DEVELOPMENT OF LARVAE OF ASCARIS SUUM FROM THE THIRD TO THE FOURTH STAGE IN A CHEMICALLY DEFINED MEDIUM

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(Received 29 June 1976)

Abstract—STROMBERG B. E., KHOURY P. B. and SOULSBY E. J. L. 1977. Development of larvae of Ascaris suum from the third to the fourth stage in a chemically defined medium. International Journal for Parasitology 7: 149-151. Third stage larvae of Ascaris suum, recovered from the lungs of rabbits 7 days after oral infection with eggs, were cultured to the fourth larval stage in chemically defined, low molecular weight medium. The medium consisted of tissue culture medium 199, supplemented with glucose and glycyl-histidyl-lysine and gassed with a mixture of N₂ CO₂ O₂ (90:5:5). Growth and development in this medium were similar to that in media supplemented with whole serum or with a serum dialysate.

INDEX KEY WORDS: Ascaris suum; in vitro culture; chemically defined medium; glycyl-histidyl-lysine.

INTRODUCTION

There have been many attempts to cultivate the larval stages of Ascaris suum in vitro (reviews in Silverman, 1965; Taylor & Baker, 1968). Sylk, Stromberg & Soulsby (1974) cultured third-stage larvae from the lungs of rabbits to the fourth stage, but their medium included an undefined component, serum. This paper reports the culture of third-stage larvae to the fourth stage in a chemically defined medium.

MATERIALS AND METHODS

Source of larvae. New Zealand white rabbits (2.5-3.0 kg) were infected per os with 100,000 embryonated eggs of A. suum. The larvae were killed on the 7th day after infection and the larvae were collected from the lungs by the Bearmann technique and treated as previously described (Sylk et al., 1974).

Culture media. Four culture media were used, one containing whole serum (complete culture medium), one containing a dialysate of serum (serum dialysate medium), one containing a defined tripeptide mixture (defined medium) and one which lacked any serum component (control medium). The complete culture medium consisted of 19.2 ml of medium 199 (Grand Island Biological Co., New York), 0.8 ml of swine serum, 0.45 mg glucose (total of 19.65 mg glucose in medium), 6000 units of penicillin and 6 mg of streptomycin. A low molecular weight component of serum (serum dialysate) was obtained by adding an equal volume of distilled water to swine serum and passing this through an Amicon Filter (Model 52) using a filter membrane (UM 10) with a 10,000 molecular weight exclusion limit. A volume equal to that of the whole serum was collected. The serum dialysate medium was prepared by replacing the 0.8 ml of serum in the complete culture medium with 0.8 ml of serum dialysate. The defined medium was prepared by substituting 400 ng of glycyl-l-histidyl-l-lysine acetate tetrahydrate (Calbiochem) for the 0.8 ml of serum in the complete culture medium, while the control medium substituted 0.8 ml of saline for the serum in the complete medium. The media were sterilized by passage through a 0.45 µm Nalgene filter and were dispensed into sterile French Square bottles (200 ml capacity). Third stage larvae were introduced into each culture bottle, which was then gassed with a mixture of N₂ CO₂ O₂ (90:5:5), stoppered and incubated at 37°C on a roller drum.

Experimental design. Following preliminary studies which demonstrated that whole serum, serum dialysate or tripeptide were necessary for growth and development, a series of replicate cultures (2000 larvae per 20 ml of culture medium) was then used to compare growth and development of larvae in the complete culture medium and in the defined medium. Thirty-three cultures were set up for each type of medium (complete and defined) and at least three cultures per day were harvested from each type of medium on days 0, 2, 3, 4, 5, 6, 7, 8 and 12 of incubation. A minimum of 100 larvae were evaluated for growth (length) and development (larval stage) for each sample. Six replicate cultures of each medium (complete, serum dialysate and defined) were prepared with a gas phase of air and were examined at intervals over a period of 12 days to determine the necessity of the mixture of N₂ CO₂ O₂ for ecdisis and growth. When air was substituted for the gas mixture the pH was increased by 0.2. To examine the effect of density on development, replicate cultures (6-10) each containing 1000, 2000, 3000, 4000 and 5000 larvae in each medium were maintained 12 days and the percent ecdisis at each density was determined.

Identification of larval stages. Larvae were harvested by sedimentation and fixed at 90°C in alcohol-formalin-
acetic acid (Cable, 1960). The stages were identified according to the criteria of Nichols (1956) and Douvres, Tromba & Malakatis (1969).

RESULTS

Larvae obtained from the lungs of rabbits on the 7th day post infection underwent the third ecdysis to the fourth stage in all three culture media (complete medium, serum dialysate medium and defined medium) but not in medium which lacked a serum component (control medium). The percentage moulting to the fourth stage and the mean length of larvae after various times in culture in the complete medium and in the defined medium are presented in Table 1. There was no appreciable difference in the mean length or the percent moulting of larvae cultured in the defined medium compared with larvae cultured in the complete culture medium. All larvae appeared normal at the end of the culture period. In the serum dialysate medium the percentage that moulted to the fourth stage was the same as in complete medium, but growth was not measured. As has been previously described (Sylk et al., 1974) larvae in the different media were observed in the process of moulting and numerous empty sheaths were seen free in the culture media.

None of the larvae moulting to the fourth stage or increased appreciably in length when they were cultured in various media in which the gas phase was air. However, the larvae in such media remained viable for the 12-day culture period.

Larval development was found to be density dependent in the defined medium. At a density of 3000 per culture, 86% of the third-stage larvae underwent the moult to the fourth stage, while at 1000, 2000, 4000, or 5000 larvae per culture, the percentage moult was 81, 83, 28 and 11, respectively. It was observed that the larvae which did not moult increased very little in length. However, at all these larval concentrations in the complete and serum dialysate media, 80-95% of the larvae underwent the moult to the fourth stage and there was no evidence of density dependence.

DISCUSSION

Previous studies (Sylk et al., 1974) have demonstrated that third-stage larvae recovered from the lungs of guinea pigs or rabbits can be cultured to the fourth stage in medium 199 with the addition of glucose, swine serum and gassed with \( \text{N}_2-\text{CO}_2-\text{O}_2 (90:5:5) \). The synchronous development of larvae in such cultures has provided an opportunity to examine products produced by the larvae which might play a part in the immune response of the host to the parasite. However, the presence of complex and undefined high molecular weight components (e.g. serum) in the medium compromised the identification and quantification of the parasite derived components. Removal of the high molecular weight component(s) of the serum proteins by selective filtration, as has been done for mammalian cell culture (Gwatkin, 1960; Metzgar & Moskowitz, 1960) and for the cultivation of *Echinococcus granulosus* (Herd, Chappell & Biddell, 1975) has permitted the use of a medium supplemented with an ultrafiltrate of serum (serum dialysate medium) which supported growth and development of larvae in a manner similar to medium supplemented with whole serum. Though the serum dialysate medium offered substantial advantages in the examination of the culture fluid for parasite derived material, it was nevertheless still undefined.

A further refinement in the culture technique was possible with the replacement of the serum ultrafiltrate by a low molecular weight serum component (three amino acid complex) which has been shown to be necessary for the growth and development of liver cells (Pickart & Thaler, 1973; Pickart, Thayer & Thaler, 1973). A comparison of larvae cultured in medium supplemented with the tripeptide or with whole serum showed no appreciable differences in

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\begin{array}{|c|c|c|}
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\text{Day} & \text{Complete medium} & \text{Defined medium} \\
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0 & 1227.6 \pm 151.7 \dagger (0) & 1221.4 \pm 80.0 (0) \\
2 & 1391.9 \pm 170.6 (0) & 1348.5 \pm 129.9 (0) \\
3 & 1452.7 \pm 226.2 (4) & 1475.3 \pm 163.6 (0) \\
4 & 1547.5 \pm 206.1 (18) & 1613.7 \pm 288.8 (20) \\
5 & 1623.3 \pm 297.7 (32) & 1596.7 \pm 325.0 (36) \\
6 & 1696.8 \pm 275.0 (46) & 1715.7 \pm 348.8 (42) \\
7 & 1822.9 \pm 390.4 (74) & 1826.0 \pm 379.9 (68) \\
8 & 2113.9 \pm 494.9 (80) & 2001.6 \pm 438.4 (80) \\
12 & 2143.5 \pm 393.2 (88) & 1995.1 \pm 469.4 (81) \\
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\end{array}
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*Mean of a minimum of three samples, each consisting of a minimum of 100 larvae examined.

†S.D.

‡Numbers in brackets are the percentage of the total numbers of fourth stage larvae on the day indicated.
Development of *Ascaris suum* larvae in a defined medium

the mean length or the percentage of larvae that moulted between the two culture media. Growth was associated with moulting in both media. All larvae which moulted had also increased in length; conversely, larvae which did not grow did not moult. Sexual differentiation on day 8 in complete culture medium had been reported previously (Sylk et al., 1974). No sexual differentiation was observed on day 12 in the defined medium. However, gonad formation was observed in 24-day cultures.

The rate of growth and the rate of moult of larvae in the complete culture medium in the present study was lower than that observed in the previous report (Sylk et al., 1974). The previous studies used larvae from the lungs of guinea pigs or rabbits 7–10 days post infection whereas the present study used larvae harvested at 7 days post infection. It has been noted previously (Sylk et al., 1974) that larvae harvested earlier than 7 days after infection failed to undergo ecydysis or to grow in medium which would support growth and development of larvae harvested later in the infection. Hence 7 days would appear to be the minimum time at which larvae will continue to develop satisfactorily when removed from an *in vivo* to an *in vitro* system. This may be dependent upon time or may be dependent on the accumulation of products required for growth and ecydysis *in vivo*.

A more extended period of adaptation to *in vitro* conditions seems necessary for the younger larvae (7-day) and, perhaps, reflects the need for the physiological mechanisms which control development to become fully functional, which may be slowed by *in vitro* cultivation. Larvae obtained from the lungs of rabbits at 8 or 9 days post-infection developed faster than 7-day larvae but slower than 10-day larvae (unpublished data). In the present study larvae were harvested on the 7th day as this provided larger numbers than would be available if the larvae were harvested later.

The necessity for a serum derived supplement for growth and development was illustrated in the control medium in which the larvae were unable to moult or grow but remained alive for the 12-day culture period. The synthetic tripeptide used in this study replaced the serum component.

The need for an appropriate gas phase was also demonstrated. Larvae in cultures which contained air as a gas phase were unable to moult or grow, while those receiving the gas phase (N₂–CO₂–O₂) did so. This may be a consequence of a lower oxygen tension or increased carbon dioxide tension or both.

The density of larvae in culture was found to be very important in the tripeptide supplemented medium, while this did not appear to be a factor in media supplemented with whole serum or serum dialysate. The reason for this is unclear at present and the phenomenon requires further investigation. Preliminary studies indicate that it is not associated with exhaustion of the supply of tripeptide in the medium. Perhaps the metabolism of the larvae is more efficient and some other medium component becomes limiting.

These studies have demonstrated that an appropriate *in vitro* physiological environment can be provided for the synchronous moulting and growth of larvae of *A. suum* by a totally defined, low molecular weight, culture medium. This is the first instance in which this synthetic tripeptide has been used to enhance the essential processes of a metazoan. This culture medium will now permit a more satisfactory approach to the examination of parasite derived materials which may serve as antigens in the immune response to the parasite or lead to a better understanding of parasite metabolism. This defined culture system may lead to a better understanding of the process of moulting and the host–parasite relationship.

Acknowledgements—The authors would like to thank Mr. Derek Muncey and Mrs. Rosetta Goss for their technical assistance. This work was supported, in part, by U.S.P.H.S. Research Grant AI-06262 and a University of Pennsylvania Faculty Research Grant, 74–36.

REFERENCES


