



MAINTENANCE OF ADULT ONCHOCERCA VOLVULUS IN VITRO¹

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1. INTRODUCTION

Living adult *Onchocerca volvulus* free from host tissue are needed for various biological, biochemical and immunological investigations. Recently Schulz-Key et al. (1977) have succeeded in isolating living worms from nodules by careful digestion of the nodular tissue with collagenase. With some modifications of this technique we have now been able to isolate living worms for temporary maintenance in vitro. This opens the way for work on the following lines:

- (1) Studies on the biochemistry, metabolites, and enzymes of the parasite with a view to understanding the mechanisms of its pathogenicity, and to elucidating the mode of action of chemotherapeutic compounds upon its metabolism;
- (2) Screening substances for chemotherapeutic action in vitro;
- (3) Keeping worms in the laboratory for experimental transplantation to laboratory animals;
- (4) Providing a reliable transport system for adult parasites when supplying other institutions with living material for further research.

2. DESCRIPTION OF THE ISOLATION OF WORMS FROM NODULES, USING COLLAGENASE

The parasitic material for this investigation came from 29 patients in southern Togo. For the in vitro culture of worms and for transplantation experiments it was necessary to use an aseptic technique throughout the process in order to avoid contamination of the culture solution. Eighty-seven nodules were excised in regional hospitals and transported to our laboratory at room temperature in containers filled with sterile medium 199, with Hank's salts, glutamine, sodium bicarbonate, and 0.16 mg/ml gentamicin.

2.1 Preparation of the nodules

To avoid damaging the worms before digestion only the obviously loose enveloping layers of the nodules were carefully removed with scissors under the dissecting microscope. Conglomerate nodules were not separated for it was often found that loops of female worms were in connexion with neighbouring sections.

2.2 Concentration of the collagenase solution

For the digestion of the nodular tissue the concentration of the collagenase (Boehringer, No. 103586) used on the first day of incubation was varied individually, depending on the size of the nodule and the thickness of its wall, from 0.3 to 1.0%. Small nodules and those with visible worms were treated with lower concentrations of collagenase. As soon as the worms or sections of them became transparent, human serum (from cases negative or positive for *O. volvulus*) was added at an increasing concentration of 15-30%. For small nodules weighing less than 0.5 g it was opportune to add the human serum right from the beginning. The collagenase solution was changed for the first time after the nodules had been incubated for 24 hours, and, depending on the progress of digestion, the concentration of the enzyme was usually considerably reduced on the second day. As the cuticular structures of the parasites may themselves contain collagen it was necessary to remove the worms from the collagenase solution as soon as possible. Worms which were still not completely isolated could usually be kept in culture medium without enzyme. The previously initiated digestion tended to continue spontaneously and the relics of human tissue still adhering to the surface of the worms usually dissolved after a few days and could then be removed with tweezers or rinsed off with fluid from a pipette.

2.3 Temperature for digestion

The temperature was an essential factor influencing the time of digestion and the viability of the worms. At a temperature of 35°C in a waterbath the digestion was accelerated, but the process of digestion had to be controlled constantly to avoid too long an exposure of free worms to the enzyme solution. Nodules kept at room temperature (28-30°C) needed at least 24-48 hours before worms could be isolated. Even from large nodules it was possible to obtain living worms after four to five days of incubation at room temperature. However, with large nodules it was not possible to use the higher temperature for the worms usually died after two days of incubation. The most practical method was to maintain the nodules at 35°C during the first day of incubation and then to keep them at room temperature subsequently.

2.4 Definition of "living worms"

Adult female *O. volvulus* are sedentary parasites closely embedded in the nodular tissue. It is presumed that they are immobile in the host - perhaps with the exception of the anterior part which may move during the process of mating. Contrary to free living filariae, such as *Litomosoides carinii* or the male worms of *O. volvulus*, female worms of this species often remained motionless in the culture medium and only showed movements after certain stimuli. Often only some loops of the worms showed slow and rather ponderous movements. For this reason it was necessary to find criteria additional to movement for the definition of "living worms" among the females.

Active movements. The ability to make active movement was of course the best indication that a female worm was alive. Living females often reacted immediately by making movements when they were transferred into a less suitable medium, such as saline or isotonic phosphate buffer (pH = 7.2). Movements could also be provoked by transferring the worms to a cooler medium or by a few seconds shaking of the test-tubes in which they were maintained.

Living worms showed regular contractions of the walls of the uterus in vitro. In those parts of the uterus which contained oocytes the uterine contractions were often revealed by the to and fro movements produced in these cells. The direction of their movement would change sometimes after seconds, sometimes only after several minutes. Usually the direction of movement of the floating oocytes in both branches of the uterus was the same, but antagonistic movements were sometimes observed. In those parts of the uterus which were closely packed with further developed embryos these movements could not be found. Contractions of the walls of the uterus were often the last signs of life that could be detected in moribund females.

Signs of recent death in female worms. Evidence of recent death in totally motionless females was sometimes provided by the presence of live microfilariae still moving slowly in the uterus.

2.5 Yield of living worms after collagenase digestion

Even with careful use of the collagenase technique most of the worms originally living in the nodules could not be isolated alive (Table 1). From 87 nodules of different sizes and consistencies only about 32% of the male and 23% of the female worms were viable enough after isolation to survive for at least three days in culture. Some worms which seemed to be motionless and dead at the first viewing recovered in a few hours after being transferred to the culture medium containing 30% of human serum.

Small nodules with a weight less than 1 g, and the small satellites of bigger nodules, provided the highest yield of living worms suitable for the maintenance in vitro.

About two-thirds of the female worms isolated intact contained uterine microfilariae. However, the majority of those females without embryos survived the digestive process, and small, probably young females showed the best viability.

3. MAINTENANCE OF THE WORMS IN VITRO

3.1 Culture conditions

For the maintenance of the worms, in vitro medium 199 was used at pH 7.2, as indicated above (see section 2). The worms were kept in sterile 10-ml glass tubes filled with 2 ml of medium, and loosely covered with aluminium foil. Human serum from Onchocerca positive or negative patients was added at a concentration of 15 or 30%. Some of the test-tubes contained human albumin (Boehring OBDA 44) at a concentration of 15% instead of the human serum. No special gas phase was used.

The worms were kept at room temperature (28-30°C) or in a waterbath at 35°C. The culture medium was changed every day, and the worms were washed in sterile culture solution before being placed in the fresh medium. To assess the daily output of microfilariae the culture solution used during the previous 24 hours was centrifuged. Some of the worms which contained eggs only were checked daily for the development of embryos.

3.2 Survival of worms

There was no difference in the survival of worms kept at room temperature and those kept at 35°C. More than three-quarters of the worms kept in vitro survived for more than one week in the culture medium (Table 2). The median survival period was 10 days for male and 11 days for female worms. The longest time of survival was 26 days for a male and 32 days for a female.

The survival of 15 male and 12 female worms kept in culture medium containing human albumin instead of human serum was only seven days on average.

The addition of gentamicin as an antibiotic prevented any bacterial growth in the culture solution. The growth of fungi was found in only a few test-tubes.

The pH in the tubes containing male worms soon rose to alkaline values, while it remained constant for 24 hours in tubes containing female worms.

Decreasing viability of the worms was indicated by the slowing and cessation of movements and, in female worms, by the slowing of contractions in the wall of the uterus. In the third week of maintenance a slight detachment of the cuticle and a shrinking of the organic structures could be observed in some female worms.

3.3 Development of embryos and output of microfilariae

No development of embryos in the uteri could be observed under our conditions of maintenance. In culture medium containing human albumin only single microfilariae were released (nine females with microfilariae observed). In test-tubes containing serum of Onchocerca positive patients a single female worm could release up to 667 microfilariae per day, but the total number of microfilariae released in culture from a single female was never more than 1500 (seven females observed). In tubes with 15% serum from Onchocerca negative patients the maximum output was about 1000 microfilariae per day (five females observed); but in all worms the output of microfilariae decreased markedly after a few days in culture and again the total output of a single worm never exceeded 1500.

4. DISCUSSION

Since complete human cell culture media have become available from the pharmaceutical industry, the cultivation of filarial worms has become less laborious. For our studies medium 199 with Hank's salts was found to be a suitable basic culture medium containing not less than 52 ingredients.

In all in vitro trials with filarial worms the survival of the adults has been very limited. For Dirofilaria immitis a survival of only eight days was achieved by Taylor (1960). Weinstein & Sawyer (1961) succeeded in maintaining Dirofilaria uniformis for up to 21 days. Litomosoides carinii could be kept in simple Ringer solution with horse serum for up to two weeks (Hawking, 1954). Wenk et al. (1978) kept the same parasite in medium 199 with different sera for up to 23 days and were able to study the development and output of microfilariae in vitro.

In the present trials single male and female O. volvulus were able to survive for 26 and 32 days respectively, and the median life spans of the two sexes were 10 and 11 days. These relatively long survivals were obtained in spite of the potentially damaging process of collagenase digestion. The survival of the worms was sufficiently long for several investigations to be carried out. Living worms could be successfully transported from Africa to Europe, where special investigations, such as the blockage of pathways in energy metabolism by suramin (Walter & Schulz-Key, 1980) could be performed in vitro.

Wenk et al. (1978) found that when female L. carinii were maintained in culture nearly all eggs and developmental stages continued their development to microfilariae, which were then released in vitro within one week. In no female could they find evidence of a continued production of eggs. Nor did they observe any evidence of reinsemination which seems to be necessary for the continuous production of microfilariae in vivo (Johnson et al., 1974).

In our studies the investigation of the development of uterine microfilariae in vitro is not yet completed. Preliminary results show a relatively low output of microfilariae per day. Wenk et al. (1978) found that the release of L. carinii microfilariae in vitro may be accelerated or enhanced by serological factors. A serum factor has also been shown to suppress the production of blood-circulating microfilariae of Dipetalonema viteae in vivo (Haque et al., 1978). For the skin-dwelling microfilariae of O. volvulus no factors influencing the production and output of microfilariae in man are known, and no regulatory mechanisms influencing the production of embryos have been described. Therefore, it is not known whether the low output of microfilariae observed in our trials is within the range found in the human host, or whether it is very low on account of sub-optimal culture conditions or serological factors.

In our in vitro studies of adult O. volvulus continued development of eggs to microfilariae in utero was not observed. However, when isolated female worms containing only fertilized eggs were transplanted into laboratory hosts, their contained embryos continued to develop and microfilariae were released in vivo after about 10 days. This shows that the process of collagenase digestion did not inhibit embryogenesis.

5. SUMMARY

Eighty-seven Onchocerca nodules removed from 29 patients in southern Togo were digested with collagenase and the adult parasites were isolated for maintenance in vitro. Medium 199 with additional human serum served as culture medium. Forty-three (32%) males and 53 (22%) females were isolated in sufficiently good condition to survive on average 10 days (male worms) and 11 days (female worms). The maximum survival was 26 days for a male and 32 days for a female. The development of embryos in the females was not observed in vitro. The maximum output of microfilariae was about 1000 per day.

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RESUME

Quatre-vingt-sept nodules onchocerciens, prélevés sur 29 patients, dans le sud du Togo, ont été digérés à la collagénase, et l'on a isolé les parasites adultes pour les conserver in vitro. On a utilisé comme milieu de culture le Milieu 199, additionné de sérum humain. Quarante-trois (32 %) mâles et 53 (22 %) femelles ont pu être isolés dans des conditions suffisamment bonnes pour survivre, en moyenne, 10 jours (vers mâles) et 11 jours (vers femelles). La survie maximale a été de 26 jours pour un mâle et de 32 jours pour une femelle. On n'a pas observé de développement d'embryons chez les femelles in vitro. La production maximale de microfilariaes a été d'environ 1000 par jour.

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TABLE 1. NUMBERS OF MALE AND FEMALE ADULT *O. VOLVULUS* ISOLATED BY COLLAGENASE DIGESTION FROM 87 NODULES TAKEN FROM 29 PATIENTS IN SOUTHERN TOGO (WEST AFRICA). THE NUMBERS ISOLATED ALIVE, AND THOSE SUITABLE FOR USE IN VITRO CULTURE, ARE SHOWN ACCORDING TO THE WEIGHT OF THE NODULE

	Weight of nodule (grams)				
	0.2-0.5	0.6-1.0	1.1-2.0	> 2	0.2-10.0 (total)
No. of nodules examined	32	29	17	9	87
	No. of male worms (% of total)				
Total number of intact males	29	37	34	33	133
Intact males per nodule ¹	0.9	1.3	2.0	3.7	1.5
Isolated with signs of life ²	24 (83%)	22 (60%)	16 (47%)	24 (73%)	86 (65%)
Used for <u>in vitro</u> maintenance ³	15 (52%)	14 (38%)	2 (6%)	12 (36%)	43 (32%)
	No. of female worms (% of total)				
Total number of intact females	37	73	63	59	232
Intact females per nodule ¹	1.2	2.5	3.7	6.6	2.7
Isolated with signs of life ²	18 (49%)	43 (59%)	26 (41%)	11 (19%)	98 (42%)
Used for <u>in vitro</u> maintenance	12 (32%)	24 (33%)	13 (21%)	4 (7%)	53 (23%)

¹ Worms presumed to have been alive before digestion of the nodule.

² Includes living worms suitable for in vitro culture, living but damaged worms, and worms believed to have died during or just after the process of digestion, i.e. showing signs, of very recent death (cf. section 2.4).

³ Worms having survived in vitro for more than three days.

TABLE 2. SURVIVAL OF ADULT *O. VOLVULUS* IN VITRO AFTER BEING ISOLATED FROM NODULES BY COLLAGENASE DIGESTION

	No. of worms examined	Survival of worms (% of total)				Median survival in days
		Surviving at end of				
		Week 1	Week 2	Week 3	Week 4	
Male worms	43	34 (79%)	12 (28%)	5 (12%)	0	10
Female worms	53	39 (74%)	15 (28%)	8 (15%)	2 (4%)	11