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Evaluation of *in vitro* Culture Conditions of *Trichinella spiralis* Newborn Larvae

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Abstract

Aspects of *in vitro* culture conditions of *Trichinella spiralis* newborn larvae were evaluated as follows. When maintained for 6—7 days at 37°C in 5% CO_2 atmosphere in Tissue Culture Medium 199 (M199) supplemented with 10—30% FBS (fetal bovine serum), or 30% serum from rodents or primates, newborn larvae grew from 108 to 135 μm in body length. However, less growth was observed in M199 alone or in 10—30% artificial bovine serum. Larval survival rate in M199 containing 20% FBS was 100% for the first 4 days of cultivation, but declined sigmoidally after that time. The glucose content of M199 with 20% FBS containing 10,000 newborn larvae per ml decreased by 20% of the initial value on day 9. Also, the concentrations of alanine and aspartic acid in culture media changed. Such changes in concentrations were more remarkable in cultures of mature muscle larvae than with newborn larvae. It is concluded that M199 with 20% FBS is useful for *in vitro* culture on newborn larvae.

Key words: *Trichinella spiralis,* newborn larva, *in vitro* cultivation, *in vitro* growth, glucose, amino acids

Introduction

An improved technique for collecting a large number of newborn larvae *in vitro* was provided in a previous report (Takada & Tada, 1988). To date, no information on *in vitro* culture conditions of newborn larvae have been published. In this study, the growth rate and survival of newborn larvae in culture medium supplemented with various kinds and concentrations of sera, glucose and amino acids were examined to determine the optimal *in vitro* culture conditions for newborn larva.

Materials and Methods

Preparation of newborn larva: The stock infection of the Iwasaki strain of *T. spiralis* was maintained in ddY-mice as previously described (Takada *et al.* 1985). Small intestines of mice were opened longitudinally in 0.85% NaCl solution on day 6 after oral infection with 20 muscle larvae per g body weight. The intestines were incubated for 2 hr at 37°C to collect adult worms using a modified Baerman's apparatus. Axenic newborn larvae were obtained from adult worms by cultivation with antibiotics for one day, according to our previous report (Takada & Tada 1988). The number of newborn larvae used was counted in a plastic dish with 2 mm grids (60 × 15 mm; Corning).

Preparation of muscle larva: For recovery of muscle larvae, the carcasses of mice that had been infected with *Trichinella* for 30 days were artificially digested in 1% pepsin — 1% HCl solution for 1 hr at 37°C. A suspension of these larvae were added to a spinner flask and collected from its bottom.

Growth and survival rate: Newborn larvae were cultivated in Medium 199 (M199; Difco) alone or with (a) 10, 20 and 30% fetal bovine serum (FBS; Gibco), (b) artificial fetal bovine serum (Nu-S; Collaborative) or (c) 30% heat-inactivated serum from the ddY-mouse, Chinese hamster, Japanese monkey (*Macaca fuscata fuscata*) or human. Incubation was carried out at 37°C in a humidified 5% CO_2 atmosphere.

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Growth rates and survival *in vitro* were observed for about 2 wk. The density newborn larvae was 65 per 100 μl-well in a 96 well culture plate (Corning). This represented an average number of newborn larvae produced by a female adult. The body length of newborn larvae (N = 20) from triplicate wells was measured daily using an ocular micrometer and light microscopy (× 100). Larvae were previously fixed in 10% formalin for 10 min. In determining survival, microscopically observed nonmobile, arc-shaped larvae were judged to be dead.

Glucose and amino acids: Newborn and muscle larvae were cultured in M199 containing 20% FBS at a density of 650 and 10,000 per 1 ml-well using a 24 well culture plate with flat-bottom (Corning). Incubations were for 9 days at 37°C in 5% CO_2. All of the supernatant media were stored at 4°C before biochemical assays. The glucose content in supernatant fluid, before and after cultivation, was assayed by TBA-880 Autoanalyzer (TOSHIBA; HK method). Sixteen amino acids (alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine and valine) in the same materials was assayed using an 835 Amino Acid Analyzer (HITACHI).

Statistics: Student's t-test ($p < 0.05$) was used to evaluate the significance of differences between mean values.

Results

Effect of serum supplementation on larval growth

The mean body length (N = 120) of newborn larvae just after being produced by adult worms was 108 ± 2.3 (SE) μm. No significant growth was observed when newborn larvae were cultured in M199 alone. However, the length increased up to 135 μm when larvae were cultured in M199 containing 10, 20 or 30% FBS for 6—7 days. The increase amounted to an average of 25% of the initial body length. The body length also increased to 135 μm also in M199 containing 30% of serum from ddY-mouse, Chinese hamster or Japanese monkey. Mean body length of newborn larvae increased to 120 μm in M199 with 20 and 30% Nu-S, but did not increase in the medium with 10% Nu-S (Fig. 1-A, B and C).

Survival rate

There was a tendency for newborn larvae to survive longer in M199 with 30% primate serum than with 30% rodent serum (Fig. 1-B).

Newborn larvae never survived more than 2 days on M199 alone. In M199 containing 20% FBS of most manufactured lots, survival rate of newborn larvae was maintained at about 100% through 4 days of culture. After that time survival declined sigmoidally until day 12. But a rapid decline of survival rate was found in M199

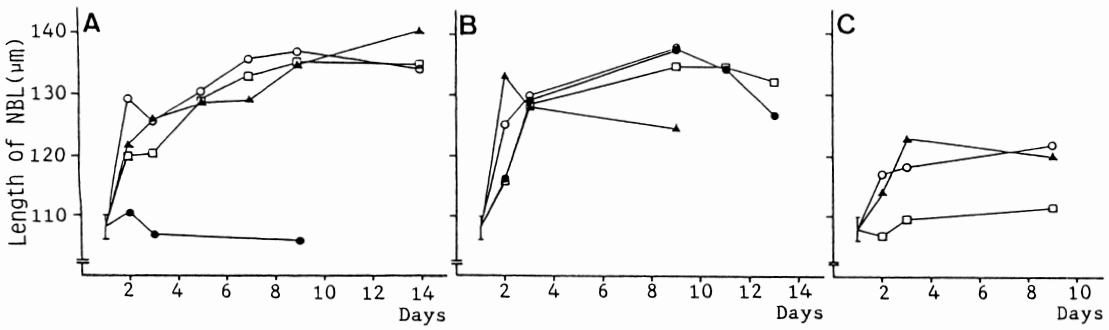

Fig. 1. The longitudinal growth of newborn larvae during the course of cultivation in M199 supplemented with various kinds and concentrations of sera.
A: fetal bovine serum (FBS), 0% (●), 10% (□), 20% (▲) and 30% (○) B: Each of 30% serum from ddY-mouse (○), Chinese hamster (▲), Japanese monkey (□) and man (●) C: Artificial FBS (Nu-S), 10% (□), 20% (▲) and 30% (○).

with 20% FBS of a lot examined here, namely, that survival rate showed more than 90% until day 3 and then rapidly decreased to as low as 7% on day 5 (Fig. 2).

Change in glucose content

Initial glucose concentration in M199 alone was 105 mg per dl. The glucose concentration in media with 10—30% FBS, 10—30% Nu-S, and 30% serum of ddY-mouse, Chinese hamster, Japanese monkey and man was 87—99, 108—116, and 112, 102, 83 and 93 mg per dl, respectively (Fig. 3).

In incubates of 650 newborn larvae per ml, no significant uptake of glucose was detected, while with 10,000 larvae/ml 20% of the initial glucose was consumed by day 9 (Fig. 4). On the other hand, muscle larvae at 650 per ml utilized 32% of the initial glucose content by 9 days. At 10,000 per ml, larvae consumed 88% of medium glucose by day 4 and 97% by 9 days (Fig. 4).

Change in amino acid content

Amino acid content did not change significantly in culture supernates of 650 newborn larvae per ml maintained for 9 days. With 10,000 newborn larvae per ml, alanine increased by 23% and aspartic acid decreased by 40% over 9 days (Fig. 5-A). There were no significant changes in the other components. When amino acid contents in culture medium were analyzed in the low den-

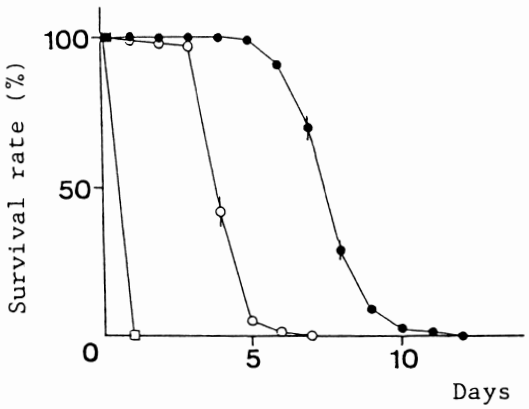

Fig. 2. Survival rates of newborn larvae during the course of cultivation with M199 alone (□) and containing each of 20% FBS of two different lots (●, ○).

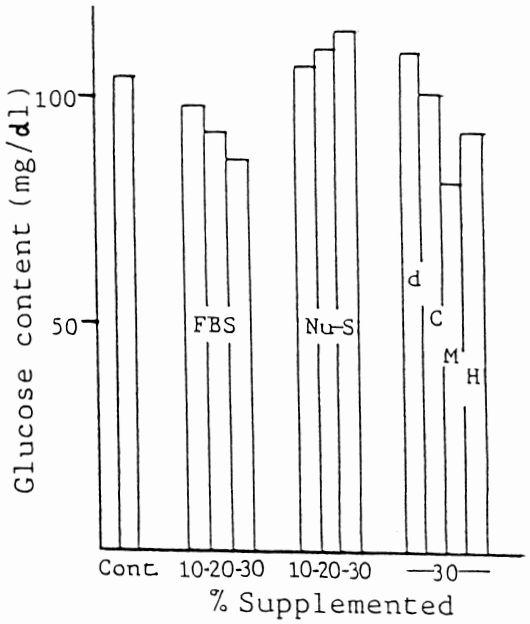

Fig. 3. Initial glucose content in M199 containing various kinds and concentrations of sera.
Cont: medium alone d: ddY-mouse serum C: Chinese hamster serum M: Japanese monkey serum H: man serum.

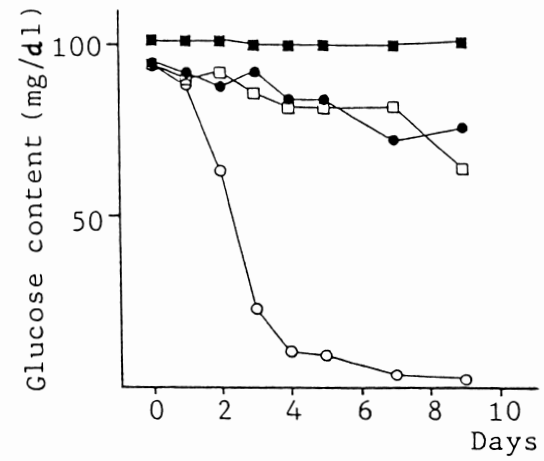

Fig. 4. Changes in glucose content during the course of cultivation in M199 with 20% FBS.
Newborn larva: 650 per ml (■) and 10,000 per ml (●) Muscle larva: 650 per ml (□) and 10,000 per ml (○).

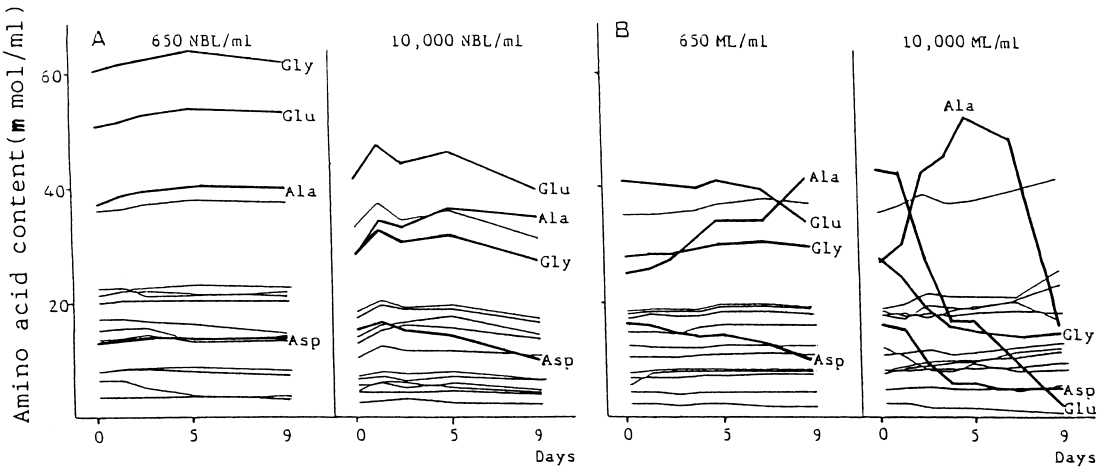

Fig. 5. Changes in concentrations of 16 kinds of amino acids during cultivation of larvae in low and high densities in M199 with 20% FBS. A: newborn larvae B: muscle larvae. Ala: alanine Asp: aspartic acid Glu: glutamic acid and Gly: glycine.

sity of 650 muscle larvae per ml, it was found that alanine gradually increased by 67%. On the contrary, aspartic acid and glutamic acid decreased by 38% and 17% respectively for 9 days. In the high density of muscle larvae, almost all of amino acid contents significantly changed throughout cultivation, especially alanine constantly increased from 27.3 to 52.0 m mol per ml (190% of initial content) for 5 days and then adversely decreased to 15.4 m mol per ml (50% of initial content) on day 9. Aspartic acid, glutamic acid and glysine decreased by 66%, 95% and 46%, respectively (Fig. 5-B).

Discussion

Salt solutions to which are added minute amounts of nutrient material (Keily 1914; Levin 1940) and some synthesized media (Kim 1962, Meerovich 1965) have been used to support the development and the molts of muscle larva into adult worm *in vitro*. Although some workers have described various immunological experiments involving newborn larvae (Ortega-Pierres *et al.* 1984, Gansmuller *et al.* 1987, Ching Hua Wang and R.G. Bell 1988), there have been no detailed reports of *in vitro* culture conditions for this stage.

In Medium 199 containing 10—30% FBS, newborn larvae grew from 108 to 135 μm in body length within 7 days and then terminated their growth. In contrast, they showed less growth in M199 alone or supplemented with 30% Nu-S. The limited growth of 25% of the initial body length of newborn larvae corresponded with the result reported by Despommier *et al.* (1975). They observed a stabilization of newborn larvae 2—3 days after intramuscular inoculation. Since such stabilization probably arose from stage-specific changes of both a physiological and a biochemical nature (Stewart and Read 1974, Farris and Harley 1977), it is possible that the *in vitro* growth observed herein was equivalent to that occurring during early intracellular stages *in vivo*.

Newborn larvae cultured in M199 with 30% Nu-S containing the largest amount of glucose showed less growth and poorer survival (Fig. 3 and 4) than those in medium with 30% FBS containing the least amount of glucose. These results indicate that a difference in glucose content is not responsible for the difference in larval growth and survival rate despite the fact that glucose is the essential energy source for the worms. It is suggested that some growth factor(s) in each serum

tested affects in *in vitro* growth of newborn larvae (Fig. 1). An observation similar to the situation observed in the present study for newborn larva had been made for muscle larva by Trakanov (1964), who described that serum from pig and rabbit was more suitable than bovine serum for cultivation. On the other hand, we found that the survival rates varied not only with concentrations of FBS added, but also with different lots of FBS. The factors in animal serum that affect the survival rate of newborn larva remain to be resolved.

The moderate changes in glucose content and quantitative composition of amino acids in the incubate 10,000 newborn larvae per ml were linked to the survival rate of the worms. The survival rate was 100% until day 5 with decrement thereafter (Fig. 2). Therefore, the present findings on glucose and amino acids consumptions reveal a certain aspect of nutritional requirement of newborn larvae.

It seems that newborn and muscle larvae metabolized glucose and amino acids similarly. The glucose uptake by newborn larvae showed only one-fifth of that by mature muscle larvae, and alanine and aspartic acid characteristically fluctuated in both newborn and muscle larvae. Regarding glucose and amino acids consumption of muscle larvae in the high density of 10,000 per ml, alanine content rose to a peak on day 5 postcultivation and then rapidly declined. Also, the glucose in the medium was rapidly consumed. Ninety percent of the initial glucose was utilized within the first 5 days. It is, therefore, probable that transamination is concerned with the metabolism of glucose, since the rapid increase of alanine was associated with the decrease in glucose levels in the medium. A similar situation mentioned above is seen with eggs of *Schistosoma japonicum* (Kawanaka *et al.* 1983) and of *Angiostrongylus cantonensis* (Takahashi *et al.* 1985) cultured *in vitro*.

These findings indicate that M199 supplemented with 20% FBS is useful in investigating the development of circulating and early intracellular stages of *Trichinella in vitro*. It is not necessary to supplement the medium with nutrient glucose and amino acids for the cultivation of newborn larvae for 2 weeks. These results contribute to our knowledge regarding the successful *in vitro* culture of *Trichinella* newborn larvae.

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