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PERSPECTIVES ON THE IN VITRO CULTURE OF FILARIAE

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SUMMARY

A primary constraint in the culture of human filariae is in obtaining starting material—either microfilariae (mfs), which infect invertebrates, or third stage larvae (L3s), which are infective to humans. Cryopreservation methods which partially overcome this difficulty have been developed for both mfs and L3s. Complete development of mfs to L3s outside an intact host was obtained recently when mosquito thoraces infected by *Brugia malayi* (24 h after the bloodmeal) were maintained in vitro. In another recent study in which no host tissues were present, a semidefined culture medium was used to investigate the properties of reduced glutathione (GSH) that stimulate early development of *Onchocerca lienalis* mfs. An extended cysteinyl backbone and a free sulfhydryl were identified as the key structural elements provided by GSH. Stimulation also required the presence of low and high molecular weight components of serum as well as oxygen. Molting of *Onchocerca* spp. L3s to the fourth stage at the rate of 50 to 70% has been reported by several researchers. Key factors identified in those successes have been temperature and serum lot. Improved long-term viability occurred with cellular co-culture. Beneficial effects of co-culture were shown to be due both to cellular conditioning of the medium as well as to lowered dissolved oxygen levels as a result of cellular metabolism. With the use of cell-conditioned medium and decreased incubator oxygen levels, long-term viability of *Onchocerca* larvae in culture exceeded that previously reported. Recently, *Brugia malayi* adults of both sexes were cultured from L3s using a semidefined medium supplemented with human serum. Many of these sexually matured adults mated and produced viable microfilariae.

Key words: filariae; in vitro; *Onchocerca*; *Brugia*; culture; GSH.

The World Health Organization (WHO) has estimated that more than 200 million persons are infected with filariae, which can result in debilitating diseases such as bancroftian filariasis (elephantiasis) and onchocerciasis (river blindness). This latter disease, affecting nearly 11 million persons, is the second leading cause of infectious blindness in the world; at least 663 000 persons are irreversibly blind as a result of chronic onchocerciasis. Thus, filariases have been designated by the WHO among the key diseases targeted for control efforts. In addition, *Dirofilaria immitis* or "dog heartworm" causes cardiac congestion in dogs and has spread to large portions of the United States due to efficient transmission by a variety of mosquito species. Control of filariasis is complicated because transmission by blood-sucking insects can amplify both the number and intensity of infections.

As few acceptable animal models are available for investigating the biology and treatment of human filariasis, the development of in vitro culture techniques would serve to provide research opportunities for several areas, including vaccine development, drug testing, and metabolic studies (24). In addition, insight into important biochemical and nutritional aspects of worm development may be suggested by the components that produce successful culture even though that success may be derived empirically.

In this examination of the potential for in vitro culture of filariae, I will not attempt to include discussion of all filariae, but will focus on species that are either of major importance in human and animal health or serve as commonly used experimental models (Table 1). Within that subset of filariae, I will primarily be discussing those parasites that have been used for in vitro culture in the recent past.

In vivo, filariae progress through seven morphologically distinct developmental stages: three are unique to their mammalian hosts and two occur only in the blood-sucking arthropod. Microfilariae and third stage larvae (L3s) are the infective stages and thus exist in both hosts. Microfilariae are ingested from infected vertebrates by arthropods (usually insects) during blood-feeding. Shortly after entering the new arthropod host, the very motile microfilariae (mfs) move to a specific anatomical site where they become less physically active and initiate differentiation. This process can be recognized by the physical changes that characterize the first stage larva (L1); the long, slender mf becomes shorter and wider. Two molts produce the second stage (L2), then the third stage larva (L3) after 6 to 14 days, depending on the filaria species. L3s are physically active and move from their site of differentiation and growth to their host's mouthparts. L3s initiate or intensify infection in vertebrates when they move to the new host from the vector's mouthparts while it is blood-feeding. Within the new environment, each species of L3s moves to a specific anatomical site, where further differentiation occurs. Successive molts release a fourth larval stage (L4) and a juvenile form, which undergoes further growth and sexual development before reproductive capability is attained. This process of maturation can take from 6 to 12 mo. in human infections (2). After multiple inseminations, millions of embryos can be produced by a fertile filariid female during a lifetime of as many as 8 to 15 yr for some species.

TABLE 1

FILARIAL PARASITES—SOME MEDICALLY IMPORTANT SPECIES THAT HAVE BEEN CULTURED IN VITRO

Taxon	Invertebrate Host	Site of Development	Vertebrate Host	Site(s) of Development
Onchocerca lienalis	black fly	thoracic muscles	bovine	skin & gastrosplenic ligament
Onchocerca volvulus	black fly	thoracic muscles	human	skin and in nodules
Wuchereria bancrofti	mosquito	thoracic muscles	human	blood & lymphatic system
Brugia malayi	mosquito	thoracic muscles	human, other animals	blood & lymphatic system
Dirofilaria immitis	mosquito	malpighian tubules	dogs, cats	circulatory system (heart)

Fully developed embryos, designated mfs, are released from the uterus of gravid females. They either retain the egg membrane (for example *Brugia* or Wuchereria spp.), or shed it within the uterus (*Onchocerca* or *Dirofilaria* spp.) and thus are termed "sheathed" or "unsheathed" mfs. Although all mfs of a species within the host may seem to be identical morphologically, apparently physiologic changes must occur before they can successfully parasitize their invertebrate host. For example, mfs taken from nodular tissues surrounding adult female *O. volvulus* do not develop to L3s when inoculated into black flies. This is in contrast to mfs obtained from the subdermal region, where they subsequently locate in readiness for uptake during black fly feeding.

Efforts to culture filariae in vitro have been in progress for a number of years but, in spite of some apparent breakthroughs, routine culture of complete or partial cycles that normally occur within invertebrate or vertebrate hosts has not been obtained. Initiation of culture usually begins with mfs or L3s. Infective mfs can be obtained from mammalian hosts in either peripheral blood (*Dirofilaria, Brugia, Wuchereria*) or superficial skin snips (*Onchocerca*). To obtain L3s for culture, the appropriate insect may be inoculated by feeding on an infected mammalian host or by intrathoracic injection of mfs (for example, *see* 10). When worms have developed to L3s, they can be collected from the mouthparts or the crushed body of the vector, rinsed of tissue, and then used for initiating culture. Cryopreservation techniques have been developed for mfs of several filariids to provide a ready supply for culture of that particular stage or for infecting insects (9,11). The success of freezing procedures was confirmed by the ability of cryopreserved mfs to complete development to L3s after ingestion or inoculation into the appropriate vector. Cryopreserved L3s also have been recovered as active larvae that develop to mature adults when inoculated into suitable mammalian hosts (12). Not only do such cryopreservation methods allow cultures to be initiated in synchrony, they also provide starting material without the need for each research investigator to maintain insect colonies or vertebrate hosts.

Parasite localization within distinct hosts and tissues at different stages of development suggests that particular microhabitats provide unique components essential to growth or differentiation or both. Depending on the species, mfs migrate to intracellular locations in the Malpighian tubules (*Dirofilaria immitis*), fat body or thoracic muscles (*Onchocerca, Brugia, Wuchereria*) shortly after being ingested. Some cue, either physical or metabolic, may be available to signal the location of the appropriate developmental niche. The presence of critical developmental factors that are localized to a specific anatomical site was suggested in experiments by Nayar et al. (15). They demonstrated that conditions needed to support development of *D. immitis* mfs to L3s were uniquely associated with Malpighian tubules of genetically susceptible hosts. Transferring Malpighian tubules (24 h after infection) with mfs from a nonsusceptible host to the hemocoel of a susceptible host did not support significant development to the L3 stage, whereas reciprocal transfers, i.e. infected tubules from susceptible to nonsusceptible hosts, provided the required physiologic niche to complete normal development. Thus, all components needed for development were provided within the cellular milieu of the appropriate Malpighian tubule. Further support for a tissue-specific requirement was provided by the demonstration that mosquito thoraces maintained in vitro, after invasion in vivo by *Brugia malayi* or *B. pahangi* mfs, could support development to the L3 (14). When mfs were cultured extracellularly in a similar environment they developed only to the second stage. Dietary supplementation of mosquito hosts with *p*-aminobenzoic acid (PABA) and an antibiotic and antimycotic solution beginning 6 days before infection significantly increased the final number of L3s produced. In addition, components required to maintain the excised, infected thoraces included a biphasic culture medium composed of an agar layer base covered by a 50:50 mixture of Schneider's and Grace's media, which was supplemented with 20% fetal bovine serum (FBS) and 1% solution of antibiotic and antimycotic. In each case, growth to L3 was associated with an intracellular site of development. Thus a focus on the specific physiology and metabolism of Malpighian tubules or thoracic muscles should provide important insights into the environment required to support growth and development in vitro of the different filariae normally inhabiting those sites.

Host nutrition has been shown to influence directly parasite development both in vivo and in vitro. Oral supplementation of adult *Aedes aegypti* mosquitoes with folic acid or the subunit, PABA, increased the number of L3s that developed both in vivo (18) and in thoracic muscle organ cultures (14). Nutrients and metabolic conditions likely to occur in higher concentrations in insect tissues where worms develop, such as trehalose, folic acid, proline, lipids, and oxidative metabolism within thoracic muscles, should also be investigated for their effects in vitro.

Along those lines, initial development of *Onchocerca lienalis* microfilariae to late first stage larvae in response to glutathione (GSH) was studied (17). Titration of GSH showed that peak effects occurred when the tripeptide was added at 15 m*M*, which is similar to normal intracellular levels of eukaryotic cells. Using various structural analogues, the effects of GSH were shown to be contributed by its free sulfhydryl group and an extended cysteinyl backbone. Cysteine was not limiting because the addition of that amino acid alone was not effective, nor was the GSH effect neutralized when inhibitors of gamma glutamyl transpeptidase were present. Addition of specific sugars such as glucose, fructose, or trehalose to the L-15

based medium further augmented the GSH effect (16). The FBS component was examined by fractionation of sera into three subsets based on molecular weights (<10 000, 10 000 to 30 000, and >30 000 Da). Testing the effects of each subfraction alone, or in combination, led to the identification of the <10 kDa and >30 kDa fractions as contributory to the positive effects of FBS. Further analysis showed that the high molecular weight component of FBS could be replaced by very high levels (>1 m*M*) of Zn^{++} or Mn^{++}. Taken together, these studies suggest that GSH supplementation of FBS-containing medium served to augment the non-protein thiol pool that is known to be utilized in a variety of eukaryotic cellular reactions.

Movement of L3s from the arthropod vector to new mammalian hosts presents these worms with a drastic change in environment. The most immediate and obvious change is an increase in temperature that perhaps initiates a heat shock response (23). Heat shock protein (HSP) 70 genes were identified in a genomic library of *Onchocerca volvulus* by screening with sera from persons in endemic filariasis areas (20). As an increase in temperature to 37° C is a requirement to initiate development of L3s to fourth stage larvae (1), perhaps heat shock proteins play a role in that process and are released during molting, whereupon host antibodies are produced.

L3s also encounter oxygen tensions within new sites of residence (skin or lymphatics) that are low and may be critical for continued development. In vitro culture of the skin-dwelling rodent parasite, *Acanthocheilonema* (=*Dipetalonema*) *viteae*, was shown to be enhanced by oxygen tension between 20 and 50 torr, the range that occurs subcutaneously (8).

Molting and growth of L3s and maintenance of L4s and adults of several filarial species can be enhanced by the use of worm and cell co-culture (4,5,21,22). One of the cell lines used more commonly for that purpose is the Rhesus monkey kidney cell line ($LLCMK_2$). In our studies of worm and cell co-culture we recorded decreasing levels of dissolved oxygen in the medium associated with the formation of the cellular monolayer (4). Decreased oxygen in the medium bathing the cells was also maintained after the monolayer was formed, even though the incubator was continuously gassed with 95% air:5% CO_2. We speculated that the lowered oxygen levels might partially account for beneficial effects of monolayer-filariae co-culture.

The beneficial effects of reduced oxygen on parasites cultured in vitro could be demonstrated, however, only in the presence of other changes associated with cellular metabolism. Cell-conditioned medium, in the presence of normal (95% air, approximately 20% oxygen) or reduced oxygen (9% air, approximately 2.5% oxygen), significantly enhanced the growth and development of *Onchocerca* larvae over that achieved in non-conditioned medium (4). In the presence of cell-conditioned medium and reduced oxygen, worm development equaled or exceeded that achieved in worm-cell co-cultures. It has not been determined whether the cellular effects were the result of removal of a toxic substance(s), the addition of a growth enhancing component(s), or both. Development of worms to mature adults was not achieved in that system, suggesting that a microenvironment more similar to the one where the worms locate in vivo may be required. Perhaps medium conditioned by keratinocytes or fibroblasts or both would provide metabolites or growth factors of different composition or concentrations required by filariae that undergo differentiation and growth subcutaneously.

Success in culturing mammalian stages of filariae also seems to be highly dependent on unknown factors in serum. Commercially available FBS has been used most commonly as a supplement for those cultures. Variation in diet and health status of the cow, in addition to the gestational age of the fetus, are obvious factors affecting the composition of fetal serum. In an attempt to produce a serum of consistent composition, many suppliers pool several collections of sera taken at various stages of fetal gestation. Careful prescreening of sera must occur, however, to identify a lot of FBS that supports growth and development equal to levels previously recorded. For example, the first report of culture of a filaria from L3 to young adult (*A. viteae*) was accomplished using a single lot of FBS (7). Equivalent success with other FBS lots has not been reported. Development in vitro to young adults from L3s of *B. malayi* was also reported using an $LLCMK_2$ cell co-culture system (13), but was apparently also dependent on serum factors, as similar results have not been reported by others. Indeed, the batch of FBS used was identified by one researcher as "the most important factor" for successful in vitro culture of filariae (5).

Perhaps host-specific factors may be required that are not ordinarily present in FBS. Such an interpretation is suggested by a recent report that L3s of *B. malayi* developed to sexually mature adults in a fairly simple culture system if supplemented with human serum (19). The basic media and conditions used for these cultures (the actual source of human serum was not identified) were identical to those that supported development of *Wuchereria bancrofti* only to fourth stage larvae (6). It will be interesting to determine if success of this culture system is limited by fairly unique components of the specific human serum used or if culture can be achieved routinely with a broad range of human sera.

Although the promotion of growth and development in vitro of organisms such as filariae is a complex task, research toward those ends is warranted in light of the potential usefulness of such culture techniques. Because filariae have highly specific sites of development within insect and mammalian hosts, successful culture in vitro may require the simulation of microenvironments that reflect those unique associations (3).

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