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Stage-specific Monoclonal Antibodies to the Potato Cyst Nematode *Globodera pallida* (Stone) Behrens

KAREN D. BACKETT,[1,2] HOWARD J. ATKINSON,[1] AND JOHN M. S. FORREST[2]

Abstract: Using standard hybridoma technology and hierarchical screening, monoclonal antibodies (MAbs) were obtained with specific reactivity against two developmental stages of *Globodera pallida*. The procedure was based on enzyme-linked immunosorbent assay (ELISA) with homogenates prepared from second-stage juveniles, young adult females, and potato roots. Hybridomas were formed by fusing myelomas with splenocytes derived from mice immunized with either infective juveniles or females of *G. pallida*. About 600 hybridoma lines were screened from the fusion involving the mouse immunized with juveniles. Two MAbs (LJMAb1 & 2) were identified with high reactivity toward second-stage juveniles but no reactivity with either potato roots or females of *G. pallida*. A total of 630 cell lines was screened from the corresponding fusion involving the spleen of a mouse receiving immunogens from adult female nematodes. One MAb (LFMAb1) was obtained with the required specificity against only adult female *G. pallida*. This work extends the application of monoclonal antibodies in nematology from valuable probes for research and species identification to recognition of developmental stages. These specific MAbs have potential value in plant breeding programs for screening for resistant lines unable to support nematode development.

Key words: ELISA, *Globodera pallida*, monoclonal antibody, potato cyst nematode, stage-specificity.

Kohler and Milstein (14) pioneered the routine production of monoclonal antibodies (MAbs). Such specific antibodies are more suited for long-term diagnostic use than those of polyclonal origin, because unlimited quantities of uncontaminated, specific antibody of homogeneous affinity can be produced. An immunized Balb/c mouse may produce as many as 10–40 $\times$ 10^6 clonotypes, although often many fewer B-cells are activated (23) and antibodies against the more antigenic components of the immunogen predominate. A potential limitation of the MAb approach with complex immunogens is difficulty in prediction of the number of hybridomas that must be screened to obtain a MAb with a desired reactivity. Nonetheless, this use of complex antigens has been a frequently valuable approach in producing specific antibodies for pathogen detection in virology, bacteriology, and to a certain extent mycology (11).

Atkinson et al. (2) used an immunological screen to describe a wide range of reactivities for MAbs raised to complex antigens prepared from second-stage juveniles (J2) and females of *Heterodera glycines*. Similar success has also been achieved for *Meloidogyne incognita* (7,13). The first reported MAbs against potato cyst nematodes were developed following use of species-specific proteins purified from eggs of *Globodera rostochiensis* or *G. pallida* as the immunogen (20). The approach has proven of value for species recognition under field conditions (21).

Several structures of nematodes change with development, including the reproductive system and cuticle (4). Stage-specific differences have been found in proteins of free-living *Caenorhabditis elegans* (6) and also in proteins associated with the cuticle of *Meloidogyne* species (17,19). Atkinson et al. (1) produced several specific MAbs against J2 and female *H. glycines* and subsequently obtained one specific to an intestinal organelle of the J2 before invasion and a second that was specific to the cuticle of the adult female (1). Stage-specific MAbs have also been obtained for *Meloidogyne* spp. (7).

The purpose of our investigation was to raise stage-specific antibodies to *Globodera pallida* using complex immunogens and thereby to provide a novel basis for subsequent identification of parasitic develop-

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[1] Centre for Plant Biochemistry and Biotechnology, University of Leeds, Leeds LS2 9JT, England.

[2] Scottish Crop Research Institute, Invergowrie, DD2 5DA, Scotland.

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ment on host plants. This approach may facilitate discrimination of resistant and susceptible plant lines in potato plant breeding programs.

Materials and Methods

Preparation of immunogen and antibodies: Air-dried cysts of *Globodera pallida* (pathotype $Pa_{2/3}$) from stock cultures maintained at the University of Leeds were incubated in a screw-top glass jar on a 30-μm-pore nylon mesh at 20 C for 4 weeks in 3–7 ml of potato diffusate (22). The second-stage juveniles (J2) emerging from cysts were collected beneath the mesh, and their number was estimated (ca. 100,000 individuals per injection) using a counting slide (22). They were centrifuged at 13,000*g* for 2 minutes and added to an equal volume of 400 mg/ml sucrose. Following recentrifugation, debris-free nematodes were collected at the interface between the two solutions for immediate use. For production of adult females, potato plants (*Solanum tuberosum* cv. Desiree) were grown in soil infested with *G. pallida* (1 cyst/10g soil). After 25 days, young adult females (ca. 1,000 individuals for each injection) were hand-picked from the tap water-washed roots, collected over liquid nitrogen, and stored at −70 C.

Immunogens were prepared from homogenates of young adult female and freshly hatched J2 of *G. pallida* ($Pa_{2/3}$). Nematodes were homogenized (using a 0.1 ml glass homogenizer, Jencons Scientific, Leighton Buzzard, England) in PBS-Tween (137 mM NaCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 0.05% Tween 20, pH 7) and then centrifuged in this solution at 11,400*g* for 5 minutes. Three 6-week-old female Balb/c mice were each injected interperitoneally with a volume of the supernatant containing 100 μg protein, for both immunogen preparations. Two uninjected mice were used as controls. There were four injections at intervals of 3 weeks. For the first injection, the protein was combined with an equal volume of Freund's complete adjuvant, to give a final volume of 700 μl. Incomplete adjuvant was used for the second and third injections, and PBS was used for the final immunization. Tail bleeds were taken 10 days after the second and third injections, and the titre of polyclonal antiserum was measured with enzyme-linked immunosorbent assay (ELISA). For each fusion experiment, the two mice with the highest specific antibody titre were used.

Cell fusions were performed with a polyethylene glycol procedure (10,11) using the P3 X 63Ag8.653 myeloma cell line. Dulbecco's Modified Eagle's medium (Gibco, Uxbridge, England) was used for culturing hybridomas.

ELISA screens: The MAb produced by each cell line was assayed in a differential ELISA screen. The antibody-containing supernatant from the cell lines was collected every 2 days and stored in 0.01% sodium azide at −20 C (8). A series of microtitre plates (NUNC immunoplates, maxisorp, Gibco) was coated with 100 μl/well of 0.5 μg/ml protein (purified in the same way as described for the injections) obtained from J2 of *G. pallida* ($Pa_{2/3}$), young adult female *G. pallida* ($Pa_{2/3}$), J2 of *G. rostochiensis* (Ro1), or potato roots (MAbs were not tested against adult females of *G. rostochiensis*). These proteins were added in coating buffer (15 mM Na_2CO_3, 35 mM Na_2HCO_3, pH 9.6) and stored at 4 C for a minimum of 24 hours before use. Coated plates were then assayed with the following procedure. Plates were first rinsed twice with PBS and then incubated at room temperature with 100 μl/well of 1% bovine serum albumin (BSA) in PBS for 30 minutes on an agitation platform (Rotatest shaker, Luckham Ltd., Burgess Hill, England). Plates were rinsed with PBS and incubated with 50 μl of 1:2 diluted hybridoma culture supernatant in PBS-Tween; two wells were used per test clone for 1 hour. After further rinses in PBS-Tween, plates were incubated for 1 hour in 100 μl/well of a goat–anti-mouse alkaline phosphatase conjugate diluted 1:2500 in 1% goat serum in PBS-Tween, for 2 hours. Plates were again rinsed five times

and then incubated in PBS-Tween for 30 minutes before five more rinses with PBS. Finally, 100 µl/well of 1 mg/ml *p*-nitrophenyl phosphate (PNPP) in 10% diethanolamine buffer (pH 9.8) containing 0.05 M $MgCl_2$ was added. The color reaction was measured photometrically at 405 nm using a standard densitometer (Multiscan, Bio-Rad, Richmond, CA) at 2, 16, and 23 hours. The following controls were used on each plate: no primary antibody (culture medium only), a nonspecific primary antibody, and 1:500 dilution of the specific polyclonal serum obtained after the fusion.

Cell lines were cultured if they showed a positive response to *G. pallida, G. rostochiensis,* or both species and no reactivity to potato root homogenate. The isotype of MAbs from positive cell lines was determined using a standard kit (Serotech, Oxford, England). Cell lines were subcloned twice by dilution and stored under liquid nitrogen (8). Cell lines were named with the prefix "L," to denote production at University of Leeds; "J" or "F" for antibodies raised against J2 or adult female nematodes respectively, and "MAb" to signify monoclonality.

Immunofluorescence: The specific antigens recognised by LJMAb1 and 2 were localized in second-stage juvenile nematode sections (*G. pallida* $Pa_{2/3}$) by immunofluorescence (1). This method was not used on adult females because previous work had revealed this technique to be unsuitable for adult female material (2). Conditions were first optimized using a polyclonal antibody to the tetrapeptide phe-met-arg-phe-amide (FMRFamide), which is known to react with the nematode nervous system (3). Whole nematodes were fixed in 0.1 M phosphate-buffered 4% paraformaldehyde (pH 7.2) for 18 hours before washing three times in M9 buffer (42 mM Na_2HPO_4, 22 mM KH_2PO_4, 86 mM NaCl, and 1 mM $MgSO_4 \cdot 7\ H_2O$) (5). Approximately 100 individuals were cut into sections 0.1–0.3 mm thick and incubated for 2 hours in rabbit anti-FMRFamide antibody (Cambridge Biochemicals Research, Cambridge, England) diluted 1:1000 with PBS-Tween, 0.2% saponin (Sigma, St. Louis, MO) to provide positive controls. After three PBS-Tween washes, the nematode sections were incubated for 2 hours in an anti-rabbit IgG fluorescein isothiocyanate conjugate (FITC, Sigma) diluted 1:50 with PBS-Tween. After rinsing in PBS-Tween, sections were mounted in a fluorescence stabilizer, Citifluor (Agar Scientific, Stansted, England) before observation. Immunofluorescence was examined using an Olympus BH-2 microscope fitted with an epillumination attachment, a blue excitation filter (B460), and a secondary filter (EY455) with a band pass >460 nm.

Immunolocalization of MAb reactivity was based on these protocols. About 50–100 nematode sections were incubated in each dilution (0.5–100%) of the test hybridoma supernatant in PBS-Tween–0.2% saponin solution overnight before rinsing and incubating in an anti-mouse IgG FITC conjugate diluted 1:20. All other steps were as described in the previous paragraph. Positive controls consisted of a series of nematode sections incubated in the FMRFamide polyclonal antibody. Negative controls were as follows: omission of primary antibody, omission of secondary antibody, and a MAb primary antibody without reactivity with nematodes plus secondary antibody.

Results

Because the time course of color development varied between individual supernatants, absorbance was recorded at intervals to ensure that individual color reactions were measured after development of a detectable color and before the optical density exceeded the range of the densitometer. The polyclonal serum recovered from experimental mice had a higher specific titre than the control mouse in both fusion experiments. ELISA screening of approximately 600 hybridoma cell cultures (from a total of 1,000 plate wells) in the fusion based on J2 as the immunogen

identified two cell lines named LJMAb1 and LJMAb2 that were specific to protein from J2 of *G. pallida* (Fig. 1). No MAbs were produced that showed high affinity to protein from J2 of *G. rostochiensis*. The fusion based on females as the immunogen yielded approximately 630 hybridoma cell lines (from a total of 1,000 plate wells) of which 287 produced antibody. Nematode-specific antibody was produced initially by 58 lines. After subcloning and further specific screening, one high-affinity line specific against young adult females (*G. pallida* $Pa_{2/3}$) was obtained (LFMAb1). The three MAbs (LJMAb 1–2 and LFMAb1) were isotype IgM.

With anti-FMRFamide as the primary antibody, a bright FITC fluorescence of nerve ganglia and neurons was produced, indicating that internal penetration of antibody had been achieved. Both LJMAbs showed bright specific staining of the surface cuticular annulations but showed no reactivity with the internal structures of the nematodes. There was no observable reactivity in the negative controls.

Discussion

Both fusions produced high-affinity MAbs with specific reaction against the immunogen and some antibodies with a broader, non-stage-specific reactivity. All members of this latter group and several other MAbs were not stably produced by the cell clones. Unfortunately, the genetic instability of hybridomas is a common problem (9,15).

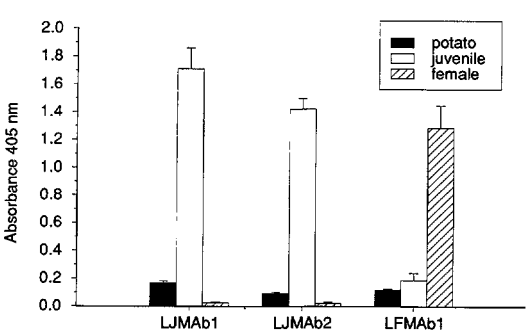

FIG. 1. Comparison of reactivity in ELISA of three monoclonal antibodies (LJMAb1, LJMAb2, and LFMAb1) against potato roots, homogenates of juveniles and females of *Globodera pallida* (bars represent mean ± SE).

About 600 hybridomas established in about 1,000 plate wells used for both fusions. The Poisson distribution suggests that at this frequency, most wells contained a single cell line (8). Of these, 30% would be expected to produce MAbs using donor spleens from mice (8), a number in close agreement with the 287 antibody-producing hybridomas identified in the second fusion. This provided a set of 58 MAbs that did not cross-react with potato roots, and eventually one enduring cell line of the required specificity (LFMAb1) was subcloned by limiting dilution. This low frequency of success from a near-optimal fusion emphasizes the unpredictable outcome of fusions based on the use of complex immunogens. Other workers have experienced similar difficulties (9), and more than one fusion is frequently necessary to produce a MAb of interest. One essential need is a rapid and definitive screen that is able to select a subset of interesting hybridoma lines before labor-intensive subcloning. These problems may be lessened by selecting a protein(s) of interest as immunogen (20), when such a protein is known and likely to prove satisfactory.

Infective J2 of *G. rostochiensis* were used in the screens because it was considered necessary to obtain species-specific antibodies. It is essential for future MAb utilization in plant screening programs that false results do not arise by cross-reactivity following mistaken use of *G. rostochiensis*. Both LJMAb1&2 are specific to surface antigens of *G. pallida*, and further work may establish that these MAbs recognize distinct antigen(s) to that used previously to raise species-specific antibodies (20). Possibly LJMAb1&2 also have potential for routine recognition of *G. pallida* from soil samples (18,21). If so, then production of a collection of species-specific MAbs for *G. pallida* that recognize distinct antigens could help resolve problems in assignment of populations to particular species or species mixtures. Because the antibodies rec-

ognize antigens that differ between species, the MAbs may provide a basis for discerning intraspecific variation in *G. pallida*.

Stage-specific proteins have been identified for several *Meloidogyne* species (18), and stage-specific MAbs have been reported for *H. glycines* (1,2) and *Meloidogyne* species (7). This work and our development of stage-specific MAbs for *G. pallida* suggest that such antibodies could be obtained for any plant-parasitic nematode of interest. The three MAbs selected in this work did provide the required basis for stage-specificity without cross-reactivity with potato host roots. Therefore, they have the potential for detecting J2 and young female stages in planta. The two LJMAbs may allow detection of plant invasion. *Globodera* spp. normally enter roots of both resistant and susceptible potato lines irrespective of subsequent establishment of either a compatible or incompatible interaction. Therefore LJMAb1–2 may provide a basis for ensuring that differences are not due to dissimilar invasion rates arising from experimental error. They may also help identify unexpected bases of resistance to invasion. LFMAb1 is the primary antibody of interest for future work on screening for plant resistance, because all known resistant responses for potato plants occur before females develop.

Present methods for potato plant bleeding are laborious, especially at the assessment stage. Clearly, the three antibodies could provide the basis for an ELISA that is suitable for routine use. ELISA tests are widely used in diagnosis, require only generally available equipment, and provide a basis for rapid and inexpensive analysis. The system could be developed for use on plants grown from both tubers and seed. At present, plantlets grown from true potato seed do not provide reliable material for resistance screening, because they often do not support full development of females (16). This problem could be eliminated by a reliable ELISA analysis following nondestructive sampling of small amounts of nematode-infested root tissue from plantlets. Plantlets established as resistant from such a test could then progress without delay in a breeding program. Further work is in progress to investigate the potential of these MAbs for use in breeding programs for potato plant resistance.

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