Journal of Nematology 29(2):168–172. 1997.

Maintenance of Dispersed Reproductive Cells from Male and Female *Ascaris suum*[1]

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Abstract: In vitro cultivation of tissues and cells provides an experimental methodology to define and manipulate physiological mechanisms that are not possible with in vivo techniques. Tissues from the germinative-growth zones of adult *Ascaris suum* gonads were excised and minced, and then enzymatically dispersed and transferred to an artificial, perienteric fluid-fetal calf-serum-medium complex. Cells were maintained in a viable state for 8 days, with medium replacement every 48 hours. During this period, morphological changes in the gonadal cells included decreased size, dedifferentiation, and degeneration. Two indices of metabolism, evolution of $^{14}CO_2$ from radiolabelled glucose and reduction of the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium), decreased by approximately 50% and 60%, respectively. The in vitro procedures developed provide the first opportunity to examine specific cellular functions of nematode reproductive tissues over an extended period of time.

Key words: Ascaris suum, cultivation, nematode, parasite, reproduction.

Tissue culture techniques have been developed to examine cells independent of systemic variation during homeostasis and the stress of experimental manipulation. More specifically, tissue and cell culture technology has been used to elucidate inter- and intracellular activity, environmental aberrations, genetic regulation, and the formation of cellular products (Freshney, 1994). Advances in most areas of physiological research have been accelerated by the development of in vitro organ, tissue, and cell cultivation.

Most nematodes are minute with a fibrous exterior cuticle, thereby obviating routine and reliable excision of specific tissue types. In contrast, the swine intestinal roundworm, *Ascaris suum,* is large; females measure 20 to 50 cm in length, and males measure 15 to 30 cm (Noble et al., 1989). Furthermore, the sexes easily are distinguished by tail morphology, worms are abundant at many swine abattoirs or by experimental inoculation of pigs, and intact adult parasites can be maintained outside the host for several days.

The nematode reproductive tracts are tubular structures in which germinal cells develop (Bird and Bird, 1991). Doran (1961) successfully cultivated an organ explant from the germinal cord of female *Ascaris lumbricoides.* After 6 days, cells began growing out from the original explant, and cellular growth was maintained for more than 45 days, although no evidence of cell division was evident. Similarly, Ogura (1989) demonstrated outgrowths of ovarian tissues from the rodent nematode parasite *Angiostrongylus cantonensis* for up to 26 days during which automotility, i.e., migration of cells, was evident.

This study was conducted to develop procedures for the maintenance of reproductive tissue cells from *A. suum* and the assessment of the tissue's relative viability over time. Because of the phenomenon in nematodes of eutely (constant number and position of most cells) (von Ehrenstein and Schierenberg, 1980), reproductive germinative tissue at the most distal portion of the tract, mitotically active for the continuous production of gametes, was selected to attempt cultivation. Potentially, cells from these tissues might maintain mitotic competency during in vitro cultivation.

METHODS AND MATERIALS

Parasites: Adult *A. suum* were obtained from pigs at a local abattoir, rinsed of host intestinal debris in warm tap water, and maintained at 37 °C in artificial perienteric fluid (APF) with 0.2 M Na acetate (Del Cas-

Received for publication 2 October 1996.

[1] Mention of a proprietary product does not constitute an endorsement or a recommendation for its use by the U.S. Department of Agriculture.

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The authors acknowledge the able assistance of Lisa Carr, Theresa Goldman, and Marilyn Stanfield.

tillo and Morales, 1967) supplemented with 0.1 M glucose. To maintain 100 active worms for up to 6 days in vitro, 2 liters of APF were changed twice daily. Prior to dissection, worms (n = 20) were sexed, separated, and incubated in 1 uM chloramphenicol solution at 37 °C in a shaking water bath for 1.5 hours.

Preparation of cells: Under aseptic conditions in a surface-sterilized hood (Forma Scientific, Marietta, OH), worms were placed in a petri dish on a gauze pad. A longitudinal incision was made to expose the reproductive and intestinal tracts. The distal germinative/growth zones of the reproductive tracts were excised, finely minced, and placed in warm APF on a slide warmer at 37 °C until all tracts were dissected.

Minced germinative-growth zones from 10 worms were placed in a sterile, conical 50-ml tube with 40 ml of APF and collagenase (0.25 mg/ml, Sigma Chemical Co., St. Louis, MO). Tubes were placed in a shaking water bath at 37 °C for 30 minutes, with multiple inversions of the tube every 10 minutes. After partial collagenase digestion, cells were filtered through a sterile 100-um nylon cell strainer into another 50-ml conical tube. Cells were washed in 40 ml of Hanks' Balanced Salt Solution (Sigma Chemical Co., St. Louis, MO) plus a 5% (v/v) antibiotic-antimycotic solution (penicillin and streptomycin-amphotericin B, Gibco, Grand Island, NY). The cells were centrifuged at 75*g* for 10 minutes at 37 °C without braking, and then the supernatant was aspirated; this wash cycle was repeated two times. Cells were suspended in 50 ml of final medium composed of 74% APF, 20% fetal calf serum, 5% antibiotic-antimycotic solution, and 1% sterile-filtered pseudocoelomic fluid, which was drained from adult worms. Periodically, cell viability was assessed with trypan-blue exclusion staining in a hemocytometer (Freshney, 1994). Cultures were adjusted to 1×10^5 cells/ml and maintained in 75-cm^2 cell culture flasks in an incubator at 37 °C and 5% CO_2. Culture supernatant was replaced with final medium on alternate days for 8 days.

CO_2 metabolism: Cells were incubated for 4 hours in APF without glucose. Next, cultures were centrifuged at 75*g*, and supernatants were aspirated. Cells were resuspended in APF with U-[^{14}C]glucose (0.00625 mCi/flask; 265 mCi/mmole, ICN Radiochemicals, Irvine, CA), placed in side-arm flasks with a suspended hyamine-hydroxide trap, and incubated at 37 °C in a water bath for 4 hours. Cultures were terminated by the addition of 5% trichloroacetic acid to the medium and then refrigerated overnight. Radioactivity trapped by hyamine hydroxide was counted in a Beckman LS6000 scintillation counter, corrected for quench, and converted to disintegrations per minute (DPM) (Allen and Danforth, 1984). Cell cultures were rinsed onto tared filter paper, dried at 30 °C for 24 hours, and weighed at room temperature.

MTT reduction assay: The tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) is reduced in living cells by NADH-dependent dehydrogenase activity. Cells, which were suspended in the final culture medium, were dispensed into 24-well plates each day for a week. MTT (50 µl, Sigma Chemical Co., St. Louis, MO) was added to each well, and plates were wrapped in aluminum foil and incubated for 37 °C for 4 hours. After the addition of 0.2 ml of dimethyl sulfoxide, cells and media were removed and read immediately at 570 nm in a spectrophotometer (Comley et al., 1988; Freshney, 1994).

Statistics: Data from metabolic indices among days were subjected to analysis of variance and Duncan's multiple-mean comparisons (SAS Institute, Cary, NC).

Results

Cell cultivation and morphology: The majority of dispersed reproductive cells of both male and female worms remained viable for 7 days in culture, although there were progressive signs of degeneration and dedifferentiation, such as decrease in size, loss in morphologically distinct features, and cell death. Evidence of cell replication, i.e., increased cell concentration or appreciable cellular adhesion, did not occur in cultures of gonadocytes from either sex.

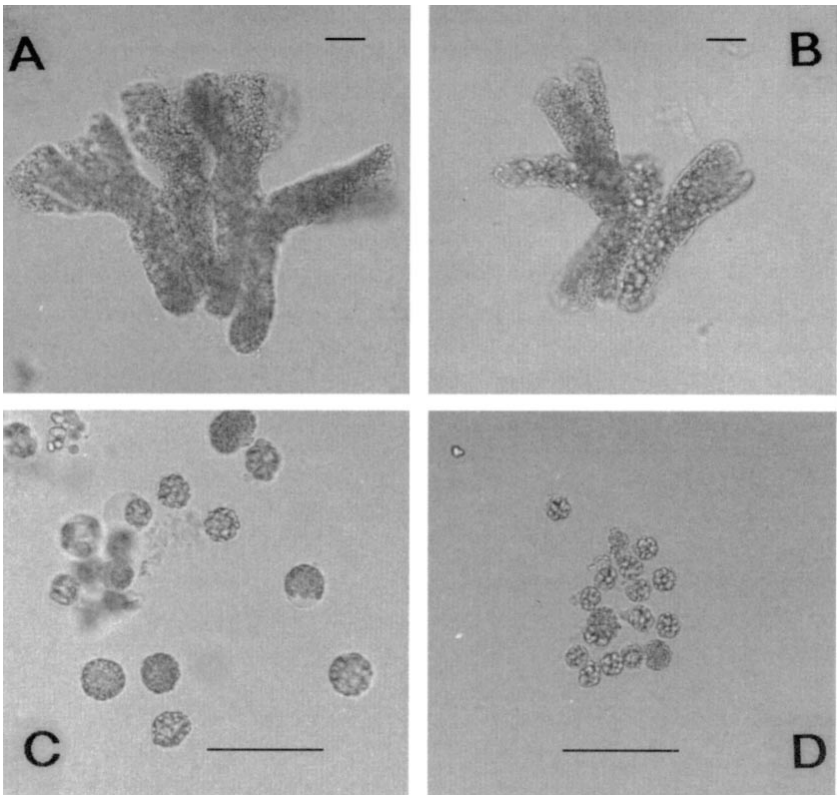

FIG. 1. *Ascaris suum* reproductive germinativum after enzymatic dispersal. Bars are 20 µm. A) Four cells from the ovary on day 1 of cultivation. B) Four cells from the ovary on day 8 of cultivation. C) Several cells from the testes on day 1 of cultivation. D) Several cells from the testes on day 8 of cultivation.

On the first day of cultivation, ovarian germinative cells were conical with a fimbriated edge (500 × 250 µm; Fig. 1A) and viability exceeded 92%. After 7 days, the cells were columnar with reduced or no fimbriation and 35% smaller (Fig. 1B); viability was 55%.

The testicular germinative cells originally were uniform, small (70 µm), and spherical with a crenulated surface (Fig. 1C). Viability was 86%. After 7 days in culture, the cells had shrunk by 30%, accentuating the crenulations (Fig. 1D), and viability was reduced to 58%.

CO_2 metabolism: The rate of conversion of radiolabelled glucose to $^{14}CO_2$ decreased to 55% of initial values in the ovarian cell cultures after 4 days in culture but maintained that level for the last 4 days of culture (Fig. 2). In contrast, $^{14}CO_2$ production by the testicular cells increased by 20% on day 3 and then progressively decreased for the remainder of the cultivation period until $^{14}CO_2$ production was 50% of initial values.

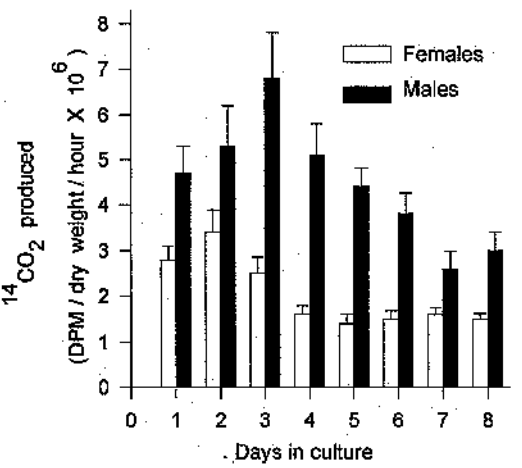

FIG. 2. Metabolic activity of *Ascaris suum* reproductive cells each day during 8 days of cultivation, as indicated by disintegrations per minute (DPM) obtained from $^{14}CO_2$ recovered during a 4-hour period of incubation in culture medium containing U-[^{14}C]glucose. Brackets above bars indicate SE for four replications ($P \leq 0.05$).

MTT reduction assays: Metabolism as indicated by the reduction of MTT progressively declined over the cultivation period in germinative cell cultures from both sexes (Fig. 3). Absorbencies in media from ovarian and testicular cells were both 60% lower at the termination of the culture when compared to initial values.

Successful maintenance of germinative cells for 8 days critically depended on the development of procedures in two areas. The first was the optimal concentration and duration of collagenase treatment. Enzymatic digestion to the point of complete cellular dispersion reduced viability. Hence, after optimal digestion (30 minutes at 0.25 mg/ml) undispersed cells remained and had to be filtered out. Effective filtering limited the amount of tissue that could be processed during a single digestion to six reproductive tracts.

Secondly, survivorship of the cells beyond a few days was found to be significantly improved by inclusion of pseudocoelomic fluid and a high percentage of fetal calf serum. Because this nutrient-rich medium is subject to bacterial and fungal growth, and the tissues are derived under aseptic but not sterile conditions, repeated changes in the medium were required to maintain microbicide potency. Fungi were a frequent contaminant without this regimen.

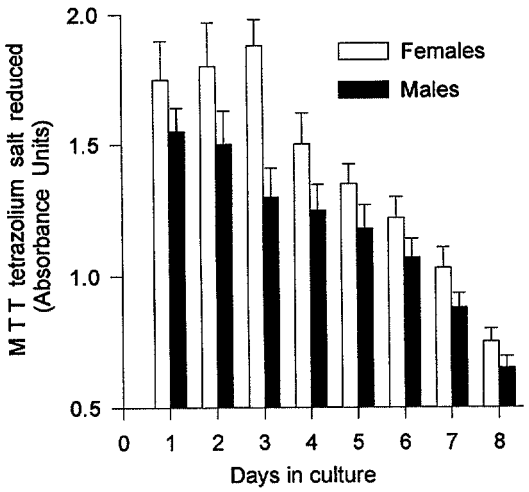

FIG. 3. Absorbance readings of culture medium and *Ascaris suum* reproductive germinative cells in multiwell plates incubated with MTT for 4 hours on each of 8 days of cultivation. Brackets above bars indicate SE values for eight replications per sex each day ($P \leq 0.05$).

DISCUSSION

In organ cultures of nematode female reproductive tracts, Doran (1961) and Ogura (1989) each described an increase in cell size, suggesting that association with the basement membrane might be required to prevent dedifferentiation and size reduction experienced with dispersed cells. Matrix interaction, cell-cell interactions, and soluble factors have been defined as critical components in many systems (Freshney, 1994). Propagation of nematode germinal cell lines might be dependent on identification of these components.

The similarity in the temporal profiles of the two independent indices of metabolic function, glucose metabolism and MTT reduction, suggests that the first 3 days of cultivation are relatively constant in physiological processing. The progressive decline in these parameters after the third day coincided with morphological dedifferentiation and degeneration. Therefore, under these cultivation conditions, the initial 72 hours appear optimal for experimental manipulations or definition of metabolic events.

Maintenance of gonadal cell cultures from *A. suum* for several days could provide a system to define several aspects of nematode physiology including regulation of ecdysteroid production (Fleming, 1985), steroidal synthesis and manipulation (Chitwood, 1987), physiology of senescence (Rothstein, 1980), and control of gametogenesis (Gamble et al., 1995). Adaptation of our techniques to other nematode tissues also might facilitate production of nematode antigens, testing of chemical and biological control agents, and definition of biochemical pathways. Control of the cellular environment offers the investigator of nematode biology valuable opportunities in basic and applied research.

LITERATURE CITED

Allen, P. C., and H. D. Danforth. 1984. The effects of *Eimeria acervulina* infection on the metabolism of

chick duodenal tissue. Veterinary Parasitology 14:105–115.

Bird, A. L., and J. Bird. 1991. The structure of nematodes. 2nd ed. San Diego, CA: Academic Press.

Chitwood, D. J. 1987. Inhibition of steroid or hormone metabolism or action in nematodes. Pp. 122–130 *in* J. A. Veech and D. W. Dickson, eds. Vistas on nematology. Hyattsville, MD: Society of Nematologists.

Comley, J. C. W., M. J. Rees, and A. B. O'Dowd. 1988. The application of biochemical criteria to the assessment of microfilarial viability. Tropical Medicine and Parasitology 39:456–459.

Del Castillo, J., and T. Morales. 1967. The electrical and mechanical activity of the esophageal cells of *Ascaris lumbricoides.* Journal of General Physiology 50:603–629.

Doran, D. J. 1961. In vitro survival of germinal cord tissue of *Ascaris lumbricoides* var. *suum.* Journal of Parasitology 47:890.

Fleming, M. F. 1985. *Ascaris suum:* Role of ecdysteroids in molting. Experimental Parasitology 60:207–210.

Freshney, R. I. 1994. Culture of animal cells. A manual of basic techniques. 3rd ed. New York: Wiley-Liss.

Gamble, H. R., R. H. Fetterer, and J. F. Urban. 1995. Reproduction and development of helminths. Pp. 289–305 *in* J. J. Marr and M. Müller, eds. Biochemistry and molecular biology of parasites. London: Academic Press.

Noble, E. R., G. A. Noble, G. A. Schad, and A. J. MacInnes. 1989. Parasitology. The biology of animal parasites. Philadelphia, PA: Lea and Febiger.

Ogura, N. 1989. Cell culture of nematodes. Pp. 263–266 *in* J. Mitsuhashi, ed. Invertebrate cell system applications. Vol. II. Boca Raton, FL: CRC Press.

Rothstein, M. 1980. Effects of aging on enzymes. Pp. 29–46 *in* B. M. Zuckerman, ed. Nematodes as biological models. Vol. 2. Aging and other model systems. New York: Academic Press.

Von Ehrenstein, G., and E. Schierenberg. 1980. Cell lineages and development of *Caenorhabditis elegans* and other nematodes. Pp. 2–71 *in* B. M. Zuckerman, ed. Nematodes as biological models. Vol. 1. Behavioral and developmental models. New York: Academic Press.