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Conditions of *in Vitro* Culture for Development of Microfilariae of *Dirofilaria immitis*

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Introduction

The first attempt to cultivate microfilariae *in vitro* was made by Earl (1959), Sawyer and Weinstein (1963). Weinstein (1963) subsequently observed the development of *D. immitis* microfilariae and *Wuchereria bancrofti* microfilariae, to the "sausage-form" in NCTC-109 medium with various supplements. Wood and Suitor (1966) reported that microfilariae of *Macacanema formosana* developed to the third stage larvae in serially passaged *Aedes aegypti* cell cultures. Cupp and Unthank (1971) reported the development of microfilariae of *D. immitis* and *D. corynodes* to the "sausage-form" in continuous cell line culture of *Aedes aegypti*, *Culiseta inornata*, and *Aedes vexans*. However little is still known about the factor(s) which are necessary for the development of microfilariae to the "sausage-form" *in vitro*.

The present paper describes the culture conditions for the development of *D. immitis* microfilariae to the "sausage-form" in various media and media in the presence of cultured cells.

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Materials and Methods

Preparation of microfilariae samples

Blood containing microfilariae (mff) of *D. immitis* was obtained from a naturally infected dog. In order to isolate the mff from the blood, the same procedure described in detail by Ando *et al.* (1980) was employed. The collected mff were washed three times in the culture medium supplemented with or without fetal bovine serum (FBS) according to the scheme of the experiments.

Cell lines and media

The continuous cell lines derived from *Aedes aegypti* whole larvae (Peleg, 1969) and *Culex molestus* adult ovaries (Kitamura, 1970) were maintained in Kitamura's M-41 medium (Kitamura, 1970) supplemented with 10% FBS at 27 C; HeLa and BHK-21 cells were maintained in MEM or TC-199 medium supplemented with 10% FBS at 27 C or 36 C, and NCTC-109 was used either with or without FBS. Kanamycin (60 μg/ml) was added to culture media used in the present experiments. The concentration of supplemented serum indicates the final concentration in each case.

Culture of microfilariae

Cultures were made in bottles measuring 15×15×150 mm. Test samples were prepared so as to contain approximately 2×10^2

mff per 0.1 ml of test medium with or without FBS. The 0.1 ml of prepared sample was put into a bottle to which 0.9 ml of the test medium had been added. Cultures were maintained at various temperatures and the culture media were not changed throughout the experiments.

When mff cultures were made with cells, 0.9 ml of cell suspension containing 3×10^5 cells per ml was inoculated into each bottle 24 hours before the mff were added and then 0.1 ml of sample was put into each bottle. Bottles were maintained at 27 C or 36 C without renewing the culture medium through the experiments. The developmental change of mff was checked by examination with a inverted microscope.

Effectiveness of culture media

In all experiments stimulation or inhibition of the growth of mff by the respective culture media was estimated by calculating the number of living and dead mff which were showing apparent morphological changes.

The types of the first stage larvae were classified according to the presentation of Sawyer and Weinstein (1963) as follows; type I (mff-type)-typical unchanged mff plus mff with uniform increase in width, type II (intermediate)- significantly widened larvae with a distinct decrease in length, type III "pre-sausage-form" showing significant changes in both length and width, and type IV fully developed "sausage-form". The percentage of each of the above types was based on the total number of specimens per culture.

Results

Optimum pH of culture fluid on survival of microfilariae

Effect of pH of culture fluid on mff survival was investigated between the pH range

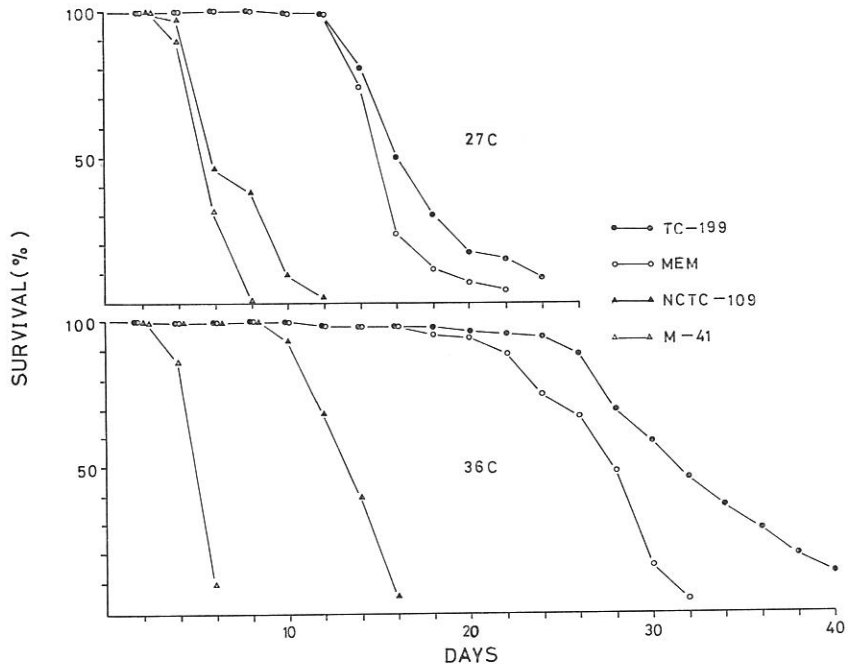

Fig. 1 The survival ratio in % of microfilariae in four media supplemented with 10% unheated FBS.
Each value indicates the mean of three replicates.

Table 1 The development of microfilariae in the presence of cultured cells

Medium*	Temp. (C)	Cell	Total† number	Development (%)‡ after 8 days LM-DM-LS-DS	after 16 days LM-DM-LS-DS	after 24 days LM-DM-LS-DS
M-41	27	—	699	1.3- 98.7-0 -0	0 -100.0-0 - 0	
M-41	27	Culex	675	100.0- 0 -0 -0	36.4- 55.3-2.0- 6.3	0 - 91.7-0-8.3
M-41	27	Aedes	632	97.2- 2.8-0 -0	7.2- 90.5-1.3- 1.0	0 - 97.7-0-2.3
MEM	27	—	675	100.0- 0 -0 -0	23.0- 77.0-0 - 1.0	1.5- 98.5-0-0
MEM	27	HeLa	598	99.2- 0.8-0 -0	98.0- 2.0-0 - 0	57.9- 42.1-0-0
MEM	27	BHK	494	100.0- 0 -0 -0	94.9- 5.1-0 - 0	6.3- 93.7-0-0
TC-199	27	—	622	99.8- 0.2-0 -0	50.2- 49.8-0 - 0	7.6- 92.4-0-0
TC-199	27	HeLa	600	99.5- 0.5-0 -0	98.8- 1.2-0 - 0	77.2- 22.8-0.0
TC-199	27	BHK	420	99.8- 0.2-0 -0	92.4- 7.6-0 - 0	51.2- 48.8-0-0
NCTC-109	27	—	610	29.1- 62.4-8.5-0	0 - 70.9-0 -29.1	
M-41	36	—	711	0 -100.0-0 -0		
MEM	36	—	704	99.7- 0.3-0 -0	98.6- 1.4-0 - 0	74.7- 25.3-0-0
MEM	36	HeLa	465	100.0- 0 -0 -0	100.0- 0 -0 - 0	0.6- 99.4-0-0
MEM	36	BHK	460	100.0- 0 -0 -0	99.8- 0.2-0 - 0	0 -100.0-0-0
TC-199	36	—	662	99.8- 0.2-0 -0	98.8- 1.2-0 - 0	95.5- 4.5-0-0
TC-199	36	HeLa	494	99.4- 0.6-0 -0	99.2- 0.8-0 - 0	97.8- 2.2-0-0
TC-199	36	BHK	665	100.0- 0 -0 -0	99.5- 0.5-0 - 0	69.2- 30.8-0-0
NCTC-109	36	—	565	98.6- 1.4-0 -0	6.2- 93.8-0 - 0	0 -100.0-0-0

* All media were supplemented with 10% unheated FBS.

† Total number of microfilariae collected from three bottles.

‡ Each abbreviation means percentage of; LM: living type I plus II, DM: dead type I plus II, LS: living type III plus IV, DS: dead type III plus IV.

of 6.0 to 8.8 with both MEM and TC-199 medium supplemented with 10% FBS at 27 C. The optimum pH supporting relative long term survival of mff was 7.2 to 7.6 in both media. In the following experiments, therefore, the pH was adjusted to 7.4±0.2 every three days by the addition of 0.5% HCl solution when the culture contained no cells, and by the addition of 5% $NaHCO_3$ when cells were present in the cultures.

Survival period in various media

Fig. 1 shows the survival ratio of mff in four respective media supplemented with 10% unheated FBS at 27 C and 36 C *in vitro*. The survival ratio and motility of mff at 36 C were higher than that at 27 C in general. Especially, mff moved as actively as in fresh dog blood up to 16 days after being transferred to 36 C in both MEM and TC-199 medium. The NCTC-109 medium supported the development of mff at 27 C but not at 36 C. No morphological change of mff was observed in the other three media at either temperature. When serum was not added, mff could survive for 1 day in M-41, 2 days in MEM, 5 days in TC-199, and 7 days in NCTC-109 medium at 36 C, but they could not develop at either 27 C or 36 C.

Development in the media with cultured cells

The development of mff in the presence of cultured mosquito or mammalian cells was examined in different media, and the results on mff survival are summarized in Table 1. The M-41 medium with mosquito cells and NCTC-109 medium without cells were effective in advancing the development of mff to type IV at 27 C (Fig. 2). Small numbers of mff developed to the "pre-sausage-form" within 120 hrs in M-41 medium with mosquito cells and within 96 hrs in NCTC-109 medium. No morphological change of mff was observed in the experi-

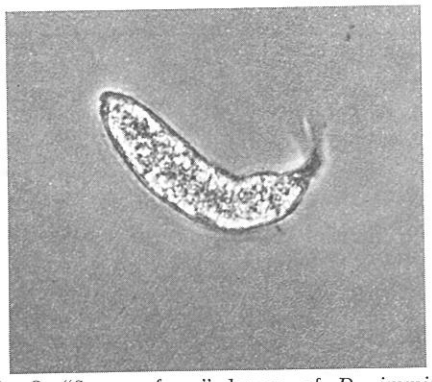

Fig. 2 "Sausage-form" larvae of *D. immitis* in a culture of *C. molestus* cells on day 10 (×482).

ments in which the other media with or without cells were used.

Cultures in NCTC-109 medium

At first, survival ratio was investigated with different amounts of unheated serum supplement. The survival time of mff was prolonged relative to the increase in the amount of serum supplement at 36 C, but no morphological change was observed. On the contrary, the survival period was reduced relative to the increase of supplemented serum at 27 C and morphological changes of mff were observed.

The number of "developing forms" relative to the serum concentration are shown in Table 2. In our preliminary experiment the number of "developing forms" was reduced slightly when heat-inactivated serum was added.

Table 2 The development of microfilariae in NCTC-109 medium supplemented with different amounts of unheated serum at 27C after 16 days culture

FBS (%)	Total* number	Development (%)			
		Type I	Type II	Type III	Type IV
20	677	58.6	19.4	14.6	7.3
10	610	56.2	14.6	17.5	11.6
5	611	39.9	21.6	22.1	16.4
2.5	613	29.4	26.9	25.3	18.4

* Total number of microfilariae collected from three bottles

In the present study it was observed that the number of "developing forms" in NCTC-109 medium supplemented with 2.5 and 10% FBS was inversely related to changes in temperature (Table 3). The number of "developing forms" increased as the temperature fell, but this increase generally did not occur under 27 C, but occasionally it was observed that the number at 24 C was lower than that at 27 C.

Table 3 The temperature-dependent development of microfilariae in NCTC-109 medium supplemented with 2.5 and 10% unheated FBS after 16 days culture

FBS (%)	Temperature (C)	Total* Number	Development (%)			
			Type I	Type II	Type III	Type IV
2.5	36	905	100.0	0	0	0
	33	733	95.6	3.3	0.8	0.4
	30	745	67.1	16.8	9.3	7.0
	27	759	32.7	27.9	23.1	16.3
	24	700	32.7	29.3	22.0	16.0
10.0	36	850	97.4	2.5	0.1	0
	33	903	88.5	7.2	3.3	1.0
	30	849	74.6	11.1	9.1	5.3
	27	922	46.4	21.6	16.7	15.1
	24	797	48.6	23.5	15.6	14.1

* Total number of microfilariae collected from four bottles

Discussion

The *in vitro* growth of *D. immitis* mff was studied under various conditions. Culture medium supplemented with unheated serum prolonged the survival period and supported the development of *D. immitis* mff. The survival ratio of mff in TC-199 medium supplemented with 10% unheated FBS was the highest among four kinds of media tested but no morphological change was observed. The NCTC-109 with serum and M-41 medium containing mosquito cells with serum were the appropriate combinations which supported the development of mff to "developing forms" in the present experiment.

The optimum amount of additional serum in NCTC-109 medium for development was found to be 2.5% of unheated FBS in our experiment, whereas Sawyer and Weinstein (1963) reported that optimum concentration was 5% and heated horse serum was much better than unheated serum. On the other hand, Klein and Bradley (1974) reported that the optimum concentration of additional heated pony serum was 20%. These data suggest that promoting and/or inhibiting factor(s) for the development of mff in individual serum might be different in quality and quantity. Klein and Bradley (1974) also reported the development of *D. immitis* mff to "developing forms" using Grace's medium supplemented with serum. In spite of having a higher surviving effect on mff, TC-199 medium did not support development, but NCTC-109 medium supported development in spite of its low effect on survival of mff. Therefore, certain substances necessary for the development of mff might be included in both NCTC-109 and Grace's medium.

This assumption was reconsidered in the experiments employing culture media containing cultured cells and it came clear that M-41 medium itself did not support the development of mff but supported the development of mff when used with mosquito cells. This result suggested that certain necessary metabolites for mff development were emitted into the medium by mosquito cells. It is also possible that the cells may have removed some substance from the medium which inhibited development. The same assumption arisen from the results by Cupp (1972) is that development of mff to the late L_1 stage was apparently enhanced by the addition of insect hemolymph. This enhancement was considered to be the effect of mosquito cells but not the hemolymph itself on immature filariae.

Another consideration arisen from our experiments was that the culture medium with mosquito cells supported the development of mff but those with mammalian cells did not.

The number of observed "developing forms" was related to the temperature conditions and optimum temperature for development of mff was below 30 C *in vitro*. Mosquitoes live well below 30 C, so body temperature of them would also be below 30 C. It is considered that this temperature-relating development *in vitro* could be the nature of mff itself.

Further studies are presently under way to have more precise information on the factors necessary for development of mff under *in vitro* conditions.

Summary

The microfilariae of *Dirofilaria immitis* were cultured in four media (TC-199, MEM, M-41, NCTC-109). Although the microfilariae survived the longest in TC-199 they did not develop in this medium. NCTC-109 medium, however, supported development in spite of the low number of microfilariae which survived in it. M-41 medium containing mosquito cells supported the development of microfilariae, but neither TC-199 nor MEM medium in association with mammalian cells did. In NCTC-109

medium the number of "developing forms" changed in relation to the additional amount of serum and the number of "developing forms" increased as the culture temperature decreased.

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References

1) Ando, K., Mitsuhashi, J. and Kitamura, S. (1980): Uptake of amino acids and glucose by microfilariae of *Dirofilaria immitis in vitro*. Am. J. Trop. Med. Myg., 29, 213–216.
2) Cupp, E. W. and Unthank, H. D. (1971): *In vitro* cultivation of filariae using continuous cell lines. U.S.-Japan Joint Parasitic Diseases Conference.
3) Cupp, E. W. (1972): Development of filariae in mosquito cell cultures. U.S.-Japan Joint Parasitic Diseases Conference.
4) Earl, P. R. (1959): Filariae from the dog *in vitro*. Ann. N. Y. Acad. Sci., 77, 163–175.
5) Kitamura, S. (1970): Establishment of cell line from *Culex* mosquito. Kobe J. Med. Sci., 16, 41–50.
6) Klein, J. B. and Bradley, R. E. (1974): Induction of morphological changes in microfilariae from *Dirofilaria immitis* by *in vitro* culture techniques. J. Parasit., 60, 649.
7) Peleg, J. (1969): Inapparent persistent virus infection in continuously grown *Aedes aegypti* mosquito cells. J. Gen. Vir., 5, 463–471.
8) Sawyer, T. K. and Weinstein, P. P. (1963): The *in vitro* development of microfilariae of the dog heartworm *Dirofilaria immitis* to the "sausage form". J. Parasit., 49, 218–224.
9) Weinstein, P. P. (1963): Development *in vitro* of the microfilariae of *Wuchereria bancrofti* and of *Litomosoides carinii,* as far as the sausage forms. Trans. Roy. Soc. Trop. Med. Hyg., 57, 236.
10) Wood, D. E. and Suitor, E. C. (1966): *In vitro* development of microfilariae of *Macacanema formosana* in mosquito cell cultures. Nature, 211, 868–870.

In vitro における *D. immitis* のミクロフィラリアの発育条件

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D. immitis のミクロフィラリアを4種類の培地（TC-199, MEM, M-41, NCTC-109）で培養した結果，生存率は TC-199 が最良であったが，いずれの培地でも 36C ではミクロフィラリアに発育は認められなかった．しかし 27C において昆虫株細胞を含む M-41 では育発が認められ，第一期後期幼虫にまで発育したが哺乳動物株細胞を含む培地（TC-199, MEM）では発育は認められなかった．NCTC-109 においても発育が認められたがその発育状況は牛胎児血清の濃度が 2.5% の時に最良であり，36, 33, 30, 27, 24C の各温度で培養した結果，温度低下につれて第一期後期幼虫に達する割合は増加し，27 又は 24C が最良であった．