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Moulting and exsheathment of the infective larvae of *Onchocerca lienalis* (Filarioidea) in vitro

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Summary

Fourth-stage larvae (L4) of the cattle filarial parasite *Onchocerca lienalis* were produced from third stage forms (L3) and maintained in vitro using a variety of culture media and feeder cell layers. Up to 50% of the L3 larvae moulted to the fourth stage in the presence of two jird *(Meriones unguiculatus)* cell lines, a monkey kidney cell line and also with Vero cells. Moulting took place 2–5 days after of the initiation of the cultures: These L4 parasites could be maintained in a healthy condition for up to 88 days, although no significant increases in size of these larvae were observed during the culture period. The medium giving the most promising results, both in terms of moulting and survival, was 199 supplemented with heat inactivated foetal bovine serum (20%) and glucose (2 mg/ml). The feeder layer cells could be replaced by fresh jird red blood cells. Moulting of parasites occurred in medium alone but the viability was reduced under these conditions.

Key words: *Onchocerca lienalis;* Filariodea; in vitro cultivation; feeder layers; infective larvae; moulting.

Introduction

Little is known of the development of filarial worms in their definitive hosts. This is especially true for *Onchocerca* species where the lack of suitable laboratory animal models and the diffculties of colonising the *Simulium* vector have made it difficult to produce infective larvae, nevertheless some success has been achieved with the latter procedure (Cupp et al., 1981). The long prepatent period of *Onchocerca* sp. makes this early invasive form of particular interest for chemotherapeutic, immunoprophylactic and immunodiagnostic studies.

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The attempts over many years to cultivate the vertebrate stages of filariae has met little success until recently (see review by Pudney and Varma, 1980). The moulting and exsheathment of infective *Dirofilaria immitis* larvae in vitro was reported by several authors (Taylor, 1960; Sawyer, 1963, 1965; Yoeli et al., 1964; Wong et al., 1980, 1981). Taylor (1960) co-cultivated the larvae with host tissue, and Wong et al. (1980, 1981) used several established cell lines from rodent, canine, bovine and porcine tissues to obtain moulting. Wong et al. (1980, 1981) also observed moulting of *Brugia pahangi* in the presence of cell lines from dogs, pigs and cattle, although Chen and Howells (1979) obtained moulting in vitro of this parasite in the presence of canine sarcoma cells only after an initial in vivo stimulation; third stage *B. pahangi* derived directly from mosquito vectors did not moult in their system. Tanner (1981) showed, with *Dipetalonema viteae* infective larvae, that an irradiated hamster kidney cell feeder layer could replace prior in vivo stimulation allowing growth and development up to the third moult in culture. Further growth of *D. viteae* through the fourth stage to the final moult, however, could only be obtained in vitro following a 6-day period in vivo. Similar achievements to these latter studies were reported by Lok et al. (1984a), Devaney (1985) and Lok et al. (1984b) working with *D. immitis, D. immitis* and *Onchocerca* sp., respectively.

Considerable progress has been made by Mak (1983) working on *B. pahangi* when he maintained infective larvae and reared them through to the fourth larval stage and then on to adults in vitro without any initial in vivo stimulation. No other workers have yet achieved the same degree of success although Franke and Weinstein (1984) obtained 96% moulting of *D. viteae* and development to young adult stage; however, the developing worms in this study were all male.

This present communication describes the moulting and exsheathment of third stage *Onchocerca lienalis* to the fourth stage in a monoplastic culture system without prior in vivo stimulation.

Materials and Methods

Production of Onchocerca lienalis infective larvae

Infective larvae were produced by the intrathoracic injection of microfilariae into females *Simulium ornatum* raised from pupae in the laboratory. The microfilariae used to infect the vector were obtained from the umbilical skin of cattle using an aseptic technique which has been previously described (Bianco et al., 1980). After injection the flies were maintained at 27°C and 85% relative humidity and fed on a 10% sterile sucrose solution containing mycostatin (100 units/ml), penicillin (200 units/ml) and streptomycin (200 g/m). Larvae were collected from the flies seven or eight days after infection when the microfilariae had developed to infective larvae stage and migrated to the head. An average of 15 larvae per fly were obtained in this way.

Isolation of third-stage larvae

S. ornatum infected by inoculation were distributed into capped tubes and dissected individually. The fly was dropped into absolute alcohol (5 sec) to kill and surface sterile it and then washed in medium to remove surplus alcohol. Medium 199 with Hank's salts (GIBCO No. 041 2350, Paisley,

Scotland) was used for the isolation of the larvae with the addition of 10% tryptose phosphate broth (TPB, GIBCO), 2 mg/ml glucose, and heat inactivated (56 °C for 30 min) foetal bovine serum (iFBS), 400 units/ml and 400 g/ml respectively of penicillin (GIBCO) and streptomycin (GIBCO), and 20 units/ml mycostatin (GIBCO) were added to the medium. Each fly was placed in a small drop of medium and the head removed and placed in a second series of drops of medium in 24 well multiwell tissue culture plate (Falcon, No. 3008). Aseptic techniques were observed throughout the proceedings. The plates were incubated for 2 h at 37 °C in air during which time the larvae escape from the mouthparts of the vector into the surrounding medium. The emerging larvae were counted and pooled in fresh medium (199 supplemented as above but with antibiotics reduced to 100 units/ml of penicillin and 100 g/ml of streptomycin). The larvae were maintained in this medium at room temperature until transferred to culture.

Culture medium

The media and the various supplements tested are given in Table 1. Erythrocytes were obtained from defibrinated jird blood which was washed twice in 199 medium, diluted to 5 ml with complete 199 medium with iFBS and used at 1:100 in the final medium). Antibiotics were generally used at concentrations of 100 units/ml 100 μg/ml. The pH of the media was adjusted to 7.3 with saturated $NaHCO_3$.

Table 1. Media, sera and other components tested for their ability to support the development of *O. lienalis* infective larvae in vitro

Component	Source
Media	
M1 Medium 199 with Hank's salts	GIBCO (041-2350)
M2 Roswell Park Memorial Institute medium 1640	GIBCO (041-2400)
M3 Leibovitz L15	GIBCO (041-1415)
Sera	
S1 Foetal Bovine (FBS)	GIBCO (011-6290)
S2 Bovine (BS)	GIBCO (031-6171)
S3 Human (HS)	GIBCO (063-6150)
S4 Swine (SS)	Flow Labs. (29-241-49)
S5 Chicken (CS)	GIBCO (033-6110)
S6 Rabbit (RS)	Flow Labs. (29-411-46)
Additives	
A1 Tryptose Phosphate Broth (TPB)	GIBCO (043-8050)
A2 Bovine erythrocyte lysate (cells lysed in an equal volume of water)	Freshly collected
A3 Proteose peptone	Difco
A4 Lactalbumin hydrolysate (LAH)	Difco
A5 2% Yeast Extract	GIBCO (061-1150)
A6 Glucose	
A7 Uridine	Sigma
A8 Jird erythocytes	
Antibiotics	
Penicillin	GIBCO
Streptomycin	GIBCO
Mycostatin	GIBCO

Feeder layer cells

The jird cell lines were derived from spleen (MUS cells), and kidney (MUK cells) of an uninfected *M. unguiculatus*, after sterile dissection of these organs followed by standard trypsinisation techniques. The cell lines were maintained in L15 medium with 10% TPB and 10% in FBS with 100 units/ml and 100 μg/ml respectively of penicillin and streptomycin using 25 cm^2 Nunc tissue culture flasks (GIBCO) at 37°C. Subcultures were made at intervals of 7–10 day when the cells became confluent, at a split ratio 1:2. These cell lines were not transformed and could only be maintained for approximately 50 passages after which new cell lines were set up. A vervet monkey kidney cell line (Vero), maintained continuously in the laboratory for ten years, was also utilised. This latter cell line is maintained in 10 oz glass prescription bottle in the L-15 growth medium, and is subcultured at weekly intervals by trypsination at a split level of 1:10.

Larval development in vitro

Monolayers of MUS, MUK, or Vero cells were produced in 96 well microtitre trays (Sterilin, England) by inoculating 0.2 ml of cell suspension in normal growth medium for the particular cells per well, 1–2 days prior to use. To set up the culture of infective larvae the growth medium was removed and replaced with 0.2 ml of the experimental medium. The L3s were placed individually in wells using a micropasteur pipette, the trays sealed with Titretek sealer (Flow labs.), and incubated at 36 °C ± 1 °C. In one series, duplicate cultures were placed unsealed in an atmosphere of 5% CO_2 in air, whilst in another, cultures were maintained at 32°C. Larval development was observed using an inverted microscope without removing them from the culture system. Initially the cultures were examined daily, and in later experiments observations were made on the first day, then at 2-day or 7-day intervals. The condition of the larvae was recorded as a) active, b) static or c) dead (i.e. static and morphologically altered), and the developmental state as a) moulted (shed cuticle present), b) incomplete moult (incomplete moults where exsheathment was not complete were not included in the moulting figure) or c) not moulted. In several experiments individual live larvae were measured using an eyepiece graticule on day 1, 7, 14 and 21 or weekly for as long as they survived (the numbers measured varied from 20 per media to 100 per experiment, with the numbers decreasing as the larvae died). At 7 days 0.1 ml of the medium was removed from the top of the well and replaced with a fresh aliquot of the medium under study. Further medium replacements were made as required (i.e. larvae still alive, condition of feeder layer, pH of the medium). A total of 1,868 L3 *O. lienalis* have been studied in this way and over 200 measured at different intervals, fixed in hot alcohol in glycerine, cleared in glycerine by evaporation and mounted in pure glycerine for more detailed morphological studies. Lengths are expressed in μm ± standard deviation and the ranges.

Results

The mean length of live infective larvae before commencement of culture was 511 μm ± 17 (range 455–581 μm), for n = 48 worms. In the series of experiments conducted there was no significant increase on this figure during the period in which they were maintained.

Moulting was first observed during an early experiment in which the larvae were introduced to the culture system in either 199 medium, RPMI 1640 or L15, supplemented with either 50% or 20% FBS in all instances, and using a feeder layer of MUS cells (a cast cuticle is in Fig. 1). Moulting took place between days 2–5 after the initiation of the culture; a break in the old cuticle appeared at the anterior end and the cuticle was then shed by a series of twisting movements. Incomplete moults were frequently seen where the L4 larvae were unable to completely cast off the old cuticle (Fig. 2).

No complete moults were seen after five days, though in some later experiments the infective larvae survived for up to 19 days in culture and these parasites appeared to have begun to moult, with the new cuticle laid down but with no sign of exsheathment (Fig. 3). The extent of moulting varied with the different media, as did survival. Medium 199 supplemented with 20% FBS gave satisfactory yields of moulting larvae and a moderately increased number of viable worms (72% active at 21 days compared with 15–56% in other media, Table 2). This combination was therefore selected for use in subsequent experiments.

Moulting was observed in cultures containing Vero cells, MUK cells and in primary cultures of MUS and MUK cells (Table 3). In all of these instances the rates of moulting were not appreciably improved by the use of one particular cell line rather than any other. Moulting was also obtained in the absence of a feeder cell layer (Table 3).

Effect of serum

Studies with heterologous serum using Vero cells as the feeder layer demonstrated that moulting can occur in the presence of FBS, bovine sera, bovine serum and heat inactivated human serum but not with rabbit, swine or chicken serum. No significant differences were found between the degree of moulting in normal or heat inactivated form of the two bovine sera. Survival of infective larvae was much reduced in the swine and chicken sera but was apparently unaffected by foetal bovine, bovine, human or rabbit sera.

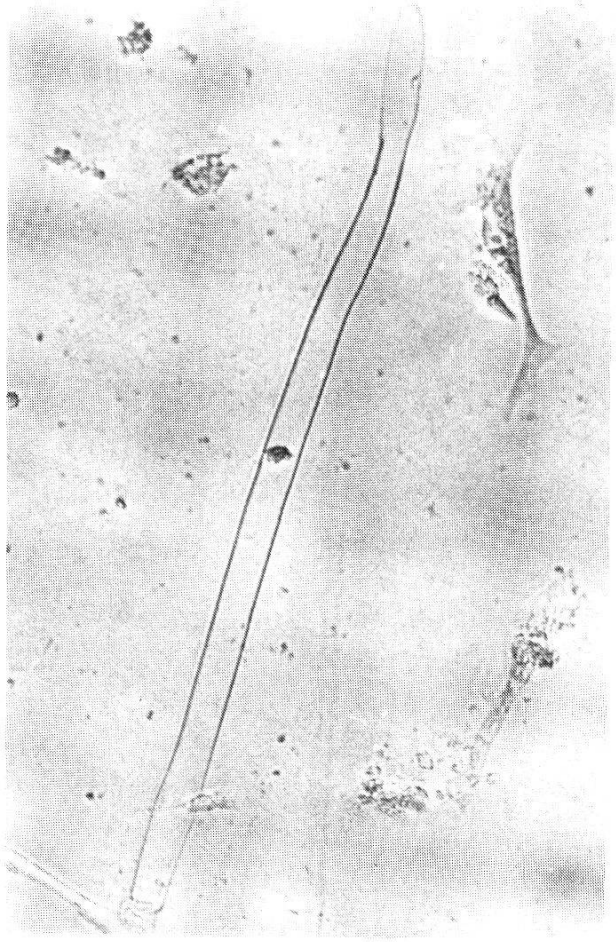
Effect of media supplements

The effect of various supplements to the media on both the incidence of moulting and also on survival of the parasites was examined. These supplements included red cell lysate, proteose peptone, LAH, uridine and yeast extract. None of these were found to have any significant or consistent effect on these parameters (Table 4). The highest viability was obtained with medium 1 (i.e. medium 199 with 20% iFBS). In this media 50% were alive at 14 days as compared with values below 40% for the other media.

The survival times of the filariae in culture varied considerably from experiment to experiment. In one experiment, for example, 86% survived for 41 days in medium 199 supplements with iFBS and TPB and glucose; this value fell to 5% still alive and apparently healthy at 88 days (see Fig. 4). The use of 5% CO_2 in air as the gas phase had no observable effect on larval development in the system used.

Viability

The sluggish movements of third stage *Onchocerca* larvae made assessment of viability by motility measure very difficult. They were scored for activity as described in the methods. There were a large number of worms that were scored



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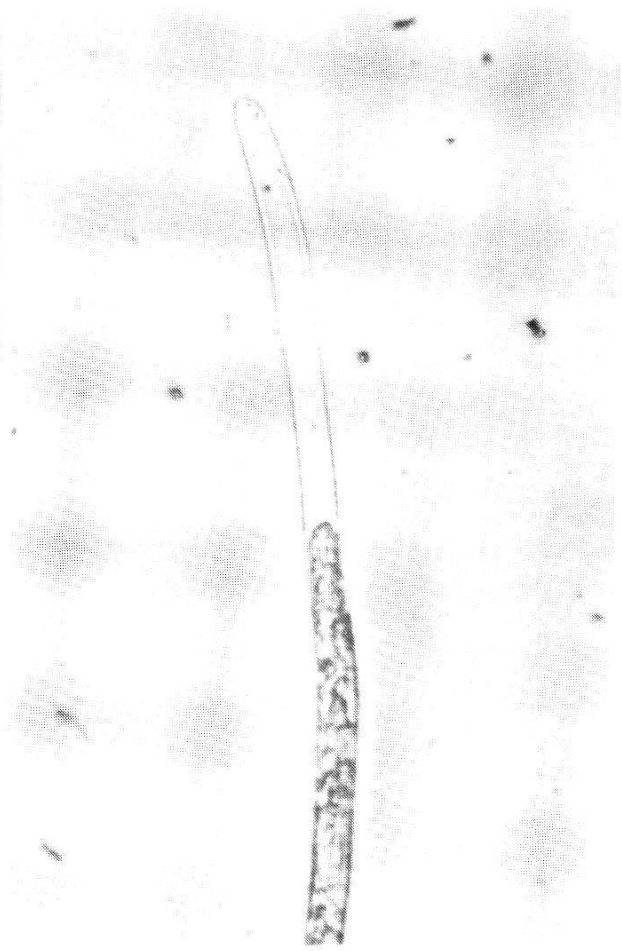

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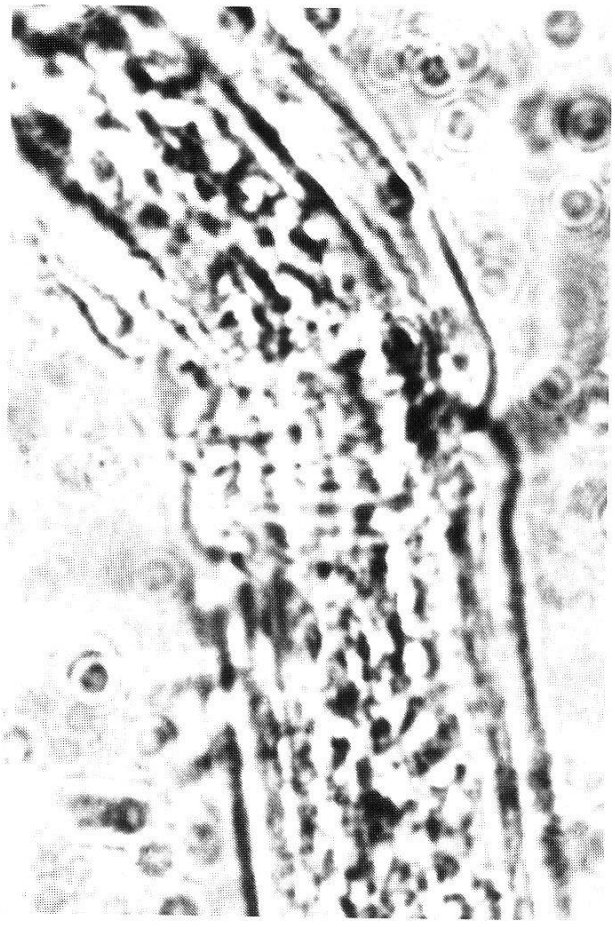

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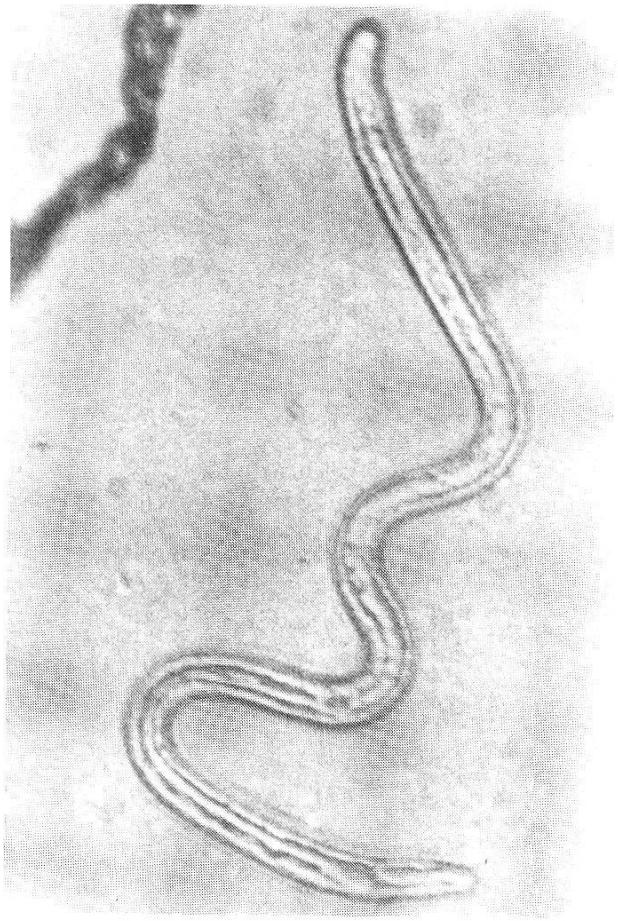

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Table 2. Moulting and survival of *O. lienalis* in the presence of a jird spleen cell line (MUS) in different media supplemented with either 20 or 50% heat inactivated foetal bovine serum (iFBS)

Medium	%iFBS	No. parasites in culture	% moult	% survival (days)					
				14	18	21	26	32	47
L15	20	19	28 (5)*	47 (9)	16 (5)	16 (5)	5 (1)	5 (1)	5 (1)
L15	50	19	5 (1)	58 (11)	37 (7)	32 (6)	–	–	–
RPMI	20	23	13 (3)	70 (16)	52 (12)	57 (13)	30 (7)	17 (4)	–
RPMI	50	19	10 (2)	63 (12)	53 (10)	53 (10)	10 (2)	–	–
199	20	18	10 (2)	83 (15)	72 (13)	72 (13)	33 (6)	11 (2)	–
199	50	19	16 (3)	68 (13)	63 (12)	47 (9)	21 (4)	–	–

* Actual number of parasites shown in parenthesis

Table 3. Moulting of *O. lienalis* L-3 larvae in 199 medium in the presence or absence of two jird cell lines observed on day 7

Medium	Without cells	MUS cells	MUK cells
1	0/8 (0)*	4/20 (20)	10/20 (50)
2	2/11 (18)	7/22 (32)	6/22 (27)
3	3/11 (27)	6/20 (30)	9/25 (36)
4	2/7 (29)	9/22 (41)	6/29 (21)
5	2/4 (50)	9/25 (36)	11/31 (35)
Total	9/41 (22)	35/109 (32)	42/127 (33)

* no. moulted/total; percentage shown in parentheses.

Medium 1 medium 199 + 20% iFBS + glucose
2 medium 199 + 20% iFBS + glucose + 10% TPB
3 medium 199 + 20% iFBS + glucose + 10% TPB + uridine
4 medium 199 + 20% iFBS + glucose + 10% TPB + 2% yeast extract
5 medium 199 + 20% iFBS + glucose + 10% TPB + uridine + 2% yeast extract

as being static as it was difficult to determine whether they were dying, just inactive, or in a quiescent period such as that reported for *D. immitis* (Sawyer, 1965) prior to a moult. During the study we observed several larvae that were completely inactive when initially checked and which moved actively when observed later. Cast cuticles were easily observed in individual wells and so the

Fig. 1. Typical configuration of cast third stage cuticle of *O. lienalis* as seen in culture (×180).

Fig. 2. Posterior end of moulting third stage *O. lienalis* larva. This moult was not completed and the larvae remained in this condition for a further 7 days (×275).

Fig. 3. Anterior end of fixed *O. lienalis* larva on 19th day of culture. The separation of the old cuticle and indentation prior to breaking can be seen, although moulting did not take place (×1240).

Fig. 4. Fourth stage *O. lienalis* after moulting has taken place on day 4 in vitro (×275).

Table 4. Moulting of *O. lienalis* L-3 larvae in the presence of a jird primary culture and cell lines in different media observed on day 7

Medium	Without cells	Jird erythrocytes	MUK primary culture	MUK cells	MUS cells
1	8/50 (16)*	27/88 (31)	11/44 (25)	12/55 (22)	0/51 (0)
5	3/51 (6)	4/77 (5)	4/48 (8)	2/60 (3)	2/56 (4)

* no. moulting shown in parentheses.

Medium 1 199 + 20% iFBS + glucose
5 199 + 20% iFBS + glucose + 10% TPB + uridine + 2% yeast extract

percentage moult within a system could be accurately determined and was regarded to be more useful than survival.

Discussion

The main observation of these studies was that *O. lienalis* infective larvae can be maintained in vitro in a healthy condition up to 88 days, with a 50% viability at around 40 days. It was also found that some parasites would moult to the fourth larval stage without the need for a vertebrate feeder layer of cells, and that the incidence of moulting was enhanced by the presence of fresh jird erythrocytes. There was a slight improvement in the subsequent survival, however, if a feeder cell layer was indeed present. These results compare favourable with those of Lok et al. (1984) who achieved 45–57% moulting with a maximum survival of 42 days for *O. volvulus* and *O. lienalis.*

The fact that moulting took place between the second and the fifth days following introduction into culture compares favourably with the time of the third moult of *O. lienalis* days in the natural bovine host (Bianco and Mustafa, unpublished observations) thus suggesting that the system may be a suitable analogy for natural events.

It was apparent from our study that the parasites may persist in these culture conditions without undergoing a complete moult although they showed cuticular changes associated with the onset of moulting. Moulting was, however, rarely seen after the comparatively short time span of 5 days. In the system used by Wong et al. (1980, 1981) moulting of *D. immitis* larvae in vitro began as early as the third day, and that of *B. pahangi* about the tenth day, and continued for as long as one month after this.

The early onset, and contracted period of the third moult in *O. lienalis* suggests that the process in this species may not require any profound physiological change, and is relatively independent of external regulating factors. By contrast in vitro moulting of *B. pahangi, D. viteae* and *D. immitis* required the stimulus of an in vivo trigger or the presence of a feeder layer of cells (Chen and

Howells, 1979; Wong et al., 1980, 1981; Tanner, 1981). The fact that moulting occurs in a cell-free system is a step towards developing a system that will facilitate studies on the nutritional requirements of parasites; experiments could be carried out in the absence of inherent complications from feeder layer systems and therefore avoid inducing variations in the results and thus difficulties of interpretation due to feeder systems. An improvement in the viability of the worms in the culture system is necessary before this can be achieved. A cell free system also has important advantages for studies of the immune mechanisms directed against early larval stages (Tanner and Weiss, 1981) and for in vitro testing for antifilarial compounds (Jenkins et al., 1980).

The lack of increase in length of cultured worms over the time period studied was not altogether surprising as the initial growth in the natural bovine host is thought to be slow. L4 larvae measured at 32 days after inoculation into cattle had a mean length of only 586 μm (Bianco, unpublished information). In fixed specimens the genital primordia were never more than a few cells. It was noted, however, that the majority of the worms examined were male, the ratio of male to female approaching 4:1, it has not been established whether this is an effect of the culture system. A similar disparity between sex ratios was observed by Franke and Weinstein (1983).

Although moulting of *O. lienalis* may not be affected by external factors, our data suggests that the subsequent survival and development requires specific conditions. In a recent review Weinstein (1986) has stressed the importance of the gas phase to filariae. The successful development in vitro of *D. viteae* depends upon the oxygen tension being within 32 and 50 mm/Hg (Franke and Weinstein, 1984). The marginal improvement of survival rate of *O. lienalis* in the presence of a feeder layer may be attributable to the changes in oxygen tension that would be brought about in the medium by the cells. There is a need for further work to be conducted to clarify this, and to determine whether controlling the gas phase could preclude the necessity of a feeder layer.

Previous studies using *O. lienalis* microfilariae from bovine skin (reported under a different taxonomic nomenclature as *O. gutturosa)* have demonstrated that the parasites behave similarly in vitro to *O. volvulus* (Pudney and Varma, 1980). Thus our studies showing that *O. lienalis* undergo the third moult in vitro provides a stimulus to the study of this parasite as a model system for the early mammalian stages of Onchocerca, including the major human parasite *O. volvulus.*

Acknowledgments

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