INVESTIGATING DEVELOPMENT OF INFECTIVE STAGE LARVAE OF FILARIAL NEMATODES

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1. ABSTRACT

Lymphatic filariasis is a parasitic disease affecting millions of individuals and one of the leading causes of disability in the world. However, considering the magnitude of filariasis as a public health problem, relatively little is known about the basic biology of the disease and its causative agents, filarial nematodes. In this paper, the biology of the infective stage larvae of the nematodes responsible for lymphatic filariasis and the approaches used to study their development are reviewed.

2. INTRODUCTION

Lymphatic filariasis is a disease which afflicts nearly 120 million people, primarily in tropical regions of the world. It is caused by one of three species of nematode parasites – *Brugia malayi*, *Brugia timori*, and *Wuchereria bancrofti* (1). *W. bancrofti* is responsible for approximately 90% of lymphatic filariasis in the world (2). The remaining 10% of cases are primarily caused by *B. malayi*. Because it can be maintained under laboratory conditions, *B. malayi* is the most widely studied nematode capable of causing lymphatic filariasis.

While not a major cause of mortality in the world, filarial parasites are responsible for high rates of morbidity in endemic areas. Physical manifestations can range from acute fevers and lymphadenopathy to debilitating chronic edema, known commonly as elephantiasis (3). Most clinical manifestations are due to damaged lymphatic vessels, believed to be the result of several additive factors, including actions of the parasite itself, inflammatory response to the parasite, and parallel opportunistic infections (4). It is estimated that over 40 million individuals suffer from gross manifestations of lymphatic filariasis, most commonly limb lymphedema and urogenital disease. As a result, lymphatic filariasis is the second leading cause of disability in the world (2).

More than half of infected individuals do not demonstrate overt signs of disease, but recent studies have demonstrated occult lymphatic pathology and renal abnormalities in these individuals. Ultrasound studies of the lymphatics of infected individuals without overt symptoms of disease revealed adult worms along with lymphatic vessel dilatation in all patients studied (5). Additional studies have demonstrated that these abnormalities are not reversed following anthelminthic treatment (6). Such observations underscore the need for interventions capable of preventing parasite establishment at the earliest possible stage of infection.

New interventions would no doubt aid current

and future treatment and control efforts, but the development of such interventions is dependent on achieving a better understanding of the parasites themselves. Many aspects of their physiology and development remain a mystery. In fact, even the most widely employed drug in the treatment of lymphatic filariasis, diethylcarbamazine (DEC), was discovered by a random screening of compounds fifty years ago and still does not have a defined mechanism of action. Further advancement of drug treatment and vaccine development would benefit greatly from an improved understanding of basic filarial biology.

3. NATURAL HISTORY OF BRUGIA MALAYI

3.1. Nematode development

Although host requirements and tissue specificities vary greatly between parasitic nematodes, there are some commonalities. All nematodes, both free-living and parasitic, follow the same basic pattern of maturation consisting of four larval stages preceding the adult form. Nematode development is punctuated by obvious transitions between life stages, which can be observed visually as a molt (7). Conservation of this life cycle pattern across species suggests that the growth and development of these organisms is closely correlated with the molting of the larval stages (8). In the life cycle of a nematode parasite, different stages pass through very different environmental conditions. A specific stimulus unique to each stage is usually required to activate the group of developmental genes responsible for life cycle progression (7).

3.2. Overview of the life cycle

The life cycle of filarial parasites requires both a vertebrate host and a blood sucking arthropod vector. There are no intervening free-living stages. In the case of *B. malayi*, the infective stage larva (L3) is transmitted by a mosquito vector during a blood meal. It enters the skin and crawls through the sub-cutaneous tissue towards a lymphatic. One week later it undergoes a molt to the L4 larval stage. This larval stage lives within the lymphatic, develops further, and molts to an adult stage worm approximately one month post-infection. The adult stage is extremely long-lived and may persist within the lymphatics for many years. Over the course of their lifespan, the adult worms mate and the females produce large numbers of live progeny known as microfilariae. The microfilariae enter the circulation and are picked up by a mosquito during a subsequent blood meal. These larvae mature within the mosquito and the cycle continues (3,9).

3.3. Infective-stage larvae (L3)

The infective stage larva (L3) is the transitional stage between the mosquito vector and the human host. Studying the requirements for the development of L3 larvae is particularly powerful approach for several reasons. Elucidating the biology of L3 larvae allows targeting the development of therapies and/or vaccines aimed at the first stage of the parasite invading human tissues – thus preventing the establishment of the parasite within the

lymphatics and subsequent damage to these vessels. Study of the L3 to L4 molt, the first major developmental event following infection of the vertebrate host, is also advantageous because this event takes place approximately one week after infection, allowing for evaluation of experimental manipulations in a timely manner. Finally, filarial L3, like the infective stages of most parasites, have essentially suspended their developmental programs and are unable to proceed to the next stage until they find a suitable environment (10). The L3 to L4 molt thus offers an easily detectable means of measuring whether a decision to proceed with the developmental program has been made.

4. *IN VITRO* CULTURE OF FILARIAL NEMATODES

4.1. *In vitro* culture as a tool for studying development

Few details are known about the requirements for development of *B. malayi* infective stage larvae. By definition, a parasite is an organism that is forced to rely on one or more elements provided by its host for survival (11). It can be hypothesized that identification of the specific host factors required for growth and survival of the parasite would aid the development of both chemotherapeutic and immunoprophylactic approaches to filariasis control. Strategies designed to prevent acquisition of these factors would hinder parasite establishment within the host. However, it has been difficult to identify specific host factors capable of stimulating parasite growth and development *in vivo*.

Many researchers have approached this problem over the years by working to design culture systems in which *B. malayi* L3 development could be completed successfully *in vitro*. One of the primary goals of *in vitro* culture systems is to mimic the *in vivo* environment, thus providing the parasite with the required elements for its development (12). As a result, these systems serve as useful tools for defining the host contributions to parasite maturation. Furthermore, *in vitro* culture systems allow development in general and larval molting in particular to be observed visually. Larvae in culture can also be readily subjected to a variety of chemical and molecular techniques without the complications of treating the host (13).

Despite the enormous utility of *in vitro* culture systems, there are a number of challenges that are somewhat unique to culturing parasitic helminths. For most parasitic helminths, little is known about the habitats they seek or the physical and chemical characteristics of the environment they encounter within the host. There are also wide variations in nutritional requirements – both between different species and even between life cycle stages of the same species. Finally, perhaps the greatest obstacle is the need for a specific trigger stimulus, presumably of host origin, before development within the host is initiated. This stimulus signals the parasite that an appropriate host has been reached (13).

Given this background, it is interesting to note that some of the first published *in vitro* systems supporting L3 to L4 molting relied upon using L3 larvae that had first

been "primed" within the vertebrate host. Chen and Howells (14) were unable to achieve molting in *in vitro* cultures of infective stage larvae of *B. pahangi* supplemented with 10% fetal bovine serum and various mammalian cell lines. In order to aid molting *in vitro*, researchers tried inoculating L3 into the peritoneal cavity of Mongolian jirds for 3-5 days. The larvae were then removed from the animals and placed in a simple culture medium. These larvae molted on day 8, the same day they would have molted had they remained within the host. This data suggested that the larvae had received a signal within the peritoneum of the host that triggered development to the L4 stage. Interestingly, however, culture of L3 in the hosts' peritoneal fluid did not promote molting.

4.2. The search for a defined *in vitro* system supporting L3 development

Since the studies of Chen and Howells (14), investigators have been searching for culture methods that do not require *in vivo* priming for successful molting. An *in vitro* system for *B. malayi* that is more suitable for studying developmental questions has been elusive. Over the past several years, numerous attempts have been made to culture the infective stage (L3) larvae of the human filarial parasite *B. malayi*, as well as the related animal parasite *Brugia pahangi*, in *in vitro* systems that promote molting to the fourth stage. While there have been reports in the literature of successful *in vitro* molting, both with and without co-culture of cells, all of these systems have required the use of serum supplementation (12,15,16,17). The standard cell culture protocol for most mammalian tissues requires supplementation with serum; consequently, most investigators seeking to develop *in vitro* culture systems for parasitic nematodes have taken a similar approach. However, the variability in serum lot and source has presented problems to those seeking to cultivate nematodes. For instance, in one of the first studies reporting successful *in vitro* molting of a filarial nematode species without cellular co-culture, Franke and Weinstein (18) reported that development of *Dipetalonema viteae* L3 larvae to the fourth stage was highly dependent on both the serum lot and the concentration of serum used.

Riberu *et al.* (16) published a report of *in vitro* development of *B. malayi* L3 larvae to sexually mature adults capable of copious microfilariae production. This human serum-supplemented system utilized commercially available reagents exclusively. No cellular co-culture was required. The length, width, and other physical measurements of the *in vitro*-derived larvae were all comparable to *in vivo*-derived larvae obtained from parallel experiments in jirds. This report generated considerable excitement; however, the results have since proven difficult to reproduce with other sources of serum. Smillie *et al.* (12) observed no molting to the L4 stage with a commercial source of human serum, but high rates of molting with fresh serum. Molting to the adult stage, however, was only seen in a small number of worms cultivated in cultures containing fresh serum from a single human volunteer; and no microfilariae were produced. Falcone *et al.* (17) similarly found that they could achieve a high percentage

of L3 to L4 molting with certain sources of human serum, but they found essentially no development to adult stage in cell-free cultures supplemented with serum.

Wisnewski and Weinstein (19) performed an extensive study on the variation of molting rates in *B. pahangi* when different sources of serum were used. They tested 19 lots of serum and found that the percentage of L3 to L4 molting observed varied from a low of 0% to a high of 93% in a system in which all other components were kept constant. They also tested a wide variety of supplements in an attempt to eliminate serum from their culture system. These included hemoglobin, insulin, transferrin, selenium, bovine serum albumin, Tween 80, and cholesterol. However, these supplements were unable to support molting. In speculating on the possible roles of serum in nematode cultures, Wisnewski and Weinstein (19) suggested that serum could serve as a source of essential nutrients, growth factors, and hormones; it could bind and protect essential nutrients that are otherwise unstable. It could also function to neutralize toxic substances in the medium or supply necessary transport factors or enzymes.

Although the studies discussed above were unable to determine what role serum fulfilled in culture, they did illustrate its potential to strongly influence L3 development. Overall, serum can serve as a rich source of nutrients for larval development and even signals for differentiation, but serum as a culture additive is almost entirely undefined. This fact makes identification of specific components required for larval development next to impossible. Furthermore, presence of serum in culture could confound the study of defined additives, such as hormones or growth factors, by interfering with or masking their effects (9). Serum also varies greatly between sources and even between lots from the same source, thus creating a major impediment to reproducibility of data (18,19). Therefore, a system for the study of parasite development and the investigation of possible therapies capable of disrupting it must, by necessity, be serum-free. A system has been recently reported in which *B. malayi* L3 successfully molted to the L4 stage in serum-free medium supplemented with polyunsaturated fatty acids. However, larvae in this system made no attempt to molt in the absence of co-cultured cells, suggesting they either could not utilize these fatty acids directly or were reliant on an exogenous signal to do so (20).

4.3. Use of co-cultured cells

The role of co-culture with various cell types in promoting development of infective stage larvae to L4 and beyond has also been a subject of much debate. A variety of different cell types have been reported in the literature to promote molting. These include monkey kidney cells (15), jird skeletal muscle cells (21), human dermal fibroblasts and Jurkat T cell leukemia line (17). It is of interest that such diverse cell types were capable of promoting molting, suggesting that the trigger for development may not be a specialized product of a given lineage of cells. However, because all of these systems required serum supplementation, the trigger for development may have come from serum, with the cell co-culture playing a

supporting role. Some researchers have found that -- when comparing cellular and cell-free cultures side by side – the presence of cells did not improve molting results significantly, particularly when cell-free cultures were incubated in an atmosphere of 5% CO_2 and nitrogen (19). This suggests that cells may aid worm development by reducing the O_2 tension in the atmosphere, providing an environment more closely resembling that found in skin and lymphatics. However, Cupp (22) found in her studies of the filarial parasite *Onchocerca volvolus* that worms molted in a reduced O_2 atmosphere only in the presence of cell-conditioned medium, suggesting that the cells provided additional products crucial to development.

5. INFLUENCE OF HOST SIGNALS

Rogers (23) hypothesized that free-living nematodes have a system of "internal secretions" regulating the timing of developmental events, but the infective stages of parasitic nematodes lack part of this system of secretions. They are thus reliant on some element provided by the host to restart their developmental program following invasion of the host. According to this theory, the host is likely to function in one of two ways to stimulate molting and development of the infective stage -- stimulating the larvae to synthesize their own "internal secretions" or providing a signal that is capable of replacing these missing "internal secretions." In all successful *in vitro* systems described in the literature to date, these signals have presumably either been present in certain batches of serum or provided by certain co-cultured cells. However, the specific signal remains to be identified.

The influence of host signals on the development of parasites has been a subject of investigation for many years. The dependency on environmental cues to trigger further development appears to be most pronounced in the infective stage (8). For the infective stages of parasitic nematodes, the stimulus for continuation of development presumably comes from the host. A parasite that locates an appropriate host environment will molt to the next stage within a set period of time after entering that host, but a parasite that fails to locate such an environment will be unable to proceed to the next stage (24). The determination of which host signals are responsible for controlling development and molting has been a difficult task. There are many characteristics of the switch to the vertebrate host environment that might function as developmental triggers for nematode parasites.

The infective stage of filarial nematodes, in particular, is affected by numerous changes and must accommodate rapidly in order to thrive in the new environment (8). For instance, *B. malayi* begins its life in the tissue of an arthropod, is deposited on the surface of the skin, and invades vertebrate connective tissues before finally ending up within the lymphatic. These locations are associated with wide variations in the physical environment, including such parameters as oxygen tension, pH, temperature, and substrate availability. The changes from one environment to the next take place very rapidly, with little time for the organism to adjust. As a result, the parasite must be equipped with a series of metabolic switches that enable it to adapt to its rapidly changing chemical and physical surroundings (25). In addition, the parasite as a foreign body is often met with an immune response on entering the host. Overall, the environment is drastically altered the moment the larva exits the mosquito, and the larva continues to encounter changes as it traverses the hosts' tissues over the next several days.

5.1. Respiratory gases

The relative levels of both oxygen and carbon dioxide vary throughout the life cycles of many parasites. *B. malayi* faces wide variations in oxygen tensions, the most drastic being the transition between its invertebrate host, the tissues of which are generally considered to be aerobic, to the subcutaneous tissues of its vertebrate host, where the partial pressure of oxygen (pO_2) is in the range of 20-43 mmHg. It is unlikely that *B. malayi* ever encounters completely anaerobic conditions, but once it has taken up residence in the lymphatics, its environment can probably be best described as micro-aerobic (25). Similarly, the partial pressure of CO_2 in the atmosphere, such as *B. malayi* L3 would encounter when first deposited on the skin, is very low (0.3 mmHg). As it invades vertebrate tissues, it is subjected to a great increase in pCO_2 within its new host, where the pCO_2 is approximately 40 mmHg. In some nematode species, an increase in pCO_2 actually functions as a trigger for exsheathment of eggs and/or larvae (7, 25). While alterations in the atmosphere generally have been unable to stimulate development of filarial L3, different gas phases have been reported to aid survival and development in the presence of other stimuli (19,22).

5.2. Temperature

The infective stage larvae also are subjected to a significant temperature increase upon invasion of the mammalian host. The transition from the arthropod vector, the temperature of which approximates ambient temperature, to the mammalian host increases the temperature suddenly to 37°C (25). While this temperature change is sufficient to activate the infective stage larvae of some helminths, it is not sufficient to trigger the development of *B. malayi* L3 larvae. Like CO_2, it might, however, be one of several requirements for initiation of development to the L4 stage. Studies on *in vitro* molting of the infective stage larvae of the filarial parasite *Dirofilaria immitis* have demonstrated that larvae survive for extended periods but fail to molt if cultured at 27°C, while parallel cultures grown at 37°C are capable of molting (26).

5.3. Nutritional requirements

Presumably, one of the major driving forces behind the development of parasitism from an evolutionary perspective is the abundance of nutrients available within the host; however, the availability of specific nutrients varies between host species and even between tissues within the same organism. The L3 larvae of filarial nematodes have fully formed guts and may begin feeding following invasion of the host, but at the time the L3 emerges from the mosquito, the esophagus is occluded by particulate material (27,28). It is possible that the range of

hosts permissive to infection by a given parasite and/or the tissues inhabited within that host may be determined by the availability of a specific nutrient. However, such specific nutritional requirements have seldom been identified (25).

5.4. Temporal requirements for host signaling

All of these changes, either cumulatively or in a set sequence, may contribute to triggering development of the next larval stage and/or necessitate metabolic changes (25). It is interesting to note that the stimulus for molting need not be present for very long in order to promote developmental progression. In some instances, exposure to a signal for under an hour is sufficient to irreversibly activate developmental processes and allow molting to take place even when the larva is subsequently transferred to a simple culture medium (8). Recent work in our laboratory has demonstrated that exposure of *B. malayi* L3 larvae to co-cultured cells capable of stimulating development for less than a day is capable of triggering molting (H. Smith and T. Rajan, unpublished data). Interestingly, the larvae are only responsive to the signal during a short time period (day 4-5 of culture) preceding the molt to the L4 stage (which takes place between days 8-10 of culture). This suggests that there may in fact be a discreet series of signals, perhaps beginning with the transition to 37°C, which trigger development. These observations correlate with previous reports demonstrating that filarial larvae can be successfully primed to molt even when removed from the host several days prior to the onset of molting (14).

It is possible that further insights into developmental requirements may be achieved by studying changes in gene expression coincident with these signals. For instance, the changes in expression of cuticular collagen genes over the course of the life cycle of the free-living nematode *Caenorhabditis elegans* have been widely studied. These studies have shown that prior to each molt a different set of individual collagen genes is expressed, but a basic temporal pattern of expression is followed for each molt. Individual genes in each set are not expressed at exactly the same time or in the exact same quantity (29,30). It is possible that similar analyses of gene expression coincident to commitment to L4 development in filarial nematodes may uncover clues to the types of signals required to initiate maturation of the infective stage larvae of these parasites.

6. OLD TREATMENTS PROVIDE NEW CLUES

Other insights to the requirements for development may be provided by the study of current therapies for filarial infections. The most widely employed drug in the treatment of lymphatic filariasis, diethylcarbamazine (DEC), was discovered by a random screening of compounds fifty years ago (31). It is extremely effective against microfilariae of several parasites, capable of reducing the number of microfilariae in the circulation of an infected human by 60% within four minutes (32). It can also be used as a prophylactic against *B. malayi* in humans and has been shown to have activity against L3 larvae in cats (33,34). However, despite the

remarkable efficacy of the drug *in vivo*, the means by which DEC eliminates *B. malayi* have not been established. When DEC is added to either microfilariae or L3 *in vitro*, worm viability is unaffected (34,35).

Pharmacologically, the best-defined activity of DEC is its ability to inhibit the production of leukotrienes via the lipoxygenase pathway of arachidonic acid metabolism. Although the precise step at which DEC is operating in this pathway is not completely agreed upon, micromolar concentrations of the drug cause an increase in 5-HETE and block production of leukotrienes B_4 (LTB_4) and C_4 (LTC_4), suggesting the synthesis of leukotriene A_4 (the precursor of both LTB_4 and LTC_4) has been inhibited (36). Hawking *et al.* (37) performed perhaps the most extensive studies on the anti-filarial activities of DEC soon after the drug's discovery. They found that cotton rats infected with *L. carinii* remained capable of developing patent infections if DEC treatment was delayed 1-2 weeks following infection, while those treated with DEC from the start of infection failed to develop patent infections. This data suggests that the drug must be present prior to the L3 to L4 molt in order to prevent establishment of infection. One possible explanation for this observation is that the larvae require a lipoxygenase product early in the course of infection and, if allowed to obtain this substance at the appropriate time, were immune to the effects of DEC-mediated lipoxygenase inhibition when the drug was administered later in the course of infection.

Recent *in vitro* data has demonstrated that, while *B. malayi* L3 larvae are not killed outright by DEC, their maturation to the L4 stage is inhibited. This observation may explain the drug's ability to prevent establishment of infection without killing larvae outright. Furthermore, other lipoxygenase inhibitors have been shown to similarly block development of infective stage larvae, suggesting that the mechanism by which DEC disrupts molting may in fact be the inhibition of lipoxygenase enzymes. Finally, inhibitors of enzymes operating downstream of lipoxygenase have also proved capable of blocking development of both *Brugia* spp. and the related filarial parasite, *Dirofilaria immitis* (H. Smith and T. Rajan, unpublished data).

Interestingly, products of the lipoxygenase pathway have also been implicated in the early development of another parasitic helminth within the human host. The parasitic trematode, *Schistosoma mansoni*, must penetrate the skin in order to infect the definitive host and then undergo a rapid transformation from free-living cercariae into tissue-dwelling schistosomulae. Upon contacting the polyunsaturated fatty acids on the surface of the host's skin, these organisms begin synthesizing lipoxygenase products that are believed to be crucial for this process (38). Salafsky and Fusco (39) demonstrated that penetration could be inhibited by the lipoxygenase inhibitor esculetin, and to a lesser extent by the cyclooxygenase inhibitor ibuprofen. Although caution should be exercised when drawing comparisons to non-nematode parasites, since parasitism is believed to have

arisen several times over the course of evolution, such commonalities are intriguing.

7. CLUES FROM DEVELOPMENTAL REQUIREMENTS OF OTHER MOLTING ORGANISMS

7.1. Free-living nematodes

Some insights can be obtained by studying the developmental requirements of well-characterized free-living nematodes such as *C. elegans*. Comparisons between the two groups may provide valuable insights, although care should be taken to also fully evaluate filarial nematodes as a distinct group before drawing conclusions (40). The evolutionary distance between *C. elegans* and the filarial nematodes is an obstacle; however, it can be argued that similarities between the two organisms are likely to be important functionally since they have been conserved through evolution (41).

Recent studies of molting in *C. elegans* have demonstrated that elimination of sterols, either by sterol-free culture or by mutation preventing sterol uptake, results in defective molting (42). It is not known whether the requirement for sterols for successful molting of *C. elegans* reflects a need for these compounds as building blocks of signaling molecules, such as steroid hormones, or if they are needed structurally for building a healthy cuticle (30). Interestingly, one of the most striking differences between parasitic nematodes and their hosts is the inability to synthesize sterols *de novo* (43,44). This inability to synthesize sterols is widespread among both free-living and parasitic helminths (43). While there have been isolated reports of *de novo* synthesis by parasitic nematodes in studies using radiolabeled precursors, including the filarial nematode *Dirofilaria immitis* (45), these results may have been confounded by the presence of contaminating microbes (44). It would be informative to determine whether a sterol requirement for molting is similarly widespread among nematodes.

7.2 Molting arthropods

One recent exciting development in the study of nematode molting is the molecular analysis linking nematodes to molting Arthropoda (46). Comparison of 18S ribosomal DNA sequences suggests that Nematoda and Arthropoda can be grouped evolutionarily into a larger classification of molting organisms known as Ecdysozona. This data suggests molting arose once in the course of evolution. As a result, there may be functional relationships between molting mechanisms in developing nematodes and those in developing arthropods.

While no conclusive data has been reported to date, there have been efforts to link the molting of nematodes to ecdysone, the steroid hormone regulating molting in insects (44). While ecdysones have been identified in several nematode species, including the filarial nematodes *Dirofilaria immitis* and *Onchocerca volvulus*, evidence of synthesis within nematode tissues has been elusive (44,47). Although their have been reports of ecdysteroids successfully promoting molting in some nematodes (48), the overall effects of ecdysones when added exogenously to various nematode species have been variable (47).

8. PERSPECTIVES

Infective stage larvae of filarial nematodes, as the transitional stage between the arthropod vector and the mammalian host, are the first to encounter the human host. Targeting of therapies and interventions aimed at blocking the development of this stage of the life cycle offers an opportunity to halt the establishment of the worm within the host and subsequent insidious damage to lymphatic vessels. However, development of new interventions requires a more detailed understanding of the driving forces behind colonization of the vertebrate host and the requirements for maturation of this stage. Important tools for studying these processes include the use of *in vitro* culture systems, studies of changes in gene expression coincident with commitment to development, analysis of mechanisms of currently effective therapies, and comparisons with other species. Continued pursuit of these avenues of research may lead to the development of new interventions effective in controlling and perhaps preventing filarial infections.

9. ACKNOWLEDGEMENTS

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