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Monoclonal antibodies (MAbs) specific to surface expressed antigens of *Ditylenchus dipsaci*

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Summary — Monoclonal antibodies (MAbs) were used to investigate differences among species and subspecies of *Ditylenchus*. MAbs raised against antigens of the oat race of *D. dipsaci* were screened by enzyme linked immunosorbent assay (ELISA) using six races of this nematode and three other species of the genus. In total 141 of 798 MAbs showed some discrimination. Four MAbs were specific to a single isolate of the oat race of *D. dipsaci* (MAbs 1-4) and recognised antigens that were associated with cuticular annulations and the lateral incisures of the nematode. At high titre MAbs 1-4 also recognised similar antigens of other species of *Ditylenchus* and races of *D. dipsaci*. Other MAbs recognised surface antigens with similar distributions whilst some were specific to just the lip region of the nematode.

Résumé — ***Anticorps monoclonaux spécifiques des antigènes de surface chez* Ditylenchus dipsaci** — Des anticorps monoclonaux sont utilisés pour rechercher les différences entre espèces et races de *Ditylenchus*. Des anticorps réagissant aux antigènes de la « race avoine » de *D. dipsaci* ont été testés (ELISA) envers six races de ce nématode et trois autres espèces du genre. Sur 768 anticorps monoclonaux, 141 au total font montre d'une discrimination. Quatre anticorps sont spécifiques d'un seul isolant de la « race avoine » de *D. dipsaci* (MAbs 1-4) et reconnaissent des antigènes associés aux anneaux cuticulaires et aux incisures du champ latéral du nématode. A forte concentration, ces anticorps MAbs 1-4 reconnaissent également les antigènes des races de *D. dipsaci* et des autres espèces de *Ditylenchus*. D'autres anticorps monoclonaux reconnaissent les antigènes de surface ayant une localisation similaire tandis que certains sont seulement spécifiques de la région labiale du nématode.

Key-words : Monoclonal antibodies, surface antigens, *Ditylenchus dipsaci*.

Conserved morphology within *Ditylenchus* makes discrimination difficult among some members of this genus of plant parasitic nematodes. In the UK three species, *D. destructor, D. myceliophagus* and *D. dipsaci*, are economically important pests and must be identified accurately. *Ditylenchus dipsaci* causes additional problems because races which are morphologically indistinguishable occur with different host ranges. One form, the giant race, is exceptional in being larger than other members of the species (Hooper, 1972). The potential of DNA probes for distinguishing these forms has been investigated (Palmer *et al.*, 1991) and this work has now been extended by the use of monoclonal antibodies (MAbs).

Previous work based on polyclonal sera has detected broad differences between organisms, and may involve many individual antibody-antigen reactions. This approach has been used for various groups of nematodes, but the results are often complex and variable and differences may be obscured (Hussey, 1979). Nevertheless, discrimination at the species level has been achieved by immunological methods within several genera of plant parasitic nematodes : *Heterodera* (Webster & Hooper, 1968; Scott & Riggs, 1971; Griffith *et al.*, 1982), *Meloidogyne* (Hussey, 1972; Misaghi & McClure, 1974), *Globodera* (Fox & Atkinson, 1985) and *Ditylenchus* (Webster & Hooper, 1968). Discrimination has been less successful at the intraspecific level. Races of *H. glycines* were indistinguishable (Scott & Riggs, 1971), as were those of *M. incognita* (Hussey, 1972). Slight differences have been noted between races of *D. dipsaci* but these may have been due to differences in the developmental stages present in antigen samples (Webster & Hooper, 1968; Gibbins & Grandison, 1968).

More recently, MAbs have been widely used in the discrimination of closely related organisms. For many animal parasites, species or strain specific antigens are known (Almond & Parkhouse, 1985) and MAbs raised to these antigens allow rapid and accurate discrimination (see Hammerling *et al.*, 1981). A MAb discriminating

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Globodera pallida from *G. rostochiensis* has also been raised against a known protein difference (Schotts *et al.*, 1990). MAbs are also of value for the study of undefined antigens, since pure antibodies can be produced in unlimited quantity from impute antigen sources (Halk, 1986). In this work MAbs were raised to a homogenate of *D. dipsaci* (oat race) and screened differentially against antigens from *Ditylenchus* species and other races of *D. dipsaci.*

Materials and methods

Nematode populations

Populations of *D. dipsaci* (giant, lucerne, narcissus, oat, and red and white clover races) were obtained from infested plants (Table 1) and subcultured onto a suitable host. The lower region of plant stems were lightly scarified and wrapped in a small amount of cotton wool. Approximately 100 fourth stage juveniles (J4) in 0.1 ml distilled water were then applied to the cotton wool support. The plants were gently sprayed with water and covered with polythene bags to maintain humidity for 48 h. Inoculated plants were maintained for a further 6-8 weeks at 15-20 °C under glasshouse conditions before the nematodes were extracted using a mistifier (Southey, 1986).

D. myceliophagus, D. triformis, and *D. destructor* were maintained monoxenically on *Rhizoctonia cerealis.* Potato dextrose agar (Oxoid) was inoculated with *R. cerealis* and incubated at room temperature for 7-10 days before approximately 100 nematodes were added. Cultures were maintained at room temperature for a minimum of 2-3 months before the nematodes were collected directly from the Petri dish lids where they accumulated in droplets of water.

Table 1. Source of *Ditylenchus* spp. populations and subsequent culture conditions.

Population	Source	Culture host
D. destructor	Potato, Sweden	*Rhizoctonia cerealis*
D. dipsaci races :		
Giant race	Field bean, RES*	Field bean
Lucerne race	Lucerne, RES	Lucerne (cv. Europe)
Narcissus race	Narcissus, Lincolnshire	None
Oat race	Dwarf French bean, Leeds	Dwarf French bean (cv. Masterpiece)
Red clover race	Red clover, RES	Red clover (cv. Redhead)
White clover race	White clover, RES	White clover (cv. Kersey)
D. myceliophagus	*Agaricus bisporus*	*R. cerealis*
D. triformis	*Rhizoctonia* sp.	*R. cerealis*

* Rothamsted Experimental Station.

Immunisation and production of MAbs

A homogenate of different stages of *D. dipsaci* (oat race) in sterile saline containing 1 % triton was used as the immunogen to inject three female BalbC mice. At weeks one and three, two intraperitoneal injections were given (200 µl of immunogen, 1 µl protein μl^{-1}, mixed with an equal volume of Freund's complete or incomplete adjuvant). A final injection at week 6 contained no adjuvant and was administered to the tail vein. Three days after the final injection two of the immunised mice were killed and the spleen cells fused with myeloma cells (line P3X6388653) as previously described (Atkinson *et al.*, 1988*a*). Serum for use as a control was also prepared from the mice by centrifugation of the blood at 11 000 *g* for 5 min.

Screening

MAbs produced were screened by ELISA against total protein extracts of *D. myceliophagus, D. triformis, D. destructor* and *D. dipsaci* (giant, lucerne, narcissus, oat and red and white clover races). Microtitre plates (Nunc immuno plates) were coated with 100 µl per well of a 0.5 µg ml^{-1} protein from the nematodes in coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate pH 9.6) and stored at 4 °C for a minimum of 12 h before use. Coated plates were rinsed twice with PBS (137 mM sodium chloride, 1.5 mM potassium di-hydrogen orthophosphate, 8 mM di-sodium hydrogen phosphate) and incubated at room temperature with 100 µl $well^{-1}$ 1 % bovine serum albumin (BSA) in PBS for 30 min. Plates were rinsed as before and incubated with the first antibody : 50 µl of a 1:5 dilution of hybridoma culture supernatant in PBS-T (PBS containing 0.05 % tween-20) and 0.1 % BSA, for 1 h. After five more rinses with PBS-T, plates were incubated for 1 h with 100 µl per well of the second antibody : goat-antimouse IgG (whole molecule) alkaline phosphatase conjugate diluted 1:2500 in 1 % goat serum, 1 % BSA in PBS. Plates were then rinsed ten times with PBS-T. Finally, to each well was added 150 µl of a 1 mg ml^{-1} p-nitrophenol phosphate, in a 10 % diethanolamine buffer at pH 9.8 and containing 0.05 M magnesium chloride. The colour reaction was measured photometrically at 414 nm using a standard densitometer (multiscan, Titertek) at 2 and 18 h. The following controls were included on each plate : hybridoma medium (no first antibody), 1:1000 dilution of serum from a non-immunised mouse and dilutions of 1:300, 1:900 and 1:2700 of the mouse-anti-*D. dipsaci* (oat race) polyclonal serum. Optical density (OD) values at either 2 or 18 h were used for analysis depending on the rate of colour development with a particular antibody. MAbs failing to give ODs > 0.2 at 18 h were discarded.

Periodate oxidation

Plates coated with nematode antigen as before were pretreated before ELISA with 100 µl per well 0.05 M

sodium acetate pH 4.5 with and without 10 mM periodic acid for 1 h in the absence of light. After two rinses with PBS-T the usual protocol for ELISA was followed, starting by incubating with 100 µl per well 1 % BSA.

IMMUNOCYTOCHEMICAL LOCALISATION OF ANTIGENS

Specific antigens were localised within the nematode by immunofluorescence, using methods previously described (Atkinson *et al.*, 1988*a*) except whole worms were cut in the fixative (1 % paraformaldehyde in PBS). A polyclonal antibody to the tetrapeptide phe-met-arg-phe-mide (FMRFamide) was used as before (Atkinson *et al.*, 1988*b*) as a control to demonstrate penetration of the nematode tissue by antibodies under these conditions. A range of dilutions of hybridoma supernatants was used (6-100 %).

Results

798 MAbs raised to *D. dipsaci* (oat race) showed a range of specificity when screened differentially by ELISA with six races of *D. dipsaci* and three other *Ditylenchus* species. Some MAbs (21; 2.6 %) were specific to a single race or species, whereas others (124;

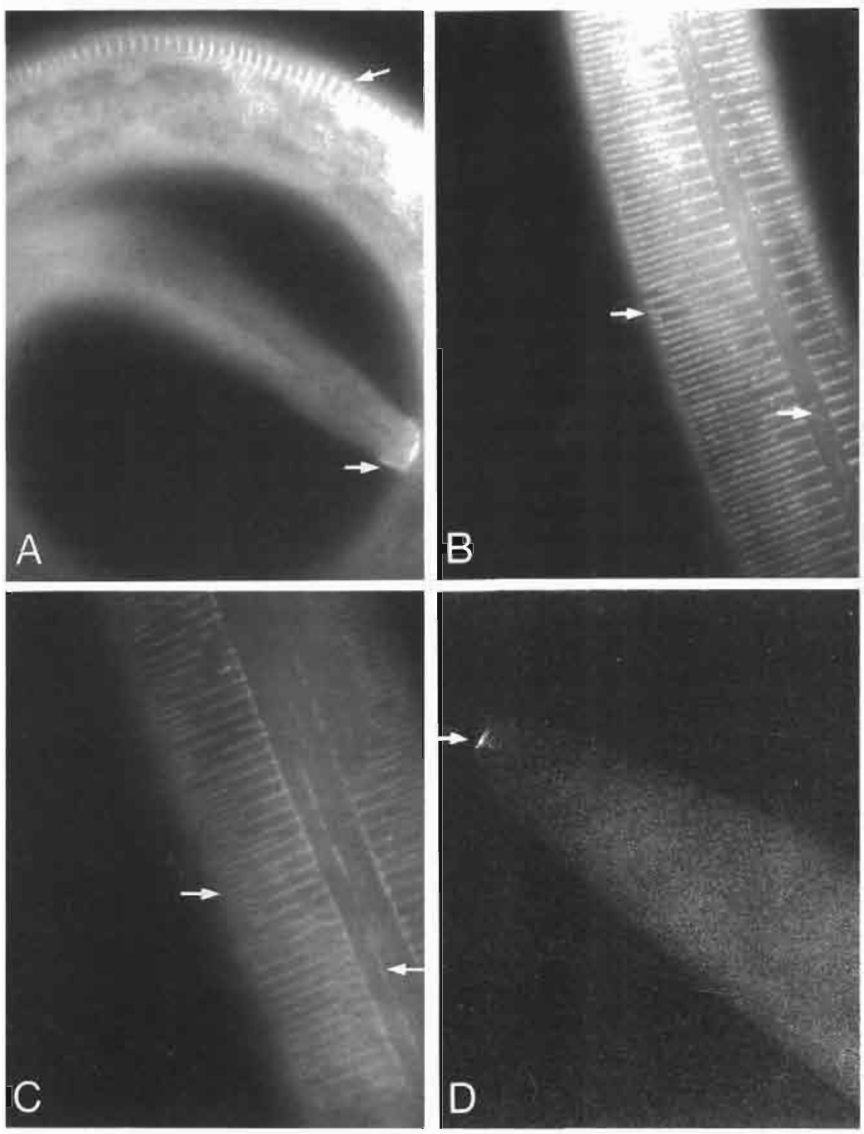

Fig. 1. Cytochemical localisation of antigens recognised by selected MAbs. *Ditylenchus dipsaci* (oat race) surface antigens as localised by MAbs 1-4 on whole nematode (A) and cut fragments of nematode (B). *D. dispaci* (red clover race) surface antigen (C) and *D. dipsaci* (giant race) antigen in the lip region (D) localised by two other MAbs. Arrows indicate sites of specific immunofluorescence. The maximum diameter of the nematodes is about 26 µm.

15.5 %) gave a quantitative but partial discrimination. Of the 21 MAbs demonstrating a unique specificity only five recognised the source antigen (*D. dipsaci* oat race) to which they were raised. The other 16 MAbs were specific either to *D. myceliophagus, D. destructor* or *D. dipsaci* (narcissus, red clover or white clover race). No MAbs were uniquely specific to the lucerne or giant races of *D. dipsaci* or to *D. triformis*. After subcloning, only four of the MAbs produced maintained their discrimination. These (denoted MAbs 1-4) were specific for *D. dipsaci* (oat race). However, when reacted with two different isolates of the same race in an ELISA, MAbs 1-4 all recognised antigen from the original isolate only. Periodate oxidation of the antigen prior to ELISA prevented recognition by the four MAbs.

The site of target antigen was investigated using fluorescent immunocytochemistry. MAbs 1-4 all recognised a surface exposed antigen present in annulations and lateral incisures (Fig. 1 A, B) of both cut and whole adults and fourth stage juveniles of *D. dipsaci* (oat race). Using undiluted cell culture supernatants, MAbs 1-4 gave similar localisation of antigen for individuals of the two other races of *D. dipsaci* that were tested (namely the red clover and giant races). However, when supernatants were diluted to 1/2, 1/4, 1/16 and 1/32 the cuticular localisation of the antigen was specific to the oat race alone. Of five other MAbs, not specific to *D. dipsaci* oat race, three bound to cuticular annulations as for MAbs 1-4, and 2 were specific to the lip region of the nematode (Fig. 1 C, D). Control reactions using anti-FMRFamide antibody to visualise nerve cords demonstrated reliable antibody penetration of cut lengths of the nematodes, but MAbs 1-4 failed to react with internal tissues of *D. dipsaci*.

Discussion

MAbs were raised against the oat race of *D. dipsaci* (oat race) and 145 from a total of 798 revealed some potential for inter- or intraspecific discrimination by ELISA, with 21 demonstrating a unique specificity. Of these some were heterospecific, recognising antigens from other *Ditylenchus* species and other races of *D. dipsaci* but not *D. dipsaci* (oat race) itself, the original immunogen. This may result from biochemical bias which can occur when MAbs are raised to an undefined mixture of antigens, since highly immunogenic but rare components of the homogenate may stimulate antibody production. Such immunogens may occur in the original homogenate at levels below that detectable by screening methods, whilst occurring more abudantly in homogenates of closely related organisms where detection is possible (Loor, 1971). The antigenic reactivity of several supernatants was lost during culture indicating that some hybridomas were unstable, possibly due to loss of vital chromosomes (Westerwoudt, 1986). However, four cell lines remained which produced population specific MAbs for one isolate of *D. dipsaci* (oat race). Previously, differences were detected, using polyclonal serum, within the genera *Ditylenchus* (Webster & Hooper, 1968) and *Meloidogyne* (Bird, 1964; Misaghi & McClure, 1974), and between races of *Heterodera glycines* (Griffith *et al.*, 1982). We show here that MAbs allow an improved level of discrimination.

Immunocytochemistry established that MAbs 1-4 recognised surface antigens which revealed annulations and lateral incisures. They may recognise the surface coat (Wright, 1987) of the cuticle, or material secreted onto the surface from sites such as the stylet, sense organs or the excretory system. All four MAbs showed similar specificities suggesting they recognised one or more antigenic determinants on the same antigen and they did not recognise internal tissues.

Periodate pretreatment of antigens resulted in a loss of recognition by MAbs 1-4 in the ELISA suggesting the antigens contained a carbohydrate group. Furthermore, the patterns of the MAbs binding to *D. dipsaci* was similar to that for the lectins Concavalin A and Lumilin, which bind to carbohydrate moieties (Durschner-Pelz & Atkinson, 1988). Glycoconjugates are surface components of plant pathogenic fungi (Dixon, 1986) and strain variation in some fungi (for example *Colletotrichum lindemuthianum*) correlates with differences in oligosaccharides which may elicit differential host responses (Anderson, 1980). Possibly, the antigens recognised by MAbs may also be involved in host recognition of nematodes. This is consistent with differences in lectin binding found for surface glycoconjugates among races of *Meloidogyne incognita* (Davis *et al.*, 1988) and success in using detergent to remove surface components of this nematode that can be resolved using SDS-PAGE electrophoresis (Robinson *et al.*, 1989).

High titres of antibody caused some binding of MAbs 1-4 to both clover races. This may indicate a general similarity between the races in the antigen with specific epitope variation as occurs in the animal parasitic nematode *Toxocara canis* (Maizels *et al.*, 1987). The site of antibody binding was common to both J4 and adults of *D. dipsaci* but both forms were recovered from plants and it is uncertain at present whether the surface is similar when this nematode is recovered from soil. Different environments have been correlated with changes of the surface components of the animal parasitic nematode *Trichinella spiralis* (Parkhouse & Clark, 1983).

The production of highly specific MAbs shows that there is potential in this approach for the diagnosis of plant parasitic nematodes. This would be the procedure of choice rather than using DNA probes especially if host recognition proves to be based on post-translational modification of proteins. The MAbs 1-4 are population rather than race specific and so their principal value would be in further work using affinity chromatography to isolate these antigens for subsequent use as immu-

nogens. This approach may provide further MAbs that recognise race-specific antigenic determinants. For reliable diagnostic use, such MAbs must be specific for the race of interest, useful for all populations and suitable for all stages of the animal.

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