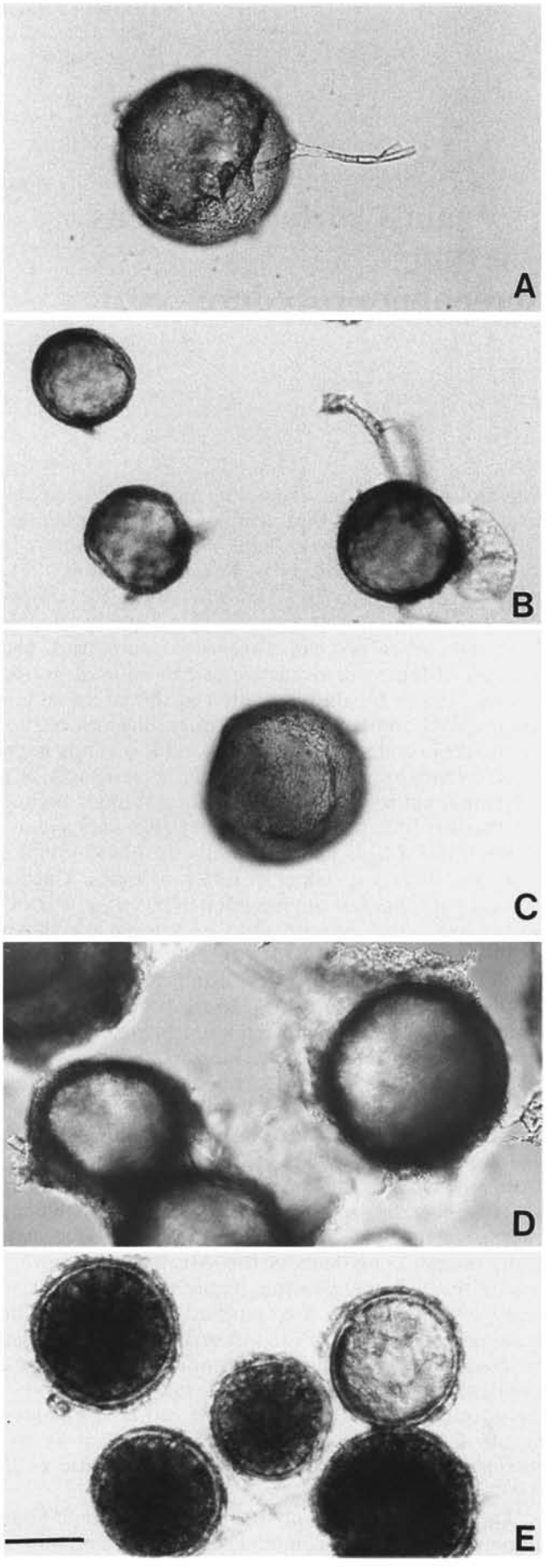


the hybridoma supernatants. Wright et al. (1987) needed more than 10^5 spores to elicit antibody production in mice. In this paper, we show that much less antigen is required for an immune response when the animals are immunized by the intrasplenic path, which was originally developed for minute amounts of highly purified immunogen on a carrier matrix (Nilsson and Larsson 1990). The amount of antigen needed for the screening process was further reduced by developing a primary screening procedure for the hybridoma supernatants based on semi-thin sections of embedded spore material. An indirect immunohistochemical staining method for light microscopy was adapted to the use of hybridoma supernatants as primary Ab. Hybridoma cells were selected that produced Abs binding selectively to the outer cell wall of the spores of the AM fungi.

Two mAbs are presented, one of which reacts selectively with the AM fungus *Glomus etunicatum* whilst the other reacts with several isolates of *Glomus*. Neither mAb shows cross-reactivities to nonmycorrhizal fungi. A further mAb was isolated that reacts with a *Bacillus mycoides* isolate originating from the surface of an AM fungus. The implications of the common occurrence of this bacterium on AM spores are discussed.

Materials and methods

Fungal material

Spores of four different isolates of AM fungi were used as immunogen in three immunization procedures. The first two isolates were obtained from aeroponic cultures at the Department of Soil Science, University of Florida, Gainesville, Fla., and were designated *G. ssp.* S328 and *G. etunicatum* S329 (Fl.). A micrograph of the latter is shown in Fig. 1A. The third isolate (Fig. 1B) was multiplied in our laboratory in a sand/expended clay culture from inoculum material originally obtained from TU Hanover (Department of Plant Diseases), designated *G. etunicatum* (Whs) and extracted by the method described by Horn et al. (1992). The fourth isolate, presented in Fig. 1C, was provided by Prof. A. Varma and was selected manually from semi-arid soils in India; it is designated *G. scintillans* (I).

Additional AM fungi were received from the same three sources for cross-reactivity tests. Fig. 1D shows a light micrograph of an isolate characterized as *G. mosseae* (Whs), which was also extracted from sand/expanded clay culture of inoculum received from TU Hanover. Fig. 1E shows spores from a soil culture inoculated with isolate S329 at Gainesville. Non-AM fungi were obtained from permanent cultures at TUM Weihenstephan (Department of Phytopathology) and soil bacteria from TU Hanover.

Fig. 1. A–E Bright field micrographs of the *Glomus* isolates used in this work. **A** *G. etunicatum* (Fl), isolate S329 from Florida, aeroponic culture. **B** *G. etunicatum* (Whs) from Weihenstephan, sand/expanded clay culture. **C** *G. scintillans* (I) from India, semi-arid soils. **D** *G. mosseae* (Whs) from Weihenstephan, sand/expanded clay culture. **E** isolate S329 from Florida, soil culture. All samples are depicted at the same magnification; *bar* = 50 μm

Immunization protocols

For the intraperitoneal immunization protocol, 14- to 16-week-old female BALB/c mice were intraperitoneally injected with 0.5 mg of crushed spores of isolate S329 in 200 µl of sterile saline solution together with an equal volume of Freund's Complete Adjuvant. The same doses of spores were given on days 29, 70, and 104 together with Freund's Incomplete Adjuvant. Three days prior to cell fusion the mice received daily booster injections of 500 spores of *G. etunicatum* (Whs) in sterile saline. These booster injections were carried out with a different AM isolate to elicit proliferation of splenic B-cells producing Ab to antigens common to both isolates.

For the intrasplenic protocol, 16-week-old female F_1(BALB/c × c57 black) mice were first intraperitoneally injected with 500 crushed spores of *G. scintillans* (I) in 200 µl of sterile saline together with an equal volume of Freund's Complete Adjuvant. After 23 weeks, 125 undamaged and surface-sterilized spores of the same antigen were applied intrasplenically. In this immunization method (subject to authorization for animal experiments, license No. 211-2531-2/92; Reg. v. Obb.), the peritoneum of the anaesthetized animal was opened, the antigen injected directly into the spleen, and the peritoneum then closed with microclamps. After 5 weeks, the same volume was again applied intrasplenically together with an intraperitoneal dose of 100 µl of Freund's Incomplete Adjuvant. The spleen cells were fused 3 days later.

The immunizations with isolate S328 were carried out by subcutaneously injecting 9-week-old female BALB/c mice with 40 µg of crushed spores in 100 µl of sterile NaCl solution (0.9%). Two and 4 weeks later, 0.5 mg of crushed spores was injected together with 100 µl of Freund's Incomplete Adjuvant per injection. Four booster injections followed 2 weeks later on a daily schedule with 0.5 mg of crushed spores each. Fusion followed 2 weeks later.

Production of monoclonal antibodies

The production of hybridomas was carried out according to the method used in our group (Giersch and Hock 1990). Briefly, myeloma cells of strain PAI-B_3AG8.I in the log phase of growth were mixed with the same number of spleen cells from immunized mice. After centrifugation at 1000 rpm for 5 min at 4°C and complete removal of the supernatant, 500 µl of polyethylene glycol, 50% (v/v; PEG 4000, Merck, Darmstadt, Germany) in RPMI 1640 (Dutch modification, Gibco, Eggenstein, Germany) containing 5% dimethyl sulphoxide was heated to 37°C and carefully mixed with the cells. After 60 s, the PEG was slowly diluted by the addition of RPMI 1640. Ten ml were added dropwise during 5 min. After incubation for 10 min at room temperature, the suspension was centrifuged at 1000 rpm and the supernatant replaced by RPMI 1640 medium containing 10% fetal calf serum (Myoclone, Gibco, Eggenstein, Germany). One half of the cell suspension was diluted 1:10 with culture medium containing 20 U/ml IL-6 (Boehringer, Mannheim, Germany) and dispersed into a 96-well microtiter plate; the other half was transferred to a cell culture bottle and stored in liquid nitrogen on the following day. After 24 h, medium containing hypoxanthine-aminopterin-thymidine was added to give final concentrations of 100, 0.4 and 16 µmol, respectively. Aminopterin was eliminated from the culture 10 to 14 days later. Hybridoma supernatants were first assayed for anti-*Glomus ssp.* specific antibodies 14 days after the fusion.

Hybridoma culture supernatants that proved positive in at least four consecutive immunogold-silver staining experiments (technique described below) were cloned by single cell deposition under microscopic control in 96-well plates. Clones were retested and positives were increased on 24-well plates and recloned to ensure population uniformity. Positives were again increased in 24-well plates and finally in cell culture bottles. Supernatants were removed every second day and frozen after aliquotation. Aliquots of the cell cultures were cryopreserved in RPMI 1640 culture medium containing 12% dimethyl sulphoxide and 20% FCS and stored in liquid nitrogen. The isotype of the Abs was determined with a double sandwich ELISA using rabbit Abs directed against the murine subclasses IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgA, IgM, and the κ and λ light chains (Biorad, Munich, Germany).

Embedding of spores

Spores of the AM fungi used in the immunogold-silver staining process for screening and cross-reactivity tests were fixed in a double aldehyde fixation in 2% glutaraldehyde and 2% paraformaldehyde according to Roland and Vian (1991), dehydrated in a series of increasing ethanol solutions (30%, 50%, 70%, 90%, 96%, 100%) and finally embedded in LR White (London Resin, London, UK).

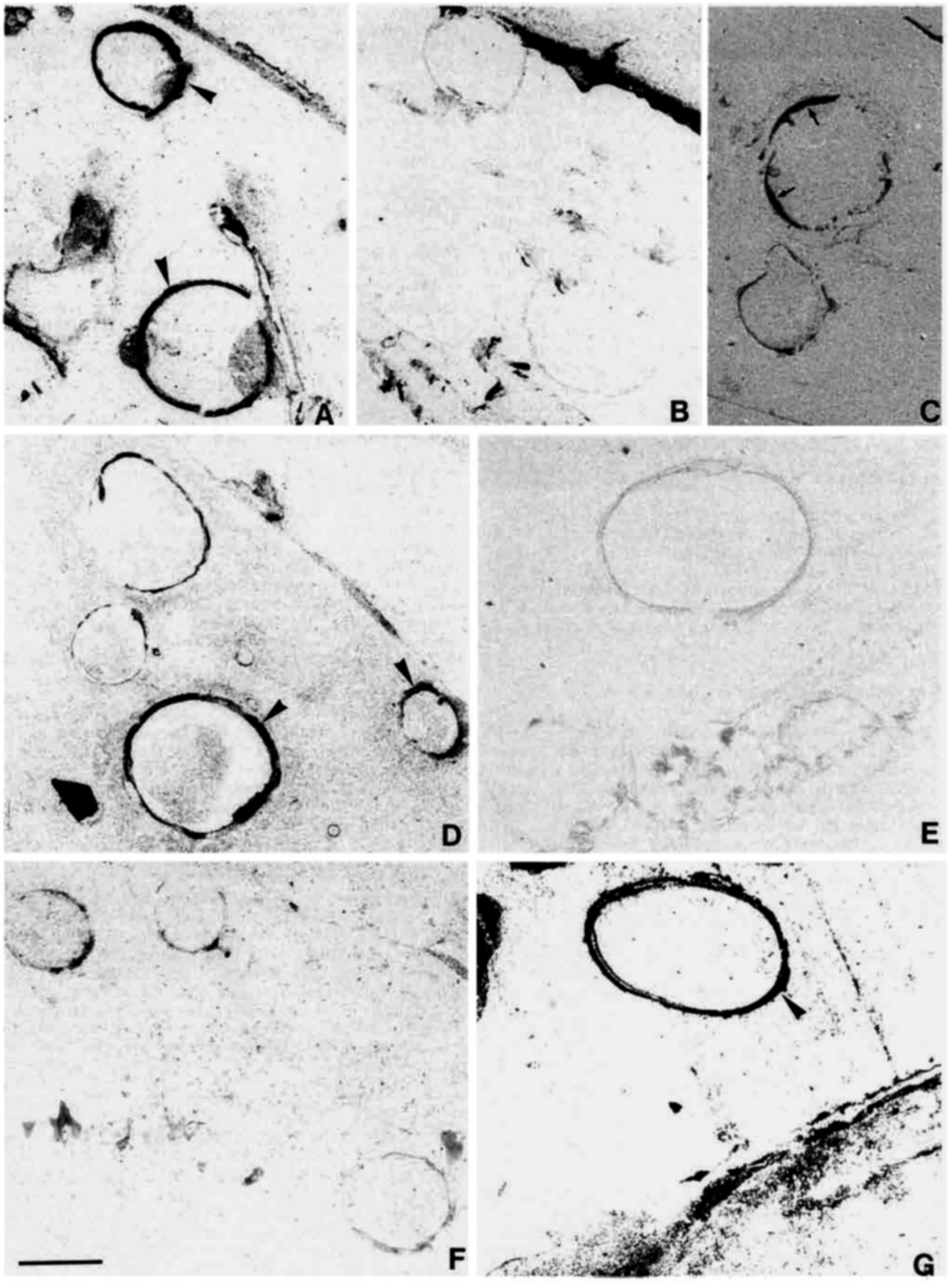
Immunogold-silver staining of semi-thin sections

Immunogold labelling was carried out according to Bonfante-Fasolo et al. (1991). A silver enhancement step made the gold label visible for light microscopy (immunogold-silver staining, IGSS). Semi-thin sections (0.5 µm) of the spores were cut with an ultramicrotome (Ultracut Manual, Reichert, Austria) and fixed onto 21-well serodiagnostic microscope slides (Menzel, Braunschweig, Germany) with poly-L-lysine solution (0.01% w/v; Sigma, Deisenhofen, Germany). The samples were rinsed five times for 2 min in PBS (phosphate-buffered saline, 0.1 M, pH 7.2) before and after the staining procedure, as well as between each step of the procedure, using a Microlab 1000 autodispenser (Hamilton, Darmstadt, Germany). The labelling procedure was as follows: (i) blocking of unspecific binding with IgGs from goat normal serum (10 µg/ml; Sigma, Deisenhofen, Germany) and incubation at 37°C for 30 min; (ii) addition of the undiluted cell culture supernatants and incubation at 4°C overnight; (iii) blocking of unspecific binding as in (i); (iv) addition of biotin-conjugated anti-mouse Ab (5 µg/ml; Sigma, Deisenhofen, Germany) and incubation at 37°C for 30 min; (v) addition of streptavidin conjugated to 5 nm gold (diluted 1:50; Sigma, Deisenhofen, Germany) and incubation at 37°C for 30 min; (vi) development with a Silver Enhancer Kit (Sigma, Deisenhofen, Germany) on ice for 10–15 min.

All dilutions in the labelling procedure were made with 0.1 M PBS supplemented with 0.1% bovine serum albumin, and all wells received 10 µl of solution at every step. Each slide carried at least one negative control using unspecific IgG from mouse (10 µg/ml; Sigma, Deisenhofen, Germany) diluted in cell culture medium. The samples were then evaluated at magnification of 20 × 10 under a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany). AM spore walls stained black to grey were counted as positive (+), all other labelling reactions were considerd negative (−).

Affinity chromatography with protein A

Some supernantants of hybridoma cultures were purified and concentrated using a protein A cartridge (MemSep; Millipore, Eschborn, Germany). The supernatants were passed through the cartridge at pH 8.5 and 4°C as described by Schuler and Reinacher (1991) and the IgG fraction was eluted at pH 2.6 and room temperature and collected in 1-ml samples. The fraction responding in a subsequent IGSS test was used for immunofluorescence experiments.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Immunofluorescence test

Ab- binding specificity and cross-reactivities were determined in an indirect immunofluorescent (IF) assay employing a biotin-streptavidin enhancer step as described previously (Sohn et al. 1990). The streptavidin was conjugated to B-phycoerythrin (Dianova, Hamburg, Germany). The individual steps of the labelling procedure were performed in analogy to the IGSS procedure except for the larger volumes used (100 µl of each solution in micro-reagent tubes). The spores were evaluated under fluorescent light with a filter combination of BP 546/FT 580/LP 590.

Enzyme immunoassay

Screening of the cell lines from the S328 (Fl)-immunized spleen and binding of mAb to soil bacteria were performed by coating the antigen to polystyrene microtiter plates (Nunc Maxisorp) with poly-L-lysine. A noncompetitive indirect enzyme immunoassay (EIA) was carried out with the mAbs of the supernatants from the hybridoma cultures using either an anti-murine IgG-specific Ab coupled to horseradish peroxidase (HRP; Dianova, Hamburg) or an anti-murine IgG-specific Ab conjugated to biotin together with Extravidin-coupled HRP (Sigma, Deisenhofen) for signal generation. Ab binding was evaluated using 3,3′,5,5′-tetramethylbenzidine as substrate for the enzyme.

Results

Production of hybridoma clones

Fusion frequencies of 4×10^{-6} were obtained for the *G. etunicatum* (Fl+Whs) intraperitoneal immunization, 3×10^{-6} for the *G. scintillans* (I) intrasplenic immunization and 5×10^{-6} for the S328 (Fl) subcutaneous immunization, as determined by the number of growing cell populations first observed after seeding. In all cases, less than 20% of the fused cells gave a positive reaction in the primary screening.

Fusion of spleen cells from intraperitoneal immunizations with *G. etunicatum* (Fl+Whs) provided 37 hybridoma cultures that reacted positively in the primary screening, carried out by immunogold-silver staining. Cell cultures that showed stable Ab production in four consecutive IGSS tests were cloned and gave three stable clones. The cell line A5B1 was selected for further Ab characterization. Fusion of spleen cells from intrasplenic immunizations with *G. scintillans* (I) produced four stable cell lines secreting anti-*G. scintillans* (I) Abs. The cell line D12F11 exhibited the strongest reaction and the highest IgG concentration in the supernatant and was selected for further characterization of its mAb.

Selection of Ab-secreting cell lines from the S328 (Fl)-immunized spleen cells was carried out in parallel by immunolabelling and EIA. Five cell lines that showed stable growth and gave a signal in both assays were further cultivated. Among them, the cell line H8F7 yielded the highest Ab concentration in the supernatant and was selected for Ab characterization.

Production of mAbs

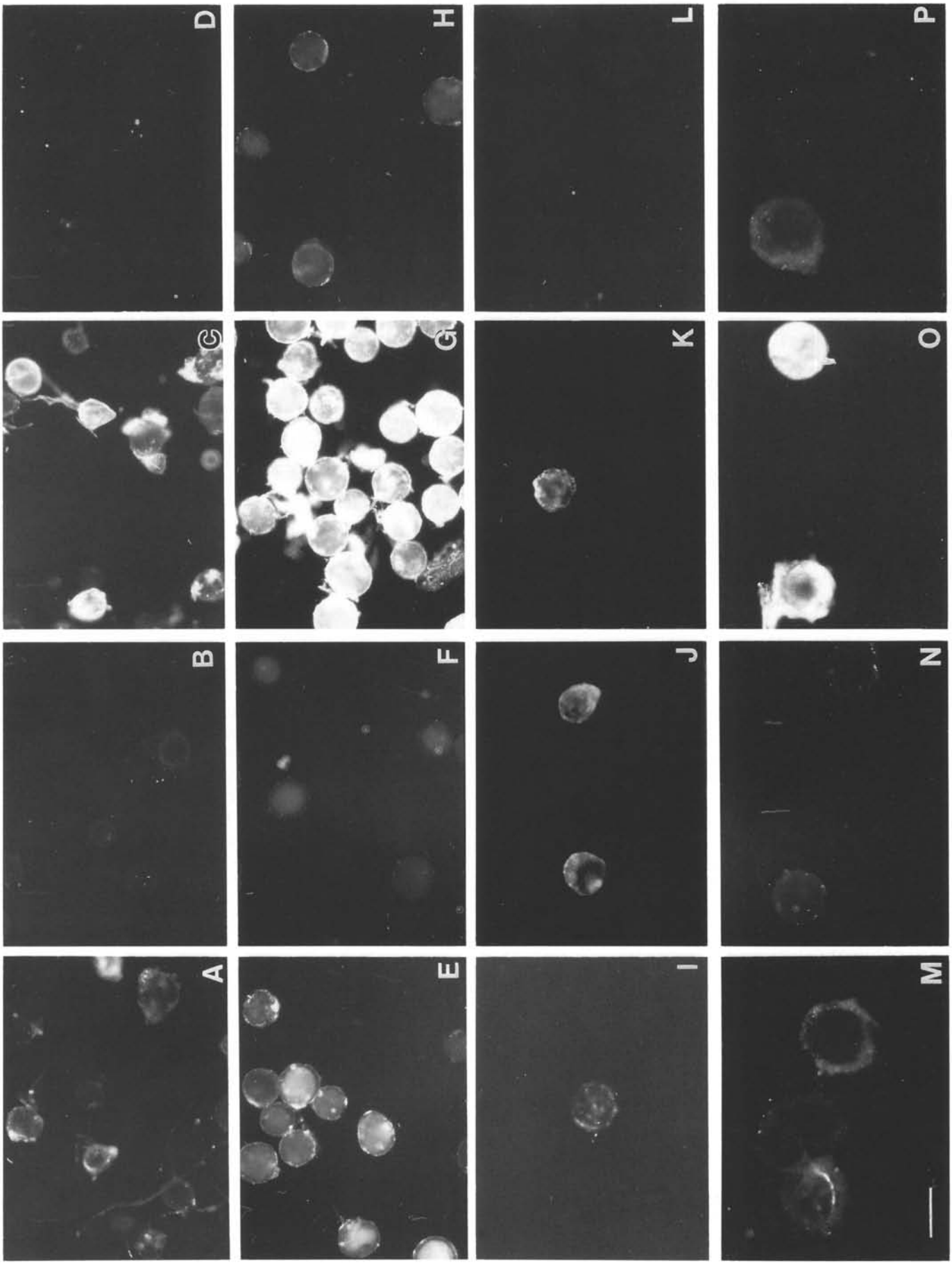
The mAb from A5B1 belongs to the IgG_{2a} subclass with κ sidechains. The mAb concentration in the supernatant is between 0.2 and 0.3 µg/ml, depending on the age of the colony and the frequency of medium change. The mAb from D12F11 pertains to the IgG_3 subclass with κ sidechains and its concentration is between 0.1 and 0.2 µg/ml. The mAb from H8F7 is of the IgM class and its titer is between 0.3 and 0.4 µg/ml. Due to these rather low concentrations, the cell culture supernatants of A5B1 and D12F11 were concentrated by affinity chromatography using protein A before being employed in the immunofluorescence tests. In this procedure, IgG from the supernatant is bound and subsequently eluted to yield a concentrated Ab solution.

Immunogold-silver staining

Figure 2 shows representative micrographs of 0.5-µm semi-thin sections labelled by IGSS during the screening process. The supernatants from the hybridoma cultures were used as primary labels. Fig. 2A is a section of *G. etunicatum* (Whs) cell walls labelled by the supernatant from cell culture A5, the origin of the mAb-producing clone A5B1. Arrowheads point to the specific labelling of the spore walls. Unspecific precipitates of silver gave a high background. However, the specific binding can easily be discerned by comparison with Fig. 2B, where a neighbouring section was labelled with unspecific murine IgG diluted in cell culture medium. Some background is visible but the spore walls are not outlined by silver precipitation. Note that the cytoplasmic content of the spores is not preserved in the embedding and cutting process. Fig. 2C shows a supernatant from the same screening not chosen for further work since the specific mAb bound to the inner surface of the spore wall (arrow). We observed this phenomenon for a number of cell culture supernatants during the screening procedure.

Figure 2D refers to a section of *G. etunicatum* (Whs) labelled with the mAb from A5B1 directed against *G. etunicatum* (Fl+Whs), while Fig. 2E demonstrates the almost negative reaction of the same mAb with a section of *G. scintillans* (I). Figures 2F and 2G show sections of *G. etunicatum* (Whs) and *G. scintillans* (I), respectively, both labelled with the mAb D12F11 directed against *G. scintillans* (I). Again, the selectivity of the mAb is demonstrated by the dark silver precipitation on the wall of the spore (arrow-

Fig. 2. A–G Immunogold-silver staining of semi-thin sections. **A–D, F** *G. etunicatum* (Whs), **E, G** *G. scintillans* (I). **A** A positive hybridoma culture supernatant gives a signal in the primary screening process. **B** When using murine IgG as control, no signal is seen. **C** A positive supernatant binds to the inner surface of a spore wall. **D** The mAb from A5B1 labels *G. etunicatum* (Whs) but not **E** *G. scintillans* (I). **F** The mAb from D12F11 shows no reaction with *G. etunicatum* (Whs) but **G** labels *G. scintillans* (I). All samples are depicted at the same magnification; *bar* = 50 µm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head). The labelling is absent in sample 2F, demonstrating that the mAb D12F11 does not recognize *G. etunicatum* (Whs).

Immunofluorescent labelling of spores

Spore isolates of four AM fungi were available in sufficient numbers and were therefore used for the validation of the IGSS results. Two isolates raised at Weihenstephan, *G. etunicatum* (Whs) and *G. mosseae* (Whs), *G. scintillans* (I) from India, and one isolate from Florida, *G. etunicatum* S329 from soil (Fl) were used as antigens in indirect IF. Fig. 3 shows the fluorescence micrographs of the four isolates [top row: *G. etunicatum* (Whs); second row: *G. etunicatum* S329 from soil (Fl); third row: *G. scintillans* (I); bottom row: *G. mosseae* (Whs)] with the three selected mAbs (far left column: A5B1; second column D12F11; third column H8F7). The far right column is a control experiment utilizing murine IgG at a concentration of 10 μg/ml in nutrient medium to detect unspecific binding and autofluorescence. The mAb from A5B1 resulted in intermediate labelling of the spores and hyphae of *G. etunicatum* (Whs) (Fig. 3A) and strong labelling of *G. etunicatum* S329 (Fl) spores (Fig. 3E). Hyphae of *G. etunicatum* S329 (Fl) were not available for testing. The mAb from A5B1 did not give a signal with the spore walls of *G. scintillans* (I) (Fig. 3I) and *G. mosseae* (Whs) (Fig. 3M). The mAb from D12F11 did not react with any isolates other than the homologous *G. scintillans* (I) (Fig. 3) in the IF experiments (Fig. 3B, F, J, N).

The third column of Fig. 3 shows the positive reaction of all four AM isolates with the mAb from H8F7. All spore isolates gave an IF signal with this mAb; this puzzling behavior suggests an antigen common to all AM so far investigated. Microscopic investigations at a higher magnification showed a patchy immunolabelling pattern, pointing to the presence of bacteria. When the reactivity with two species of soil-borne bacteria was tested in an EIA, the mAb from H8F7 gave a strong specific signal with an isolate of *Bacillus mycoides*. Spores of the AM isolate S328 (Fl), which had been used for immunization, were subsequently placed on a *B. cereus* selective agar (Oxid, Wesel, Germany). The spores proved to be infected with a bacterium able to grow on this agar. We could not achieve surface sterility of any of these isolates while retaining an IF signal with the mAb from H8F7. The controls (fourth column) lacked significant IF, showing that unspecific binding can be excluded as cause of a fluorescent signal when the mAbs are used.

Fig. 3 Immunofluorescence micrographs of spores of **A–D** *G. etunicatum* (Whs); **E–H** isolate S329 (Fl), **I–L** *G. scintillans* (Whs), **M–P** *G. mosseae* (Whs). The mAb from A5B1 (first column) gives a fluorescence signal with *G. etunicatum* (Whs) (**A**) and with isolate S329 (Fl) (**E**). The mAb from D12F11 (second column) gives a signal with *G. scintillans* (I) (**J**). The mAb from H8F7 (third column) gives a strong signal with all of the spores tested in immunofluorescence experiments (**C, G, K, O**). When unspecific murine IgG (last column) was used as control, no signal was detected (**D, H, L, P**). All samples are depicted at the same magnification; *bar* = 100 μm

Cross-reactivities

The reaction of the mAb with a variety of other soil fungi was tested using the IF protocol. Neither of the three mAb showed any labelling of soil fungi. Table 1 gives an overview of the cross-reactivity results with combinations of all mAbs with all AM isolates. The mAb A5B1 selectively labels *G. etunicatum* obtained from different sources. On the other hand, the binding of H8F7 to all AM fungi is again demonstrated. The mAb D12F11 showed a positive reaction in the IGSS experiments with *G. mosseae* (Whs), *G. mosseae* (I) and *G. macrocarpum* (I), in addition to the homologous species *G. scintillans* (I). When these reactions were checked in IF experiments with *G. mosseae* (Whs), the expected signal was not observed (Fig. 3N), probably due to the lower sensitivity of the IF test.

Discussion

Serological techniques can be employed as a valuable tool for AM research in several ways. Abs can assist in the difficult taxonomy of this fungal group when cross-reactivities are used in a serogrouping approach. Moreover, it should be possible to follow inoculated strains in their competition with autochthonous populations (Friese and Allen 1991) by Ab labelling.

When identification and quantification are required, cross-reativities to other AM species and to nonmycorrhizal fungi complicate or prevent interpretation. The polyclonal sera raised in our laboratory have all shown unspecific binding to a variety of fungal material. The use of mAbs circumvents a number of problems that arise from the considerable cross-reactivity of polyclonal sera. To date, only two mAbs have actually been raised against antigens of AM fungi. The mAbs B5 and H8 (Morton et al. 1987a; Wright et al, 1987) are highly selective for *G. occultum* showing no cross-reactions with spores of other AM fungal strains tested or nonmycorrhizal fungi. The reaction was stronger with the soluble fraction of the crushed spore suspension used in the test, suggesting an antigen located in the cytoplasm or loosely bound to an intracellular membrane. These results correspond to our finding that many of the cell culture supernatants from the primary screening gave a strong signal at the inner surface of the spore walls (Fig. 2C).

An important goal of this work was the reduction of the amount of spores required for immunization and for the screening process, since a large number of spores of a defined single-species culture exceeds the capacities of our laboratory (and probably of most other laboratories). The low amount of antigen available for testing is demonstrated by the sparse distribution of spores in most samples of Fig. 3. We therefore ap-

Table 1. The cross-reactivities of mAb A5B1 raised against *Glomus etunicatum* (Whs+Fl), mAb D12F11 against *G. scintillans* (I) and the mAb H8F7 against *G. ssp.* (Fl) were tested by immunogold-silver staining (IGSS), immunofluorescence (IF) and enzyme immunoassay (EIA)

Antigen	Source	Structure	Test system	mAb reaction		
				A5B1	D12F11	H8F7
Endogonaceae						
Glomus etunicatum	Weihenstephan, Germany	Spores	IF/IGSS	+	−	+
		Hyphae	IF/IGSS	+	−	+
Glomus mosseae	Weihenstephan, Germany	Spores	IF/IGSS	−	−/+	+
		Hyphae	IF	−	−	+
Glomus etunicatum (S329)	Florida USA	Spores	IF/IGSS	+	−	+
Glomus ssp. (S328)	Florida USA	Spores	IF	−	−	+
Glomus mosseae	India	Spores	IGSS	−	+	+
Glomus scintillans	India	Spores	IGSS	−	+	+
Glomus macrocarpum	India	Spores	IGSS	−	+	+
Glomus fasciculatum	India	Spores	IGSS	−	−	+
Glomus intraradices	India	Spores	IGSS	−	−	+
Non-AM fungi:						
Oomycetes						
Phytophthora cactorum	Weihenstephan, Germany	Spores	IF	−	−	−
		Hyphae	IF	−	−	−
Ascomycetes						
Sclerotium cepivorum	Weihenstephan, Germany	Sclerotia	IF	−	−	−
		Hyphae	IF	−	−	−
Fusarium oxysporum	Weihenstephan, Germany	Spores	IF	−	−	−
		Hyphae	IF	−	−	−
Thielaviopsis basicola	Weihenstephan, Germany	Spores	IF	−	−	−
		Hyphae	IF	−	−	−
Zygomycetes						
Mucor racemosus	Weihenstephan, Germany	Spores	IF	−	−	−
		Hyphae	IF	−	−	−
Rhizopus oligosporus	Weihenstephan, Germany	Spores	IF	−	−	−
		Hyphae	IF	−	−	−
Soil bacteria:						
Bacillus mycoides	Hanover, Germany		EIA	−	−	+
Pseudomonas fluorescens	Hanover, Germany		EIA	−	−	−

proached this problem in two ways. The amount of immunogen needed could be greatly reduced by choosing the intrasplenic pathway for immunization without great loss of fusion frequency (3×10^{-6}) in comparison to the more commonly employed immunization protocols (4×10^{-6} and 5×10^{-6}). The fusion frequency was unusually low in all immunization procedures, compared to non-AM immunizations done in our laboratory. Since the mAb concentrations in the cell culture supernatants are still too low, work is in progress to overcome this problem by scaling up the hybridoma cultures after adapting the cells to serum-free medium. Larger volumes (1–2 l) of supernatant can easily be purified and concentrated. It is also expected that a shorter incubation time after the first immunization will yield more stable mAb-producing cell lines. An additional advantage of the intrasplenic immunization is that it is the only route for introducing complete spores into the immune system of smaller laboratory animals.

The amount of antigen used for the screening process could also be reduced by choosing an immunohistochemical method that allowed us to screen the hybridoma supernatants on semi-thin sections of the antigen. The recently developed method of IGSS has been of great benefit for electron- and light microscopic research (VandenBosch 1991; Bonfante-Fasolo et al. 1991). It has also recently been used to detect antibody responses to *Aspergillus* in human sera (Reijula et al. 1992), although its use as a screening method in mAb production is novel. It offers several advantages over enzyme immunoassays or IF methods. It is more sensitive than IF and no sophisticated equipment is needed. The samples can be prepared on microscope slides which can be stored indefinitely for future reference and can be viewed in a standard light microscope. The same reagents can later be used in electron microscopy without change of protocol. In addition, screening with an immunohistochemical technique has the advantage of locating the binding site of the tested mAb. It is, therefore, possible to select hybridoma cell lines whose mAbs definitely mark the desired site, in this case the outer wall of the spores. Apart from the cellular distribution, we have no information on the nature of the antigenic determinants. Boiling the spores in H_2O or 20% methanol did not, however, diminish the binding

capacity of the mAbs. In general, IGSS is a more sensitive method than IF because the signal is enhanced by the silver precipitation. On the other hand, it is possible to employ IF in a system where complete, viable spores are to be tested. IF labelling of viable spores is, however, not suited for screening in mAb production, since the number of spores required is too high.

The antigenic determinant recognized by the mAb A5B1 seems to be species specific. The labelling of both spores and hyphae points to the presence of antigen common to both structures. The labelling pattern of the mAb D12F11 is more difficult to interpret. The IGSS signal with the homologous *G. scintillans* (I) is quite strong as are the cross-reactivities with *G. mosseae* (Whs), *G. mosseae* (I) and *G. macrocarpum* (I). The IF signals, which could only be tested within this group for *G. mosseae* (Whs) and *G. scintillans* (I), give a divergent picture. The homologous reaction is clearly visible (Fig. 3J), but cross-reaction of the mAb with *G. mosseae* (Whs) was not detected (Fig. 3N). This can be attributed to the lower sensitivity of the IF compared to the IGSS. These findings suggest a serological group consisting of several species of *Glomus,* but as long as cross-contaminations of the fungal cultures cannot be excluded as a cause of the heterologous signal, this assumption cannot at present be tested yet. A greater number of mAb exhibiting cross-reactivities with different species is required.

The mAb from H8F7 was raised using *G. ssp* S328 (Fl) as immunogen, which consists of a mixture of *G. globisporum* and other *Glomus* species. The resulting mAb was found to be directed against *B. mycoides* present on the spore surface and has shown a reaction with all AM isolates tested so far. Surface sterilization of fungal spores suppresses this reaction. If *B. mycoides* is generally associated with AM isolates, this raises the possibility that these bacteria play a role in the mycorrhiza system beyond mere cohabitation. Duponnois and Garbaye (1991) discuss the stimulating effect of several types of helper bacteria in ectomycorrhizal symbiosis and the mAb H8F7 may be helpful in further investigating this question in the case of AM fungi.

The present paper demonstrates one possible way to address problems in AM taxonomy. At the same time, it shows the need for standardization in this difficult field. The *Glomus* isolates we have received from many different sources, including the ones from our greenhouse cultures, are still of doubtful purity and uncertain taxonomic identification. The serological methods, especially the mAb apprach with its unlimited supply of Ab, can create serotyping standards to be used by other laboratories. One drawback, however, of the immunohistochemical methods presented in this paper is the difficulty of quantification of the signal obtained. We are at present attempting to quantify the reaction of these mAbs with AM spores by designing enzyme immunoassays based on chemiluminescence measurements.

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References

Aldwell FEB, Hall IR (1986) Monitoring spread of *Glomus mosseae* through soil infested with *Acaulospora laevis* using serological and morphological techniques. Trans Br Mycol Soc 87:131–134

Aldwell FEB, Hall IR, Smith JMB (1985) Enzyme-linked immunosorbent assay as an aid to taxonomy of the Endogonaceae. Trans Br Mycol Soc 84:399–402

Bonfante-Fasolo P, Tamagnone L, Peretto R, Esquerré-Tugayé MT, Mazau D, Mosiniak M, Vian B (1991) Immunocytochemical location of hydroxyproline rich glycoproteins at the interface between a mycorrhizal fungus and its host plants. Protoplasma 165:127–138

Dewey M, Evans D, Coleman J, Priestley R, Hull R, Horsley D, Hawes C (1991) Antibodies in plant science. Acta Bot Neerl 40:1–27

Duponnois R, Garbaye J (1991) Mycorrhization helper bacteria associated with the Douglas fir-*Laccaria laccata* symbiosis: effects in aseptic and in glasshouse conditions. Ann Sci For 48:239–251

Friese CF, Allen MF (1991) Tracking the fates of exotic and local VA mycorrhizal fungi: methods and patterns. Agric Ecosys Environ 34:87–96

Giersch T, Hock B (1990) Production of monoclonal antibodies for the determination of s-triazines with enzyme immunoassays. Food Agric Immunol 2:85–97

Hampton RO, Ball EM, De Boer SH (eds) (1990) Serological methods for detection and identification of viral and bacterial plant pathogens. American Phytopathology Society, St. Paul, Minn

Horn K, Hahn A, Pausch F, Hock B (1992) Isolation of pure spore and hyphal fractions from vesicular-arbuscular mycorrhizal fungi. J Plant Physiol 141:28–32

Köhler G, Milstein C (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256:495–497

Morton JB, Wright SF, Sworobuk JE (1987a) Specificity of two monoclonal antibodies against *Glomus occultum.* In: Sylvia DM, Hung LL, Graham JH (eds) Mycorrhizae in the next decade. North American Conference on Mycorrhizae, Gainesville, Fla, p 316

Morton JB, Wright SF, Sworobuk JE (1987b): Sensitivity of monoclonal antibodies to detectet propagules of *Glomus occultum.* In: Sylvia DM, Hung LL, Graham JH (eds) Mycorrhizae in the next decade. North American Conference on Mycorrhizae, Gainesville, Fla, p 317

Nilsson BO, Larsson A (1990) Intrasplenic immunization with minute amounts of antigen. Immunol Today 11:10–12

Oramas-Shirey M, Morton JB (1990) Immunological stability among different isolates of the arbuscular mycorrhizal fungus *Glomus occultum.* Abstracts of the 90th Annual Meeting of the American Society for Microbiology, Washington, DC, p 311

Perotto S. Malavasi F, Butcher GW (1992) Use of monoclonal antibodies to study mycorrhiza: present applications and perspectives. In: Norris JR, Read DJ, Varma AK (eds) Techniques for the study of mycorrhiza. (Methods in microbiology, vol 24) Academic Press, London, pp 221–248

Reijula KE, Kurup VP, Fink JN (1992) The detection of *Asper-*

gillus fumigatus antibodies in sera by immunogold-silver staining. J Microbiol Methods 15:113–120

Roland JC, Vian B (1991) General preparation and staining of thin sections. In: Hall JL, Hawes C (eds) Electron microscopy of plant cells. Academic Press. London, pp 1–66

Sanders IR, Ravolanirina F, Gianinazzi-Pearson V, Gianninazzi S, Lemoine MC (1992) Detection of specific antigens in the vesicular-arbuscular mycorrhizal fungi *Gigaspora margarita* and *Acaulospora laevis* using polyclonal antibodies to soluble spore fractions. Mycol Res 96:477–480

Schuler G, Reinacher M (1991) Development and optimization of a single-step procedure using protein A affinity chromatography to isolate murine IgG_1 monoclonal antibodies from hybridoma supernatants. J Chromatogr 587:61–70

Sohn G, Sautter C, Hock B (1990) Fluorescence immunolocalization of bound atrazine residues in plant tissue. Planta 181:199–203

VandenBosch KA (1991) Immunogold labelling. In: Hall JL, Hawes C (eds) Electron microscopy of plant cells. Academic Press, London, pp 181–218

Wright SF, Morton JB (1989) Detection of vesicular-arbuscular mycorrhizal fungus colonization of roots by using a dot-immunoblot assay. Appl Environ Microbiol 55:761–763

Wright SF, Morton JB, Sworobuk JE (1987) Identification of a vesicular-arbuscular mycorrhizal fungus by using monoclonal antibodies in an enzyme-linked immunosorbent assay. Appl Environ Microbiol 53:2222–2225