PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 146, 299–301 (1974)

Effects of Mitomycin C on Sex of *Aphelenchus avenae* (Nematoda) in Axenic Culture[1] (38092)

EDWARD J. BUECHER, EVANGELINE YARWOOD, AND EDER L. HANSEN
Clinical Pharmacology Research Institute, 2030 Haste Street, Berkeley, California 94704

Sex expression of *Aphelenchus avenae* can be influenced by environmental or chemical factors. In a strain of *A. avenae* which develops parthenogenetically at temperatures up to 28°C in axenic medium but forms males at 30°C (1), we recently showed that the masculinizing effect of increased temperature is critical to the early larval stage and is blocked in the presence of mitomycin C, a DNA inhibitor (2). We have now investigated the effect of introducing mitomycin C at different stages of growth with a view to understanding the control of this morphogenesis.

Materials and Methods. Aphelenchus avenae Bastian was maintained at 23°C in glass wool columns containing CE medium, i.e., chemically defined medium CbMM (Grand Island Biological Co., Grand Island, NY) supplemented with 25% chick embryo extract and 10% human serum (1). Experiments were started by placing mature females into BPI watch glasses containing CE medium or CE medium with mitomycin C (Calbiochem) at 5, 10, 20, or 100 μg/ml and incubated at 27 or at 30°C. Incubators were controlled ± 0.3°C. At the proper stage in development, ten eggs or larvae were inoculated into duplicate aliquots of 0.15 ml of medium in stoppered 10 × 75 mm tubes and held at 27 or 30°C for further observation. After the required interval of exposure, eggs or larvae were removed either from CE medium with mitomycin C to CE medium, or from CE medium to the mitomycin C medium. Development was observed at 7–10 days and populations at 21 days. Development and morphological details were observed also on individuals in hanging drop cultures.

Results. As indicated by the number of adults at 7–10 days, growth and development was increasingly inhibited by increased dosages of mitomycin C. In CE medium, 100% of the inoculum developed, yielding normal females at 27°C and normal males at 30°C, as found previously (1). With 5 μg/ml mitomycin C only 55% of the inoculum developed to adults. At 30°C, 33% of these adults were females, the rest males. The females expelled eggs but tended to rupture at the vulva. At 27°C all adults were females with abnormally protruding vulvas, also tending to rupture after laying a few eggs. With the level of mitomycin C increased to 10 μg/ml the male-inducing effect of 30°C temperature was completely blocked. At both temperatures, 30% of the inoculum developed to adults which were all females; the females were abnormal in that vulva was lacking in 30°C cultures, and improperly formed at 27°C, or in some cases, a normal-appearing vulva was present but the fourth molt cuticle was loosened but not shed. Embryos developed within some of these females and larvae hatched but were entrapped, leading eventually to *endotokia matricida.* A similar effect was obtained at 20 μg/ml. With mitomycin C at 100 μg/ml development was completely inhibited. Eggs inoculated into this medium hatched but larvae did not grow beyond the newly hatched L-2 stage.

The specific stage of development at which mitomycin C blocked formation of males at 30°C was examined by a series of

[1] Supported by U.S. Public Health Service Grant A1-07359 TMP from the Institute of Allergy and Infectious Diseases.

transfer experiments. Specific stages were transferred from medium containing 20 μg/ml mitomycin C into CE medium, and vice versa. The results are summarized in Table I. The first column of the table shows that mitomycin C prevented development of males if the larvae remained in the mitomycin C after the L-2 stage. The occurrence of 10% females among those removed from mitomycin C medium as eggs can be explained on the basis that a short and variable portion of the L-2 stage occurs within the egg prior to hatching.

The reverse transfer from CE medium into mitomycin C medium (second column of Table I) showed that larvae had to be exposed to the mitomycin C before the late L-2 stage in order to block development of maleness. Larvae introduced as third stage grew to males that were smaller than normal, possessing an incompletely developed bursa. Larvae transferred as fourth stage developed into normal males.

The extent of the abnormality of development in females at 30°C with 20 μg/ml mitomycin C was observed in hanging drop cultures individually inoculated with eggs. Elongation of the ovary appeared to be normal but by the fourth larval stage it became obvious that the vulval area was developing abnormally or not at all. Eggs formed by the adult female then became entrapped and frequently hatched *in utero*. As many as five developing embryos and larvae were observed within one adult, causing abnormal posterior distention of the uterus. Egg shells remained in the uterus and sometimes were pushed to the posterior end by other developing eggs. When such females with trapped, embryonated eggs were decapitated and placed in CE medium at 27°C, the eggs hatched and the larvae escaped and developed to normal, reproducing females.

Discussion. Our findings suggest that a flexible and sensitive control system for sex exists in *Aphelenchus avenae*. Except for some inhibition in development of the secondary sexual organs, the vulva and bursa, mitomycin C blocked sex reversal without interfering with gonadogenesis. In addition, surgically released larvae developed normally when transferred into CE medium, showing that the inhibitor, while preventing male formation and interfering with development of the vulva, did not prevent embryogenesis in parthenogenetic females.

Transfer experiments into and out of mitomycin C point to sensitivity of the early L-2 stage; exposure of the early L-2 stage and prehatched L-2 to the inhibitor affected development of males at 30°C. Earlier work on the temperature influence on sex development (1) showed that here too the L-2 stage was particularly affected. Unless eggs deposited at 30°C were left at that high temperature until the late L-2 stage, they would develop as females. Inversely, eggs transferred from a low temperature to 30°C after the still unhatched larvae had molted to the L-2 stage, would not become males.

TABLE I. Sex of *Aphelenchus avenae* Resulting from Incubation at 30°C in 20 μg/ml Mitomycin C at Different Stages of Development.

Stage at time of change of medium	Sex of adult after change of medium	
	Mitomycin C → CE medium	CE medium → mitomycin C
Egg	M and F[a]	F[c]
Hatched L-2	M and F[b]	F[c]
Late L-2—early L-3	F[c]	M[d]
Late L-3	F[c]	M[d]
Late-4	—	M[e]

[a] 90% Males, 10% females.
[b] 50% Males, 50% females.
[c] Abnormal females; vulva underdeveloped or absent.
[d] Abnormal males; reduced size and underdeveloped bursa.
[e] Normal males.

Since mitomycin C is a DNA inhibitor these findings suggest a need for new DNA synthesis at the L-2 stage if males are to develop at 30°C. This conforms to the postulate by Mittwoch (3) that maleness can be attributed to a burst of mitosis at an early critical stage. Other experiments (unpublished) in which there was prevention of male development at 30°C by X-irradiation of newly hatched larvae with 30,000 R, also suggest the involvement of *de novo* DNA synthesis.

Previous efforts to alter sex of *A. avenae* with stimulators or inhibitors other than mitomycin C (2), and more recently with serotonin and isoproterenol, were unsuccessful. The molecular basis for sex differentiation should however ultimately be found, most likely by correlating the effect of biochemical agents and laser microbeam surgery of particular anatomical regions controlling morphogenesis (4). Our findings suggest that the involvement of *de novo* DNA synthesis at the second larval stage must be considered in such studies.

Summary. Low levels of DNA inhibitor mitomycin C blocked a 30°C male induction system in the plant parasitic nematode *Aphelenchus avenae.* Instead, parthenogenetic females developed. These frequently lacked a fully formed vulva, but produced normal embryos. The stage at which this inhibitor was effective was the early second larval stage.

1. Hansen, E. L., Buecher, E. J., and Yarwood, E. A., Nematologica 18, 253 (1972).

2. Hansen, E. L., Buecher, E. J., and Yarwood, E. A., Nematologica 19, 113 (1973).

3. Mittwoch, U., Nature (London) 221, 446 (1969).

4. Samoiloff, M. R., Science 180, 976 (1973).

Received Jan. 2, 1974. P.S.E.B.M., 1974, Vol. 146.