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Effect of 5-Fluorodeoxyuridine on Reproduction and Aging in a Strain of Free-living Nematodes, *Rhabditidae tokai*

Kenshi SUZUKI, Yoko MORIYA and Naoaki ISHII

Department of Molecular Biology, School of Medicine, Tokai University

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To obtain age-synchronized populations of a strain of free-living nematodes, *Rhabditidae tokai*, effects of 5-deoxyuridine on reproduction and age-related survival were investigated. The following results were obtained. (1) Eggs and early larvae were much more sensitive to this drug than late larve or adults with respect to the killing effect. (2) At low concentrations (10^{-5} to 10^{-4} M), where no significant killing effect was observed, 5-deoxyuridine did not inhibit egg production, but the eggs produced in the presence of the drug were almost unhatchable. (3) In long-range observations, it was found that 5-deoxyuridine reduced significantly the life span of the nematode. It was concluded that 5-deoxyuridine is not an adequate inhibitor of reproduction in aging research, while the results postulated an interesting problem concerning egg formation or fertilization.

(Key Words: Nematode, 5-Fluorodeoxyuridine, Reproduction, Aging)

INTRODUCTION

The free-living nematodes have now been understood as very useful experimental animals for various fields of biology including aging research (1), because they have a simple structure and a relatively short life span (10). However, a problem which remains to be solved for this purpose is the establishment of a method to obtain a large number of worms with the same age, especially in studies at the biochemical or molecular level. In general, the nematode has a short generation time relative to its maximum life span. Therefore, it is easy to obtain a large number of worms in glassware as long as there is no concern for synchronization.

Various ideas have been proposed and examined to overcome this difficulty. One of these ideas was the use of inhibitors of DNA synthesis proposed first by Gershon (2). He examined 5-fluorodeoxyuridine (FUdR), aminopterine and hydroxyurea and stated that they are useful inhibitors for synchronization of *Turbatrix aceti*. However, his results were not reproduced by *T. aceti* and by *Caenorhabditis briggsae*. In both cases the life span was greatly reduced by the addition of FUdR or aminopterine to the culture medium (4). Another idea was the use of filtration to eliminate newly produced eggs and larvae (9). By using this technique, Tilby and Moses were able to produce age-related survival curves of a hermaphroditic nema-

Kenshi SUZUKI, Department of Molecular Biology, School of Medicine, Tokai University, Bohseidai, Isehara, Kanagawa 259–11, Japan

tode, *C. elegans*. There is still another method of synchronization. Hirsch and Vanderslice isolated a number of temperature-sensitive, developmental mutants in *C. elegans* (3) and one of them was used by Klass in studying some environmental factors influencing the life span (5).

To study the basic mechanism of aging, we also isolated a strain of free-living nematodes about three years ago (*Rhabditidae* sp.: tentatively called as *R. tokai*). Since, unlike *C. elegans* and *C. briggsae*, the strain is bioecious, we were able to determine age-related survival curves of unmated males and females (8). However, a much larger number of age-synchronized worms will be needed to advance studies of the molecular level. The study which will be presented in this report was carried out to determine the effect of 5-deoxyuridine on reproduction and aging in *R. tokai*. It was found that FUdR (3×10^{-5} M) does not completely prevent the production of eggs, and that the eggs produced in the presence of FUdR have a very low hatchability. However, it was also found, that FUdR reduces the life span significantly.

MATERIALS AND METHODS

The nematode used in this study was described previously (8). In brief, it is bioecious with a sex ratio (male to female) of about 1 : 15. The average length of females is 830 μm and that of males is 580 μm. A female produces about 100 eggs and, at 20°C, they hatch in 24 h to become larvae. The larvae mature in five days and produce eggs of the next generation. The mean life span of virgin females at 20°C was estimated to be about 100 days.

The method of cultivation was also described in a previous paper (8). Throughout this study, worms were cultivated on agar plates containing buffered salts and 1.7 per cent agar. *Escherichia coli* cells were given as a food source. Usually less than ten worms were cultivated on a plate (1.5 cm in diameter).

Eggs were collected and washed according to the method of centrifugation in a sucrose gradient (8). Handling and observation of worms were carried out under a microscope at a low magnification.

FUdR used was the product of the Sigma Corporation, U.S.A.

RESULTS

Killing effect of FUdR

The killing effect of FUdR was checked at concentrations ranging from 3×10^{-5} to 3×10^{-2} M using 10 to 30 virgin females on three plates. Eggs were collected and plated on the agar medium of master plates. If the plates were kept at 20°C, the eggs hatched to larvae on the next day and the larvae grew to maturation in five days, when eggs of the next generation began to appear as a result of free mating. In this study, eggs and immature worms started from the same population were used. Portions of the initial eggs and the larvae derived therefrom were picked up on new plates containing various concentrations of FUdR. If males were contaminated (the sex could not be distinguished until the third day after hatching), the plates were discarded. On the sixth day after hatching, several young adult males derived from the same population of eggs were added to each plate to examine the

reproduction ability of females. Observations were continued for 21 days after hatching. The results are summarized in Table 1.

Table 1. Killing effect of FUdR on *R. tokai*

Concentration (M)	Measurements[1]	Age of nematodes transferred to FUdR plates (days)					
		0 (eggs)	1	2	3	4	5
3×10^{-2}	(a)	17	16	15	17	18	14
	(b)	0	0	9	13	10	14
	(c)	0	0	60	76	56	100
3×10^{-3}	(a)	25	16	18	19	19	10
	(b)	0	0	17	15	19	10
	(c)	0	0	94	79	100	100
3×10^{-4}	(a)	17	30	19	20	14	18
	(b)	10	20	18	14	13	17
	(c)	59	67	95	70	93	(94)[2]
3×10^{-5}	(a)	10	25	19	18	13	18
	(b)	10	16	15	15	13	18
	(c)	100	41	(79)[2]	83	100	(100)[2]

1) The symbols in colum *Measurements* represent; (a) number of females tested, (b) number of survivors on the 21st day after hatching, and (c) per cent survival (b/a).
2) The parentheses indicate the cases where eggs were produced on any of three plates.

When eggs were directly plated on FUdR plates (zero day), they hatched almost normally at all concentrations examined. However, the larvae on plates containing 3×10^{-2}or 3×10^{-3} M FUdR remained very small and died before the 21st day. Of course reproduction did not occur. At 3×10^{-5} M, larvae grew normally and survived for 21 days, but they could not produce eggs, i.e. they were sterile. The situation was almost the same when newly hatched larvae (1st day) were transferred to FUdR plates. There was a tendency for larvae to become more resistant to FUdR as age advanced. In fact the fifth day larvae (almost mature adults) could mostly resist FUdR and survive for 21 days, although they lost the capability to produce eggs.

When the concentrations of FUdR were low (3×10^{-4} or 3×10^{-5} M), rare females produced eggs (on one plate out of three). Interestingly, however, these eggs were completely unhatchable (these cases are indicated by parentheses in Table 1). At concentrations of less than 3×10^{-5} M, reproduction proceeded normally and on control plates where FUdR was omitted, reproduction also proceeded normally. It may be concluded, therefore, that FUdR at relatively low concentrations blocks reproduction either by inhibiting the formation of eggs or by producing unhatchable eggs without a drastic killing effect. It should be noted that, since mating and the following processes were carried out in the presence of FUdR, the defects described above can not solely be attributed to the effect of FUdR on females. Furthermore, we could not distinguish whether the sterilizing effect of FUdR is

due to its effect on mating or to its effect on certain processes subsequent to mating.

To analyze the results in Table 1 in more detail, experiments were performed in which normal males and females grown in the absence of FUdR were crossed on FUdR agar or on agar without FUdR; in the former case each female was picked up on agar without FUdR and in the latter case each female was picked up on FUdR agar. The concentration of FUdR was 3 x 10^{-5} M. In both cases, similar results were obtained, i.e. in experiments in which FUdR was present only at the time of mating, 25 females out of 30 produced the normal number of eggs (about 100 eggs per a female), but they were unhatchable. In experiments in which FUdR was present during the post-mating period, 36 females out of 40 produced the normal number of eggs, but only a few (less than five per cent) of them could hatch and the rest was unhatchable. These results suggest that FUdR has very little effect on the frequency of mating and the number of eggs produced per a female, but it inhibits in some way the formation of normal, hatchable eggs when it is present at the time of mating or in post-mating culture medium. Therefore, the history of pre-mating exposure of males and females to FUdR appears not to influence greatly the inhibitory effect of FUdR.

Sex differences in the sensitivity to FUdR are still obscure at the present state of the investigation. For example, crosses were performed between FUdR-treated females and normal males or *vice versa*. After mating females were separately transferred to plates free of FUdR. In every case, crosses were performed on agar without FUdR. The results showed that the frequency of egg-laying females was decreased to 25 to 50 per cent of the control and the hatchability of produced eggs varied form one experiment to another for unknown reasons.

Effect of FUdR on the life span

The data presented above show that FUdR is effective in preventing reproduction of *R. tokai*. However, the drug may not be used in aging research unless it does not affect the life span of the nematods. To check this point, fifty female larvae were picked up on ten plates containing FUdR and the plates were kept at 30°C. Surviving nematodes were scored every two to three days. The results are shown in Fig. 1.

It is seen that at concentrations of 3×10^{-4} and 3×10^{-5} M FUdR greatly reduced the longevity. The half-life of the nematodes on control plates was 58 days, whereas that of the nematodes on FUdR-plates was 18 days and 33 days at the concentrations shown above, respectively. Therefore, in agreement with the results of Zuckerman (10), FUdR seems not to be an adequate inhibitor of reproduction for aging research.

DISCUSSION

The principal effect of FUdR on cells is believed to be the inhibition of DNA synthesis (6). Thus, it is sometimes used as a cancerostatic agent.

In nematodes, DNA synthesis is important from two aspects: one is in post-embryonic cell division and another is in the formation of germ cells. In *C. elegans*, for example, a newly hatched larvae has about 550 cells and an

adult hermaphrodite has about 810 cells (7), indicating that about 260 cells should be formed during post-embryonic growth. If the formation of these cell types is inhibited, growth as well as reproduction must be inhibited. Although post-embryonic cell division has not yet been identified in our strain, the fact that the early larvae exposed to FUdR at concentrations of 3×10^{-3} or 3×10^{-2} M remained very small until they died before the 21st day after hatching may suggest that post-embryonic cell division is also necessary in *R. tokai* for maturation.

The present results showed that low concentrations of FUdR are effective in preventing reproduction either by inhibiting production of eggs or by producing unhatchable eggs, although it is not known yet when and how the germ cell lines are developed in *R. tokai*. When animals were exposed to FUdR for a long period, the life span was shortened significantly. Therefore, the animals aged in the presence of FUdR may not be compared with animals aged in the absence of the drug. Similar results were reported by Kisiel, et al. for *C. elegans* and *T. aceti* (4).

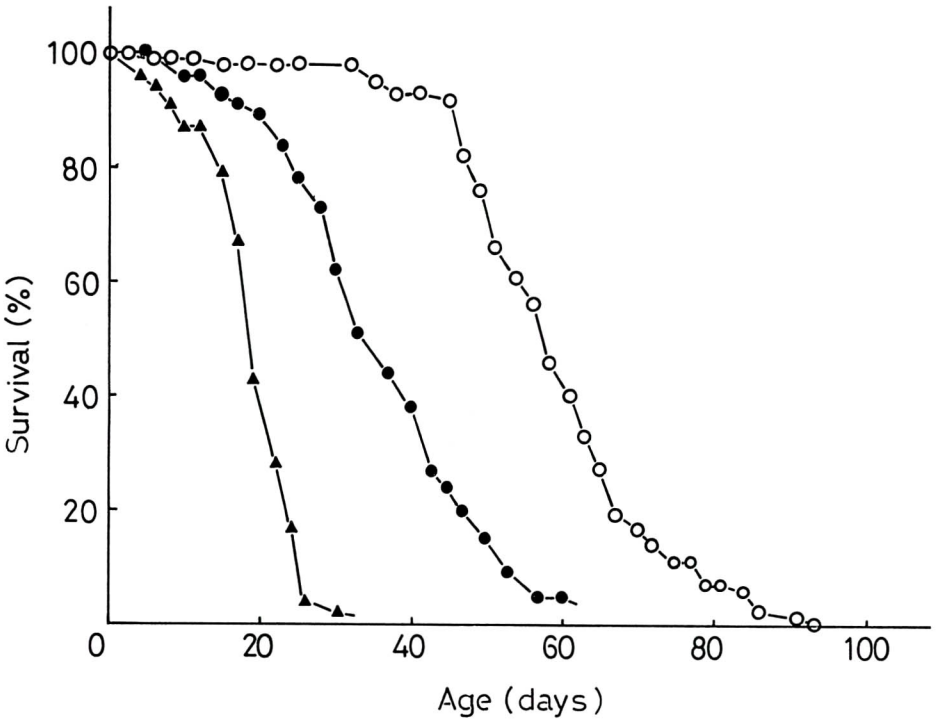

Fig. 1 Effect of FUdR on the longvity of *R. tokai*. Fifty newly hatched female larvae were cultivated on ten plates containing FUdR at 30°C. Concentrations of FUdR: 3×10^{-5}M (filled circles), 3×10^{-4}M (filled triangles), and OM (control) (open circles).

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After preparation of this manuscript a paper of Mitchel, D.H., Stiles, J.W., Santelli, J. and Sanadi, D.R. appeared in J. Geront. 34: 28—36 (1979). The results show that 5-fluorodeoxyuridine at a concentration of 4×10^{-4}M could inhibit reproduction of *C. elegans* without affecting its life span if the drug was used under carefully controlled conditions. The difference would be due either to the difference in strains or some other reasons, which is now under investigation.

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