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Selective recovery of living microfilariae from *Onchocerca volvulus* nodules

Determination of optimal conditions for their culture in vitro for excretory/secretory products

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Summary

A method for the selective recovery of living microfilariae from *Onchocerca volvulus* nodules is described. The microfilariae migrate through solidified agar gel into overlaying Hanks balanced salt solution (HBSS). The highest recovery rates of the worms were obtained with 0.3 and 0.4% agar. Optimal conditions for in vitro culture of the larvae in HBSS were established: pH range 7.0–7.5, glucose concentration 2–5 mg/ml for long term cultures, osmolality 200–309 mOsmoles/l, temperature 4° C for prolonged cultures and 24–28° C for overall best yield of excretory/secretory products (ESP). Subculturing of the larvae reduced contamination of ESP with human serum protein to minimal amounts after 9 recultures done within 96 h.

Key words: *Onchocerca volvulus*; microfilariae; in vitro culture; excretory/secretory products.

Parasitization by *Onchocerca volvulus* is associated, in man, with a myriad of clinical lesions which may be disfiguring, disabling or sometimes fatal. Treatment with filaricidal drugs is hazardous, especially in heavily infected persons (Bryceson et al., 1977). However, if this is instituted early or in lightly infected persons, the results are satisfactory and the risks to the patients minimal. Prevention by chemoprophylaxis is for the present not possible, and vector control measures are beyond the economic means of most countries endemic for this disease. Alternative methods, rapidly gaining credence, are the early detection

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of infection or re-infection and prompt treatment with filaricidal drugs, and vaccination to induce protection against one of the larval stages. Either of these involves indepth immunological studies, which so far have been limited because of lack of a suitable laboratory animal model for this infection. An alternative approach is the in vitro maintenance and cultivation of the parasite for these studies.

Schiller et al. (1979) described a method for long term in vitro culture of microfilariae using bi-phasic medium incorporating rabbit red blood cells, serum and nutrient agar. This method is suitable for morphologic studies but not for immunochemical studies on worm antigens since incorporation of rabbit protein into the culture medium and also the presence of significant human protein contaminants from unpurified worm source preclude obtaining pure excretory/secretory products (ESP). We recently showed that onchocerca supernatants (OS) obtained from in vitro culture of living microfilariae in Hanks' balanced salt solution (HBSS) contain allergens that can be employed in a sensitive, discriminatory, immediate-type hypersensitivity skin reaction for the diagnosis of on-going *O. volvulus* infection (Ngu et al., 1981). We describe here a simple method for the selective recovery of living microfilariae from nodules, and optimal physicochemical conditions for their maintenance in vitro for the production of ESP. The ESP obtained after several subculturing of the worms elicited immediate-type hypersensitivity skin reaction in onchocercal patients and was only minimally contaminated with human serum protein.

Materials and methods

Reagents. Hanks balanced salt solution (HBSS) was either purchased from Gibco or prepared as described (Hanks and Wallace, 1949) and sterilized by passage through a sterile Seitz viral filter. In a few cases HBSS containing 25 mM Hepes buffer (Gibco) was used as culture medium. RPMI was obtained from Gibco. Purified Nobel agar was purchased from Oxoid Ltd. England. All other reagents were of the highest purity available from Merck, Sigma and Prolabo (France).

Patients. Onchocerca patients were seen at the M'banjock dispensary or at the Yaounde Central Hospital. Onchocerciasis was diagnosed by the demonstration of microfilariae in skin biopsies, or by the presence of onchocerca nodules, and by a positive skin-test using onchocerca supernatants as described by Ngu et al. (1981).

Procedure for obtaining microfilariae from nodules. Onchocercal nodules were excised under aseptic conditions and freed of fat and adherent tissue. The nodules were then minced in HBSS containing penicillin (100 IU/ml) and streptomycin (100 µg/ml). 6 h later the supernatant was aspirated and the number of microfilariae per 100 µl determined by microscopic enumeration. For each set of experiments the same numbers of microfilariae in HBSS were added to melted agar to give a final volume of 2 ml, at final agar concentrations of 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1%. The worm/agar mixture was poured into individual sterile culture dishes (Falcon, 3001) and allowed to set. The agar was thereafter layered with 2 ml HBSS containing penicillin and streptomycin. 15 h later, the HBSS was aspirated, the number of emergent microfilariae determined in three samples of 100 µl from each culture dish and the total recovery rate calculated. All experiments were done in triplicate and using several batches of nodules obtained from different patients.

Determination of optimal physical and chemical conditions for the maintenance of live microfilariae in vitro for the production of ESP. Living microfilariae obtained as described above were used for the various experiments at a concentration of 1,000 microfilariae per ml HBSS-penicillin/streptomycin, or RPMI 1640 (Gibco) penicillin/streptomycin – the same batch of microfilariae being used in all cultures whenever each parameter was investigated. The parameters tested in these experiments were variables of the following:

- a) pH (6.5, 7.0, 8.5 modified by using either IM NaOH or IM HCL),
- b) temperature (0° C, room temperature: 25–27° C, 37° C, 42° C),
- c) osmolality (200, 250, 309, 350, 400, 450 mOsmoles per litre); the osmolality was varied by changing the NaCl concentration in HBSS whilst maintaining constant glucose concentration;
- d) media: Hanks balanced salt solution or RPMI 1640 (Gibco) were used.

Viability of microfilariae. Mobility and morphologic appearance of the worms were used as criteria for viability, as described by Schiller et al. (1979). Microfilariae in 200 µl of the respective incubation mixtures were counted after 6, 12 and 24 h and the percentage of living and dead worms determined. The experiments were carried out in triplicate.

Long term cultures were attempted using the optimal conditions established from the various experiments listed above. At appropriate intervals (of about 12–24 h) the microfilariae were sedimented by centrifugation at 5,000 g for 30 min and 4° C in a Beckman JA21B refrigerated centrifuge and resuspended in fresh medium.

Experiments with Onchocerca supernatants and excretory/secretory products. Onchocerca supernatants (OS) were obtained as previously described (Ngu et al., 1981); OS were essentially the supernatant from the overlaying HBSS in which the migrant microfilariae had been left for a while to metabolize.

Excretory/secretory products (ESP) on the other hand were obtained by reculturing several times large numbers of actively motile larvae under optimal conditions previously established for their survival in vitro in HBSS. Cultures were maintained at 4° C, but some were done at room temperature. The supernatants (ESP) obtained when the medium was changed at each stage of reculture were stored separately at 20° C until used. Both OS and ESP were concentrated before further studied. The protein content of OS and the various ESP samples obtained from cultures done at 4° C was determined by the method of Lowry et al. (1951). Details are given in Table 1.

Table 1. Periods of reculture of microfilariae and ESP samples

Supernatant	End of subculture (in h from start of experiment)	Protein content µg/h/100,000 microfilariae
1	1	26.6
2	2	23.2
3	3	10.0
4	16	1.3
5	19	5.5
6	36	3.5
7	48	4.7
8	72	1.7
9	96	2.9

0.5×10^6 microfilariae were cultured in 10 ml HBSS containing 1 mg glucose/ml and penicillin/streptomycin, at 4° C, pH 7.0, osmolality 309 mOsmoles/l. Viability for this experiment was >90%. The supernatants obtained, after centrifugation, at various periods of subculture were each concentrated to 2 ml before protein determination was done. No corrections were made for protein lost during concentration.

Results

Optimal agar concentration for selective recovery of living microfilariae

All microfilariae examined that had migrated through agar into the over-layering HBSS were actively motile. The optimal agar concentrations giving the highest yields (average recovery in three experiments: 37–44%) were 0.3 to 0.4%. Under our experimental conditions 0.2% agar did not solidify adequately, and so was not studied further.

Optimal physical/chemical conditions for survival of microfilariae in vitro

a) *Medium.* Maximal survival of microfilariae over a 24 h period was obtained at pH 7.0 and 7.5 in both HBSS pen./strep. and RPMI 1640 pen./strep., but the overall survival rate was better in HBSS than in RPMI at 25–27° C (Fig. 1).

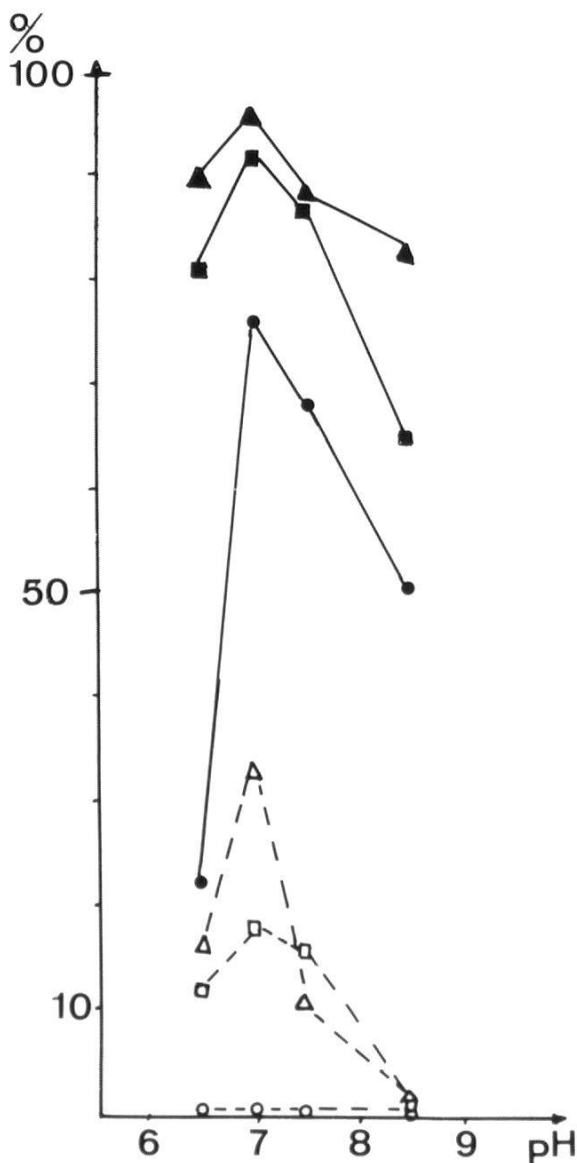


Fig. 1. Mean percentage of survival of microfilariae (2,000 per culture dish) at various pH values in Hanks balanced salt solution (solid lines) and RPMI 1640 (broken lines). Incubation times were 6 h (▲, △), 12 h (■, □) and 24 h (●, ○). Glucose concentration: 1.5 g/l. Temperature: 25–27° C.

b) *Temperature*. Over a 24 h period microfilariae cultured in HBSS survived best at 4° C, followed next by 37° C. Similar results were obtained with microfilariae from different batches of nodules.

c) *Glucose concentration*. Cultures at 4° C when examined on day 4 showed microfilariae survival rates of 19–30% in HBSS containing 2 mg/ml to 5 mg/ml. However, with some batches of nodules, survival rates of up to 90% were observed in cultures containing glucose at 2 mg per ml. When the glucose content in the medium was varied, but the osmolality kept constant and cultures done at room temperature, optimal glucose concentrations for microfilariae survival over a 42 h period were 1.5 and 2 mg/ml.

d) *Osmolality*. When the osmolality was varied, but glucose concentration constant at 2 mg/ml and culture done at room temperature, maximal survival of microfilariae was obtained in HBSS in the range of 200–309 mOsmol/l.

Prolonged maintenance of microfilariae

Using optimal conditions for survival established above, namely, HBSS pH 7.0, 2.0 mg/ml glucose, osmolality of 309 Osmoles/l, temperature of 4° C, microfilariae were cultured at a concentration of 1,000/ml in a final volume of 20 ml per petri dish. Periods of survival varied between different batches of nodules. Certain batches yielded microfilariae which were alive on the 12th day some of which appeared to have increased in size.

Discussion

The method described here for selective recovery of live microfilariae from nodular tissue offers distinct advantages of simplicity, high recovery rate of viable worms and a means of obtaining for further studies a homogeneous group of each larval stage. The differences observed between batches of nodules in the yield of migrant microfilariae is not explicable from these studies, but it is possible that viability and motility of worms from different sources vary. Heterogeneity of host immune response, both humoral and cellular, occurs with this infection (Ngu and Blackett, 1976; Ngu, 1978; Barlett et al., 1978). It is possible as a result of this that worm viability and mobility will vary between hosts. Further, some patients had taken filaricidal drugs in varying doses prior to nodulectomy. Perhaps this affects the migrating capabilities of their microfilariae through solidified agar.

From our experiments, we conclude that the pH of the medium is a very important factor affecting survival of microfilariae in vitro, pH 7–7.5 being the best range. The worms survive longer at 4° C than at higher temperatures probably because of decreased metabolic rate at that temperature. There was a wide range of osmolality at which survival occurred provided the pH of the medium was maintained between 7.0 and 7.5.

The criteria for determining viability of microfilariae was crucial in these

studies. Attempts to use exclusion of trypan blue or neutral red as a test of viability of worms was not successful probably because the filarial cuticle provides an effective physical barrier to these stains. The cited viability of 12 days for the long term cultures under established optimal conditions was the last time at which some microfilariae were seen (by at least 5 persons) to be actively motile. It is possible that from the 13th day onward, some of these worms were still alive though not showing any vigorous movement, since one observer noticed some twitching of worms at day 29. These attempts to keep microfilariae alive in vitro for prolonged periods would lead to the production of larger quantities of ESP the importance of which in immunodiagnosis, has already been described (Ngu et al., 1981).

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