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Dipetalonema viteae (Filarioidea): development of the infective larvae in vitro

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Summary

The development of *Dipetalonema viteae* third stage larvae was attempted in vitro. A monophasic culture system consisting of BHK-21 medium supplemented with 10% tryptose-phosphate broth and 15% fresh jird serum allowed the growth of previously in vivo triggered larvae, but not of those isolated from ticks. The larvae could complete the third moult and grew on as fourth stage larvae up to 5 mm. This development was comparable to that observed in vivo. The presence of an irradiated hamster kidney cell feeder layer could replace an in vivo trigger. The development of the infective larvae took place up to the third moult. Some larvae completed the moult to the fourth stage and reached a maxium length of 3 mm after 14 days. These culture systems offer the possibility to study in vitro the complete development of the infective larvae to fourth stage larvae.

Key words: Dipetalonema viteae; Filarioidea; in vitro cultivation; infective larvae; moult.

Introduction

The in vitro cultivation of vertebrate stages of filariae has been attempted for many years with very limited success. Most culture systems only allowed the maintenance of adult worms or microfilariae from the different filarial species (reviewed by Pudney and Varma, 1980). Several authors have reported the successful maintenance and partial growth of *Dirofilaria immitis* third stage larvae (Taylor, 1960; Yoeli et al., 1964; Sawyer, 1965). Taylor (1960) achieved her promising results by cocultivating host tissue and infective larvae. Recently,

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Chen and Howells (1979) reported the development of in vivo triggered third stage larvae of *Brugia pahangi* to fourth stage larvae in an analogous system consisting of dog sarcoma feeder layer cells in tissue culture medium 199. Third stage larvae directly derived from the vector did not grow in their system.

In this study we describe the successful growth of in vivo triggered third stage larvae of *Dipetalonema viteae* to the fourth larval stage in a monophasic culture system. In addition, a system with hamster kidney cells is presented which allowed the development of third stage larvae to fourth stage larvae without preceding trigger in vivo.

Material and methods

Animals. The filarial parasite *Dipetalonema viteae* was cyclically kept in the jird, *Meriones unguiculatus*, and in the soft tick *Ornithodorus moubata* as described by Worms et al. (1961). Randomly bred male jirds (40–50 g) and male golden hamsters, *Mesocricetus auratus* (strain LAKZ, 60–80 g), were obtained from the Institut für Zuchthygiene (Universität Zürich, Switzerland).

Culture medium. The main medium used was the BHK-21 (No. 171, Gibco Bio-Cult, Paisley, Scotland), i.e. the Glasgow modification of the minimal essential medium. The BHK-21 medium was supplemented with 10% tryptose-phosphate-broth (TPB, Gibco) and depending on the experiment, with varying percentages of freshly prepared jird serum (NJS) or commercially available inactivated fetal bovine serum (iFBS, Gibco). In addition, 100 units/ml resp. μg/ml of a penicillin/streptomycin mixture (Difco Lab., Detroit, Mi., USA) were added. The pH was adjusted to 7.3.

Feeder layer cells. The hamster kidney cell line BHK (C 13, H 3020 American Type Culture Collection, purchased from Gibco) was maintained in T-25 tissue culture flasks (Falcon Inc., Oxnard Ca., USA) with BHK-21 medium supplemented with 10% TPB and 10% iFBS. Subcultures were made every 6 to 8 days when the cells became confluent. In order to prevent further multiplication of the cells in some experiments, loose (i.e. not confluent) feeder layers were irradiated with 2000 rad using a Phillips RT 305 (300 kV, 2.7 mm Cu) source.

Isolation of third stage larvae from ticks. Third stage larvae (L3) were isolated sterilely from ticks as described in Gass et al. (1979). However, the medium used was the BHK-21 supplemented with 10% TPB (see above) and with 500 units/ml resp. μg/ml of the penicillin/streptomycin mixture. Pooled larvae were kept at 37° C until transfer into culture or into micropore chambers.

Micropore chambers. Micropore chambers (Millipore Corp., Bedford, Mass., USA) sealed with 5 μm Nuclepore chemotaxis membranes (Nuclepore Corp., Pleasanton, Ca., USA) were assembled, loaded with approximatively 30 L3 and implanted subcutaneously into jirds as described earlier (Weiss and Tanner, 1979; Gass et al., 1979).

Larval development in vitro. Fifty to seventy larvae were incubated in T-25 tissue culture flasks (Falcon) with 5 ml of medium and 50–70 larvae per flask. Before transferring L3 or in vivo triggered L3 (recovered from micropore chambers) into culture the larvae were washed twice in BHK-21 medium + 10% TPB with 500 units/ml resp. μg/ml penicillin/streptomycin. All the cultures were incubated at 36 ± 0.5° C in an atmosphere consisting of 90% N_2, 5% O_2 and 5% CO_2 (Carba Gas AG, composition purity ± 1%). The length of at least 40 larvae from a random sample were measured at the beginning and at the end of an experiment according to Gass et al. (1979). The cultures were observed daily and counts were made every second day of culture (CD). The following features were always recorded: (a) number of point-headed larvae (L3 as they were isolated from ticks), (b) number of round-headed larvae, (c) number of moulting larvae, (d) number of dead and immobile larvae, (e) number of shedded cuticles.

All the experiments were carried out at least in duplicates. The median and the 95% confidence limit were determined for the larval length, and the U-(rank)-test of Mann and Whitney modified for tied ranks (Sachs, 1978) was applied for the statistical analysis.

Results

Infective larvae of *D. viteae* isolated from ticks showed the characteristic pointed anterior end (Fig. 2). Their median length was 1.37 mm (1.37–1.43). The in vivo studies on larval development within jirds (Gass et al., 1979) revealed that prior to any larval growth the rounding of the anterior end of L3

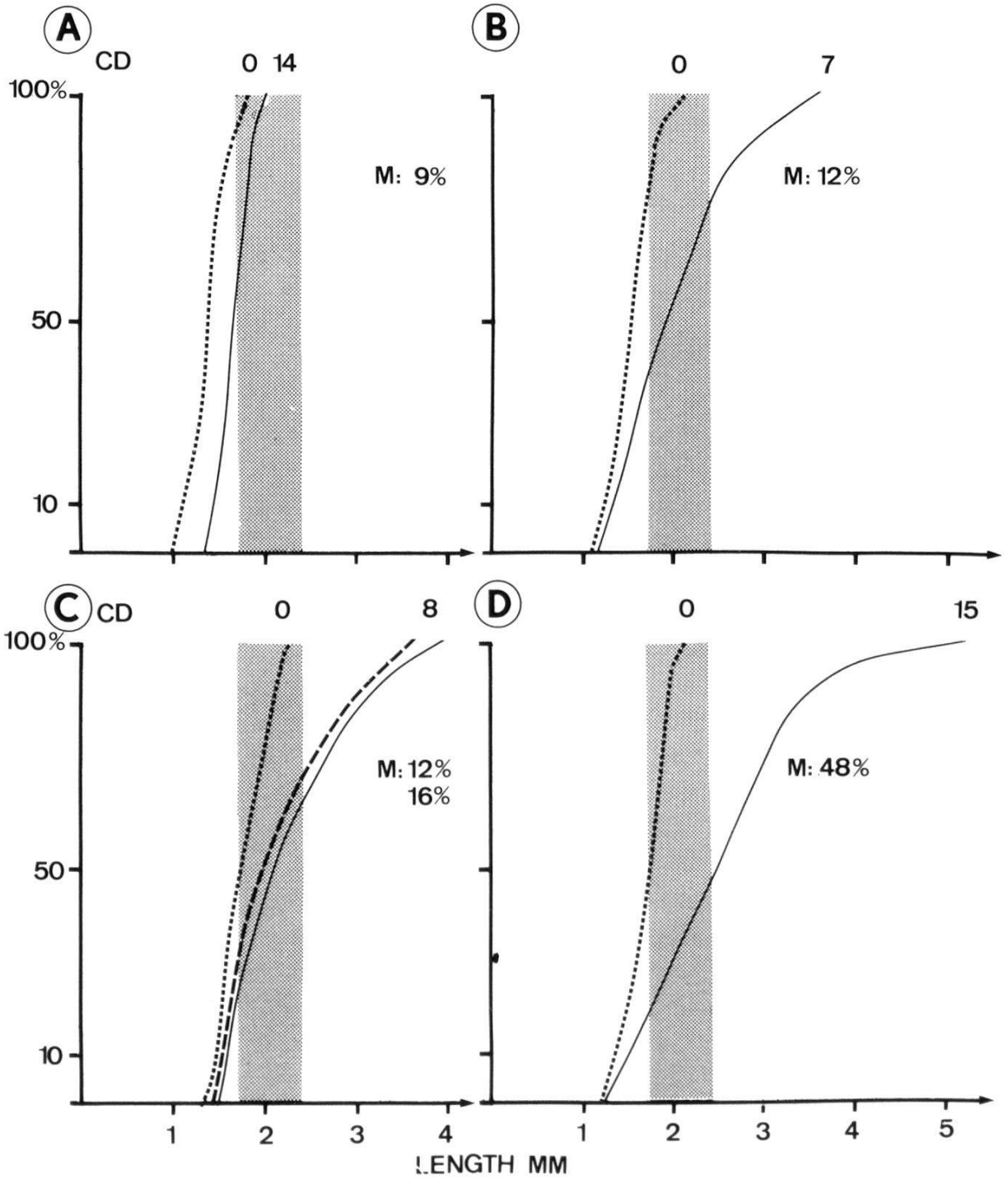
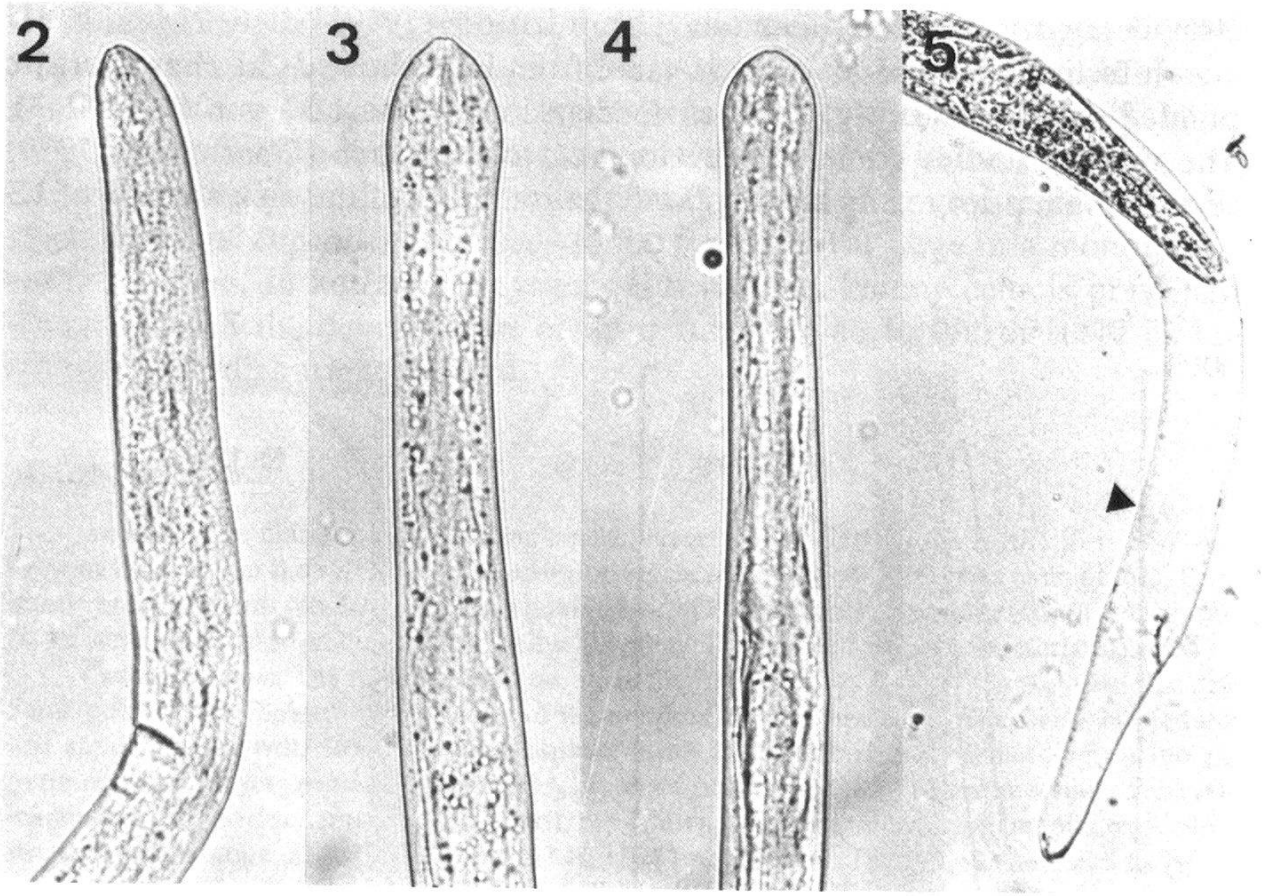


Fig. 1. The development of in vivo triggered third stage larvae of *Dipetalonema viteae* in a monophasic in vitro system. Cumulative frequency distribution of larval length (>40 larvae measured) at the beginning (· · · · · ·) and after different days in culture (CD). The shaded area indicates the range of the third moult. M = % of dead (= immobile) larvae at the end of the experiment. Larvae triggered for 2 days (A) or 6 days (B–D). In experiment C the addition of 15% fresh jird serum (——) was compared to cultures with 15% inactivated jird serum (– – – – –).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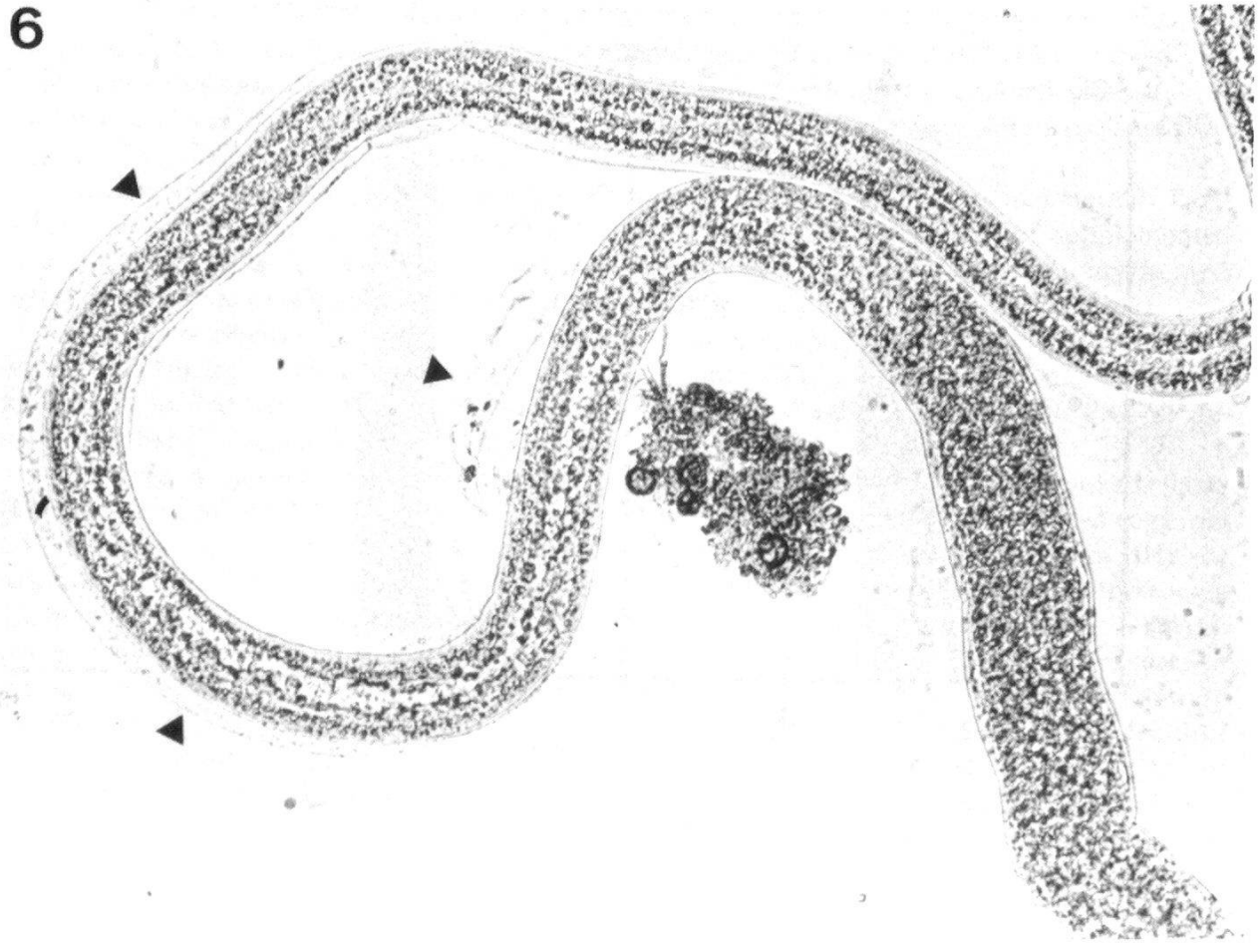
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was the main, obvious feature of infective larvae having adapted to a vertebrate host environment. Such larvae are subsequently called $L3^+$. In the same in vivo studies it has been found that $L3^+$ grow up to 1.7 mm before the third moult is observed. The length of moulting larvae lies between 1.7 mm and 2.4 mm and thus, larvae >2.4 mm are considered as fourth stage larvae (L4).

In order to initiate larval development of L3, several tissue culture media (TC 199 No. 235, CMRL 1066 No. 153G, NCTC-135 No. 135, RPMI 1640 No. 187 G, Eagle's MEM No. 189G and Earle's-MEM No. 109G, all purchased by Gibco) were cross-checked with different buffer systems ($NaHCO_3$, Hepes) and different sera (hamster, NJS, FBS) at varying concentrations (5, 10 and 20%). L3 survived only for three to nine days. None of such combinations allowed the development of L3 or even the transformation to $L3^+$. In addition, the well known tripeptid growth factor glycin-l-histidyl-l-lysin (Pickart and Thaler, 1973, cf. also Stromberg et al., 1977) did not exert any growth stimulating effect when added in a concentration of 20 ng/ml of synthetic gly-hist-lys (Calbiochem).

The best survival (up to 12 days) without any growth or transformation to $L3^+$ was observed in BHK-21 medium supplemented with 10% tryptose-phosphate broth (TPB) and 15% fresh jird serum (NJS). If the serum concentration was increased to 40% NJS, 76% of the L3 transformed to $L3^+$ within three days, but this transformation was not followed by any growth and at day 6 all the larvae were dead.

It was then decided to attempt the cultivation of L3 which had been triggered in vivo within micropore chambers implanted into jirds for 2 to 6 days. Thus, L3 underwent in vivo the transformation to $L3^+$. L3 which had been within micropore chambers for 2 days and were subsequently brought into BHK-21 medium with 10% TPB and 15% NJS only slightly grew within 14 days (the medium was exchanged on day 9) and none of the larvae completed its moult to L4 (Fig. 1 A). However, L3 exposed for 6 days in vivo grew well under the same conditions, completed their moult to L4 and grew on (Figs. 1 B–D). After 15 days in vitro, 50% of six-day-old $L3^+$ had moulted to L4, and the L4 had reached length up to 5.5 mm (Fig. 1 D). Larval mortality was moderate and only increased in cultures which were kept for more than nine days and consequently had to undergo a total medium exchange at day 7. The L4 never developed into adult worms, i.e. the fourth moult could not be observed in our system. L4 reaching a certain size (approx. >3.5 mm) became very delicate to handle and were easily affected by the procedure of the medium exchange which was necessary every 5 to 8 days. The inactivation of NJS (56° C, 30 min) did not significantly influence larval development (Fig. 1 C). When $L3^+$ and L4

Figs. 2–6. *Dipetalonema viteae* early mammalian stages in vitro. 500 ×. Point-headed third stage larvae (L3, Fig. 2) transform through a transition stage (Fig. 3) to the round-headed $L3^+$ (Fig. 4). The third moult (Figs. 5, 6), the old cuticle being shedded (▶), posterior end of a moulting larvae (Fig. 5).

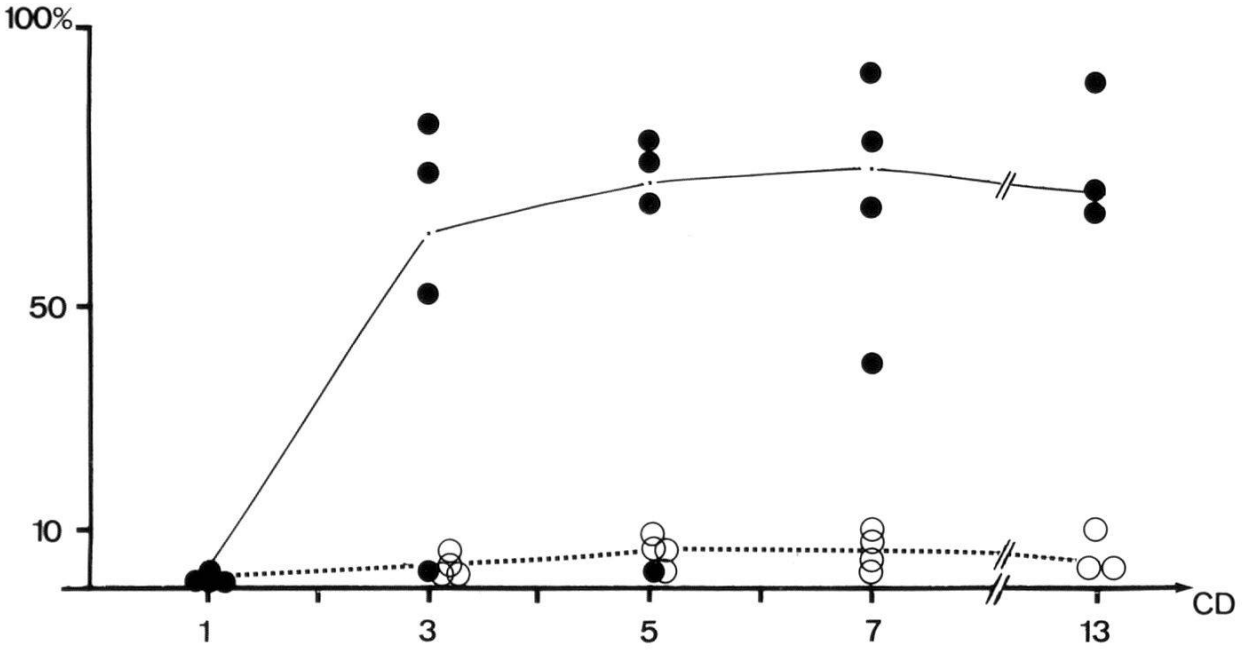

Fig. 7. The transformation of *Dipetalonema viteae* point-headed third stage larvae (L3) to round-headed L3$^+$. Median percentage of L3$^+$ (●) and dead larvae (○) after different days over irradiated hamster kidney cells and in 10% fresh jird serum. Results from 4 independent experiments with 50 to 70 larvae each (one experiment had to be terminated due to fungal contamination at day 9).

were removed from cultures at day 6 to 8 and were subsequently injected into hamsters, these hamsters became microfilaraemic and adult worms were found at autopsy (week 20 post infection).

In view of the possibility to collect excretory/secretory antigens from developing and moulting larvae, serum-free and TPB-free cultures were tested with six-day-old L3$^+$. When TPB and serum were omitted, the larvae survived only for three days. However, 10 to 23% (median 16%) of all the larvae could still complete their moult to L4. The addition of TPB (2–10%) slightly increased the percentage of moulting L3$^+$ (median 21%, range 12–66%), but allowed larval growth for 8 days in vitro.

In order to achieve a complete development of early mammalian stages in vitro, the cocultivation of host cells and L3 was attempted. L3 (Fig. 2) transformed through a transition stage (Fig. 3) to L3$^+$ (Fig. 4) in the presence of hamster kidney feeder layer cells maintained in BHK-21 medium supplemented with 10% TPB and 10% NJS or 10% iFBS (Fig. 7). L3$^+$ also significantly ($2P < 10^{-9}$, U-test) grew on under these conditions (Fig. 8). Ten percent of the larvae completed their moult to L4 in the presence of fresh jird serum.

As the medium was quickly depleted of nutrients by the fast growing feeder layer cells, the feeder layer had to be irradiated in order to stop further cell multiplication. An irradiated feeder layer could usually be used for 7 days. The transformation of L3 to L3$^+$ took place in NJS over an irradiated feeder layer – with the exception of one experiment – within 3 days (Fig. 7). If iFBS was used instead of NJS, the transformation to L3$^+$ was comparable, but the subse-

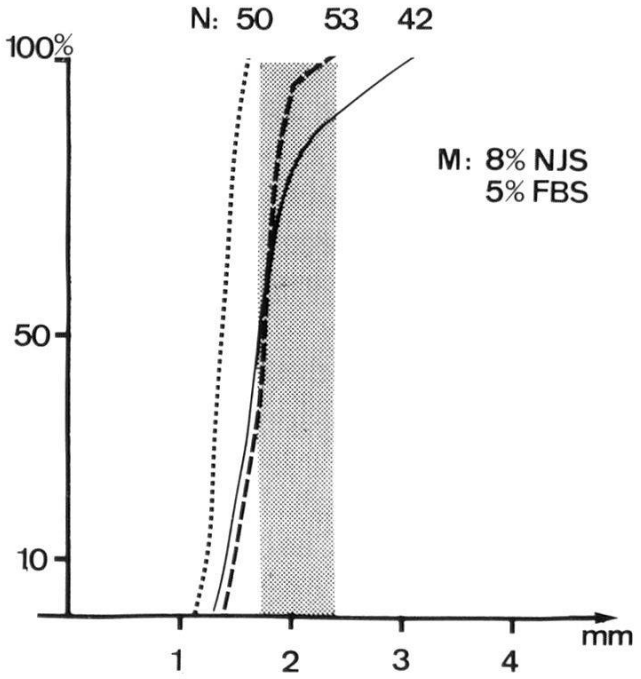

Fig. 8. The development of *Dipetalonema viteae* third stage larvae in vitro in the presence of irradiated hamster kidney cells. Cumulative frequency distribution of length of larvae isolated from ticks (·····) and of those cultivated subsequently for 14 days in fresh jird serum, NJS (——) or inactivated fetal bovine serum, FBS (– – – –). The shaded area indicates the range of the third moult. M = % of dead (= immobile) larvae, N = number of larvae measured.

quent growth of $L3^+$ was inferior (Fig. 8). Fresh FBS could not be used as such serum provoked the detachment of the feeder layer from the tissue culture vessel.

L3 which had not transformed to $L3^+$ within 7 days no longer underwent this change and remained point-headed until they died between days 9 and 12. There was always a certain percentage of larvae which was not able to adapt to the in vitro system simulating a vertebrate host environment (cf. Fig. 7, median 25%).

Discussion

The careful in vitro studies on the development of early mammalian stages of *B. pahangi* (Chen and Howells, 1979) have revealed the crucial role of the initial adaptation of infective larvae to a vertebrate host environment. L3 had to be exposed first to the host for 2 to 3 days before in vitro growth was possible in a biphasic system. In contrast to these studies we achieved the transformation of L3 to $L3^+$ of *D. viteae* in vitro either in the presence of a host cell feeder layer or with a high (40%) jird serum supplement. The second possibility, however, did not enable further growth of $L3^+$, whereas the presence of hamster kidney cells not only stimulated the transformation to $L3^+$ but also the growth up to the third moult. The growth within 2 weeks was comparable to that observed in vivo within one week (Gass et al., 1979). The growth retardation might have been

partially due to the prolonged time required for the transformation to $L3^+$ in vitro, which could finally be related to the function of the feeder layer. The feeder layers were irradiated in order to prevent further multiplication and consequently an 'overconsumption' of nutrients by the fast growing feeder layer cells. The irradiation could, however, have affected the metabolic capacities of the cells, too. Studies on the factor(s) provided by the hamster cells and the use of other feeder layers (e.g. established from jirds) would probably help to improve significantly the in vitro system and could lead to more defined conditions enabeling the complete development of *D. viteae* early mammalian stages.

The feeder layer cells are only necessary for the initial transformation and growth of L3, as the monophasic culture system described in this paper allowed the development of $L3^+$ to L4. The growth in vitro – although slightly slower – is comparable to that described for *D. viteae* larvae in vivo (Chabaud, 1954; Gass et al., 1979). In vitro grown L4 were still fully capable to become fertile adult worms as was shown with the injection into hamsters. Large (>3.5 mm) in vitro grown L4 became fragile, thus, the medium exchange was difficult and a long-term culture to fertile adult worms was not yet possible. In this regard one might ask, if the monophasic culture system lacks certain nutrients required by the fast growing L4 for the constant synthesis of an elastic multilayered cuticle.

The time required for the in vivo trigger seemed to be important. Although two-day-old L3 displayed (after removal from micropore chambers) the rounded head, they did not grow well in vitro (Fig. 1 A). Six-day-old L3, indistinguishable from 2-day-old L3, grew well. This might indicate that the rounding of the anterior end is an early morphological feature of adaptation to the vertebrate host, but that it does not alone signalize the readiness to grow. A longer exposure to the host's environment (5 to 6 days for *D. viteae*) which probably enables also fundamental metabolic changes, is necessary. The longer in vivo trigger required for *D. viteae* in comparison to *B. pahangi* (Chen and Howells, 1979) could have been due to the fact that our L3 were triggered in vivo within micropore chambers.

The addition of fresh or inactivated jird serum as well as that of TPB appeared to be essential for any growth of $L3^+$ to L4. If the serum was omitted, the third moult could still be completed by a proportion of the larvae, but the moult was not followed by a further larval growth. The serum and TPB could not be replaced by the tripeptid growth factor glycin-l-histidyl-l-lysin (Pickart and Thaler, 1973). In this respect it is interesting to point out that Stromberg et al. (1977) reported the successful development of L3 of *Ascaris suum* to L4 in serum-free tissue culture medium 199 supplemented with this tripeptid. Their system even allowed the isolation of protective antigens and, thus, supported the crucial role of excretory/secretory products and moulting fluids in the host/parasite relationship (Stromberg and Soulsby, 1977; Stromberg, 1979).

Although the BHK-21 medium alone or with only 2% TPB did not support continuous larval growth, it allowed *D. viteae* $L3^+$ to complete their moult to L4.

Thus, these conditions provide the possibility to collect probable antigens released during the third moult. In regard to this point, such culture supernatants are currently being collected and investigated. In addition, the culture systems described in this paper is being used to analyze in vitro the immune mechanisms mediating larval growth inhibition as it has been observed in infected and immunized jirds (Tanner and Weiss 1981; Tanner and Weiss, in preparation).

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