ESTABLISHMENT OF TWO CELL LINES FROM THE NEMATODE MELOIDOGYNE INCognITA (TYLENCHIDA; MELOIDOGYNIDAE)

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SUMMARY

The root-knot nematode Meloidogyne incognita Chitwood, is a pest of considerable agricultural and economic importance. As part of a study of this species, two diploid cell lines (2n=32-36) were established in vitro on two different culture media. The two cultures were obtained from mature egg-laying females and were designated MI 8C and MI 5G. Descriptions are given of the techniques used for the preparation of the primary cultures, the media and the characteristics of the cell lines obtained. The doubling time of the population was estimated.

Key words: cell lines; Meloidogyne incognita; nematodes.

INTRODUCTION

M. incognita is a phytophagous nematode of great economic importance (1,2,3). The establishment of cell lines of such a major plant parasite would be valuable for cytological, genetic and biochemical studies, and in the search for new methods to control the pest. While a large number of cultures of other invertebrate cell lines have been established [for example, many insect cell lines and, more recently, the first filarian cell lines(4)], no nematode cell line has been established to date. In view of this situation, the present attempt was undertaken.

MATERIALS AND METHODS

Sampling and inoculation of material for the preparation of the primary cultures. Mature egg-laying females of M. incognita (N. Carolina State University Race 2, No 135) were dissected out of infected tomato roots with fine tweezers and transferred to normal saline (0.9% NaCl) in universal type culture tubes. Excess liquid was removed. Females were surface sterilized for 6 min in a solution of 0.525% sodium hypochlorite, and subsequently transferred to sterile universal tubes. They were then washed twice under aseptic conditions with a large volume of sterile tap water; 5 ml of medium was added to each universal containing 15 females, and the contents removed into sterile Petri dishes with the aid of Pasteur pipettes. Females were crushed with fine tweezers and the suspension transferred into plastic 25 cm² Roux bottles and kept in the dark at 27 ± 1°C. Three to ten bottles were prepared for each of the media and their combinations.

M. incognita cells did not adhere to the surface of the flasks and for this reason 5 ml of medium were added weekly for four weeks. At the end of this period the cell suspension was transferred into sterile universals, spun down in a bench centrifuge for 10 min at 1000 rpm, the supernatant carefully removed and fresh medium added before the suspensions were transferred back to the original culture flasks. The original flasks were used again so that any cells stuck to the surface would not be lost. Whole pieces of tissue were not removed since they could possibly support growth of individual cells.

Choice and composition of the culture media. Several culture media were tested (see list of media) which support growth of vertebrate, invertebrate or plant root cultures. Media were prepared according to classical methods and kept under 4°C in the dark. Two days before use they were, where necessary, supplemented with 10% FBS and/or 5% tomato root extract (Re) and/or
5-10% *Manduca sexta* hemolymph (He). β-ecdysterone at a concentration of $7 \times 10^{-7} \text{M}$ and/or Con-A (60 μg/ml) were also added to a series of media as potential growth stimulants. Antibiotics were not used. Media were incubated at 27 ± 1°C for two days before use.

Preparation of tomato root extract and collection of *Manduca sexta* hemolymph. Tomato root systems growing under sterile conditions in PRL-4 agar in Petri dishes were collected and ground in a pestle and mortar over ice. The slurry was pressed through cotton muslin and the extract centrifuged for 12 hours at 140,000 g. The supernatant was filter sterilized and frozen. *Manduca sexta* larvae immediately prior to pupation were carefully washed with distilled water and dried on tissue paper. Prolegs were excised with scissors taking care to avoid extensive damage of the supporting tissues, and caterpillars were suspended individually over universal tubes embedded in ice. Hemolymph “contaminated” with gut contents was discarded while the remainder was pooled, heat inactivated for 5 min at 65-70°C and subsequently cleared by centrifugation at 140,000 g for 12 hours at 4°C. The supernatant was filter sterilized (0.22 μm filter) and kept frozen.

Chromosomal complement. The chromosomal complement of the newly established nematode cell lines as well as all insect cell lines kept in the laboratory was examined (1 × 10^5 cells per ml, 5 ml total). Colcemid was added to a final concentration of 0.02 μg/ml of cell suspension. After incubation for 2 h at 27 ± 1°C the cells were centrifuged, resuspended in 0.56% potassium chloride and held for 10 min at R.T. before recentrifuging. The pellet was aspirated and the cells resuspended by flicking the tube. Freshly prepared fixative (3:1 ethanol:acetic acid) was added dropwise to give a final volume of 10 ml. Cells were left in the fixative for 5 min, then collected by centrifugation and resuspended in fixative twice more. After the last centrifugation the final volume was reduced to 1 ml. Smears were stained for 20 min in a solution of 1% orcein in 50% propionic acid, and chromosomes were observed under an Olympus BH-2 microscope.

### RESULTS AND DISCUSSION

In most of the media the primary cultures did not survive for more than a period of three months after seeding. Addition of β-ecdysterone and Con-A proved detrimental as the cells lysed a few days later. A combination of certain media with 10% FBS and 5-10% He supported survival of the cultures for a period of more than five months. In these primary cultures the cells were of two different sizes: smaller spherical cells which tended to form clusters (Fig. 1 b), and larger spherical solitary cells which in certain cases appeared to divide (Fig. 1 a). In one flask of each of the following media: HL-1, Leibovitz (L-15) and Schneider’s, a new type of cell made its appearance about three months after initiation of the primary cultures. Of these, those in the HL-1 medium were eventually lost but those in L-15 and Schneider’s propagated. These new cells which grew initially from the cut edges of the gonads (Fig. 1 c,d), were larger than the ones in the primary cultures. They were of uniform size and shape, granular in appearance and generally spherical. The cell diameter was 8 μm in L-15 and 14 μm in Schneider’s medium. Soon after they appeared the other cells disintegrated (Fig. 1 e). After passage, the new cells adhered to the substratum by means of fine, long and fragile projections which branched and formed swellings (Fig. 1 f). Some of the cells adhering to the bottom of the flasks tended to elongate and become oval. The cells appeared to divide by an unequal division in which new smaller cells grew like a bud, eventually producing projections side by side with those of the “mother” cells and, if not disturbed, forming a confluent monolayer (Fig. 2 a,b,c,d). Cells propagated, although at lower rates even in concentrations of 30% Schneider’s medium. In aged cultures cells lysed leaving behind a fine network of fibers still containing some cells, and crystal-like particles. By counting the cells in monolayer colonies derived from a single cell in a given period of time, the doubling time of the population was estimated to be approximately 24 h for MI 5G and 96 h for MI 8C. Cells floating in clusters also seemed to multiply, always joined together by means of fine projections. The optimum conditions for growth of both lines were 27 ± 1°C in stationary flasks under normal atmosphere, and the cells survive liquid nitrogen freezing.

The development of cells in the L-15 medium was originally faster than in Schneider’s (a situation which was eventually reversed) though the cells in the latter medium were slightly larger than in L-15. The interval between the primary culture and the first sub-culture was about three months for both media. Passage of the cells was carried out (initially every one to three weeks) by simply shaking the flasks to bring the cells into suspension and distributing the suspension into new bottles. The rather lengthy period of adaptation to the nutrient media seems to demonstrate a selection of cell types adapted to the new environment from those originally present.

Two cell lines have therefore been obtained. These are the MI 8C in Leibovitz’s medium (Fig. 1 e) and the line MI 5G in the Schneider’s medium (Fig. 2 e). The first subculture MI 8C was obtained on September 23, 1987, and the first subculture MI 5G on September 24, 1987.

The two cell lines reported here from *M. incognita* are the first from Nematoda. Their identity was confirmed by examination of their chromosomal complement (Fig. 2 f,g) in comparison to that of all the insect cell lines.