Microreview

The role of endosymbiotic *Wolbachia* bacteria in filarial disease

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Summary

In this review, we describe the pathogenic role of *Wolbachia* endosymbiotic bacteria in filarial diseases, focusing on the host innate immune responses to filarial and *Wolbachia* products. A description of the host pathogen recognition and early inflammatory responses including TLR4-mediated signalling, chemokine and cytokine responses and inflammatory cell recruitment is provided from human studies and from animal models of filarial disease. Finally, the impact of the discovery and characterization of *Wolbachia* on filarial research and treatment programmes is discussed.

Filarial diseases

The World Health Organization estimates that filarial nematodes infect over 138 million individuals worldwide, causing morbidity, disability and economic hardship (WHO, 1992; 2000). Infection with *Wuchereria bancrofti*, *Brugia timori* or *B. malayi* causes lymphatic filariasis, a major health problem in sub-Saharan African, South America, Asia and tropical islands of the Pacific Ocean, with over 120 million individuals infected (WHO, 1992; 2000). *Onchocerca volvulus*, the parasite that causes onchocerciasis, causes subcutaneous ‘Calabar’ swellings and conjunctival irritation in response to migration of adult worms through the infected individual, *Mansonella* ssp. (*M. streptocerca*, West Africa; *M. perstans*, Central Africa and Central and South America; *M. ozzardi*, Central and South America), *Dracunculus medinensis*, commonly known as guinea-worm (West Africa, East Africa and India) and pulmonary *Dirofilaria* (in areas endemic to canine heartworm).

All human filarial nematodes undergo a complex life cycle involving an insect vector (see Fig. 1). First-stage larvae (microfilaria) are ingested during a blood meal by the insect vector. Microfilaria migrate out of the mid-gut to the thoracic flight muscles of the insect where they undergo two moults, developing into infectious L3 larvae that migrate to the salivary glands and mouth parts of the insect and are introduced into a new host during a subsequent blood meal. In the human host, the L3 moults twice to mature into adult male and female worms, producing circulating microfilariae. In onchocerciasis, adult worms are found primarily in subcutaneous nodules, although autopsy and animal studies have revealed that they are also present in deeper tissue (Duke, 1993). In contrast, in lymphatic filariasis, adult worms are found primarily in the large lymphatic vessels of the extremities. Adult worms can survive 20 years in the human host producing thousands of microfilariae each day (Duke, 1993). In onchocerciasis, microfilariae in the skin cause severe itching and depigmentation, wrinkling and sagging of the skin. In addition, lymphadenitis, which can result in hanging groin and elephantiasis. Vision loss is related to microfilariae migrating through the anterior and posterior segments of the eye. In lymphatic filariasis, the microfilariae are in the blood rather than the skin, and demonstrate either nocturnal or diurnal periodicity, coinciding with peak feeding times of the mosquito vector (Moulia-Pelat et al., 1993; Nutman, 2000).

Treatment options for filarial nematodes are necessarily limited by cost, side-effects and distribution, with no single drug being effective for all clinical disease manifestations. At the population level, the goals of a treatment pro-
Wolbachia endosymbiotic bacteria

Wolbachia are Rickettsiae-like, maternally inherited, obligate intracellular bacteria that infect many species of invertebrates (Warren et al., 1995; Stouthamer et al., 1999). Phylogenetic classification of Wolbachia is within the order Rickettsiaceae, family Anaplasmataceae, also containing the bacteria Ehrlichia, Anaplasma and Neorickettsia (Dumler et al., 2001). Subgrouping of Wolbachia is ongoing, based on recently emerging genomic information. Currently, it can be grouped into seven major clades with Wolbachia of W. bancrofti, B. malayi and Litosomoides sigmodontis in clade D, and those of Onchocerca and Dirofilaria in clade C (Lo et al., 2002). These are distinct from the clades of Wolbachia that infect arthropods (Bandi et al., 2001; Casiiraghi et al., 2001). Intracellular bacteria were identified in early ultrastructural studies of adult filarial nematodes and microfilaria (McLaren et al., 1975; Kozen and Marroquin, 1977); however, only within the past decade was the identification of Wolbachia confirmed through molecular techniques (Sironi et al., 1995; Bandi et al., 1998; Taylor et al., 1999). Wolbachia endosymbionts are found in most filarial parasites of importance to human health [B. malayi, W. bancrofti, O. volvulus, some Mansonella sp. (M. ozzardi)]. Two filarial parasite species, Acanthocheilonema viteae (infects rodents) and Onchocerca flexuosa (deer) do not naturally contain Wolbachia and are often used as experimental controls (Bandi et al., 1998; Taylor and Hoerauf, 1999). Recent reports demonstrate that the filarial nematodes Loa loa and Setaria equina do not contain Wolbachia (Chirgin et al., 2002; Buttner et al., 2003; McGarry et al., 2003). In filarial nematodes, Wolbachia endobacteria are concentrated in intracytoplasmic vacuoles within the hypodermal lateral cords of male and female worms and female reproductive organs, but are also detected by immunohistochemistry in oocytes and microfilariae (Taylor et al., 1999; Kramer et al., 2003) (see Fig. 2). Female adult worms carry the highest bacterial load, with most bacteria concentrated around the reproductive organs. Ultrastructural studies indicate that the Wolbachia endobacteria are round, with a diameter of ~0.5 μm, and are enclosed in a double trilaminate membrane and with a cytoplasm containing ribosomal-like granules (Peixoto et al., 2001). Like other Rickettsiaceae bacteria, Wolbachia is sensitive to the tetracycline class of antibiotics which is provided free by Merck (Richards et al., 2001). In this review, we will discuss the role of endosymbiotic Wolbachia bacteria in the pathogenesis and treatment of filarial disease. The reader is also referred to other reviews on this subject (Taylor, 2000; 2002; 2003; Bandi et al., 2001; Nutman, 2001; Taylor et al., 2001; Taylor and Hoerauf, 2001; Hoerauf et al., 2003a).
(tetracycline, doxycycline) and to azithromycin and rifampicin (Townson et al., 2000). Penicillin and gentamicin are not effective against Wolbachia. Partial activity is seen with ciprofloxacin, erythromycin and chloramphenicol (Smith and Rajan, 2000). There are currently no technologies that enable long- or short-term culture of these endosymbionts in cell-free medium, i.e. outside the cytoplasm of invertebrate cell lines or intact filarial worms. Wolbachia from nematodes do not appear to infect host tissues, although bacterial DNA has been detected in filaria-infected individuals after DEC treatment (Cross et al., 2001).

Although little is known about the interaction between the filarial nematode and the bacterial endosymbiont, studies focusing on the mechanism of action of anti-Wolbachia drugs indicate that Wolbachia is required for successful early moulting as well as reproduction. Studies of in vitro cultures as well as an in vivo animal model using Mongolian jirds demonstrate that the critical moulting step from L3 to L4 larvae is blocked by exposure to tetracyclines but not chloramphenicol, erythromycin or ciprofloxacin in multiple species of Wolbachia-infected worms including B. malayi, B. pahangi and D. immitis (Smith and Rajan, 2000; Casiraghi et al., 2002; Rao et al., 2002). The endosymbiont also appears to play a critical role in the reproductive capacity of the nematodes. Immunohistochemical staining of adult female nematodes for Wolbachia shows the presence of the bacteria in oocytes, morulae and microfilaria in utero, and cross-breeding studies using B. pahangi and B. malayi nematodes demonstrate that Wolbachia has a matrilinear inheritance (Taylor et al., 1999). Also, in an animal model of Litomosoides sigmodontis (a rodent parasite infected with Wolbachia), tetracycline treatment resulted in infertile yet viable adult female worms, showing degenerative oocytes and no embryos on microscopic examination and absence of Wolbachia by polymerase chain reaction (PCR) (Hoe-rauf et al., 1999). Development of L. sigmodontis was also inhibited by exposure to tetracycline. Similar results have been shown from in vivo treatment of D. immitis in dogs and B. pahangi in jirds (Kramer et al., 2003). Additionally, exposure of Acanthocheilonema vitaeae (not containing Wolbachia) to anti-Rickettsial antibiotics had no effect on filarial development or reproduction (Hoerauf et al., 1999), indicating that the effect of the antibiotic is on the bacterial endosymbiont.

Host immune responses

Initial immune interactions between the host and the filarial parasite occur within the category of an innate immune response. These responses are the first line of defence against invading pathogens and do not require gene rearrangements or clonal expansion associated with the adaptive immune responses. Chronic filarial infection results in development of B- and T-cell responses to filarial products (Cooper et al., 2001; Steel and Nutman, 2003), although the adaptive immune response to Wolbachia has yet to be determined. We have previously reviewed the role of acquired immune responses in O. volvulus (Pearlman, 1996; Hall et al., 2001a), and will focus here on innate responses.

Toll-like receptors (TLRs) are a family of at least 10 transmembrane proteins that are highly conserved in vertebrates and invertebrates, and represent a first line of defence by detecting conserved pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), PGN and CpG DNA (Takeuchi and Akira, 2001). ‘Toll’ was originally defined as a Drosophila gene product involved in development and antimicrobial resistance (Lemaitre et al., 1996; 1997). The recognition sequence of toll is very similar to that of mammalian interleukin (IL)-1 receptor, and a human type 1 Toll receptor homologue, now called toll-like receptor 4 (TLR4), was identified (Medzhitov et al., 1997; Rock et al., 1998). Taylor et al. (2000) demonstrated an LPS-like product of Wolbachia from Brugia malayi that is heat stable, reacts positively in the Limulus amoebocyte lysate (LAL) assay, can be inhibited by polymyxin B and mediates an inflammatory response via TLR4 using murine macrophages in culture.
Using a mouse model of onchocerciasis in which microfilaria antigens are injected directly into the stroma of the cornea (see Fig. 3), our laboratory has demonstrated that development of corneal inflammation is impaired in C3H/HeJ mice, containing a genetic mutation resulting in a TLR4 receptor that is truncated and inactive, compared with their wild-type C3H/HeN mice, indicating an important role for TLR4 in this disease process (Saint Andre et al., 2002).

After the initial activation of innate responses via pattern recognition receptors (PRRs) such as TLRs, the propagation of inflammation involves the production of chemotactic factors that recruit other cells to the site of infection. Extracts from adult *O. volvulus* worms have neutrophil chemoattractant properties *in vitro* (Rubio de Kromer et al., 1998). Neutrophils are one of the first cells recruited to the site of onchocercal infection and can be found in the granulomas formed around the adult worms (Greene et al., 1981; Pearlman et al., 1998; Brattig et al., 2001). Eosinophils are also recruited to the inflamed tissue but are seen later in the response (Pearlman et al., 1998; 1999a; Brattig et al., 2001). Initial studies into *O. volvulus*-induced keratitis focused on the role of eosinophils (Pearlman et al., 1998). However neutrophils are a major mediator in *O. volvulus* disease, at least in the early stages of inflammation. Studies using IL-5 knock-out mice, which do not produce eosinophils, have a sustained neutrophil infiltrate coincident with exacerbated keratitis (Pearlman et al., 1998). Interestingly, IL-5 does not interact with neutrophils directly; rather, this cytokine appears to mediate neutrophil accumulation in the granulomas around adult worms (*L. sigmodontis*) by indirectly controlling the production of neutrophil chemotactic agents such as tumour necrosis factor (TNF)-α and KC (a mouse IL-8 homologue) (Al-Qaoud et al., 2000). Further studies have demonstrated an important role for the receptors PECAM-

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**Fig. 3.** Histological sections of mouse corneas. C57Bl/6 mice were immunized with *O. volvulus* antigens as described previously, then injected intrastromally with ~5000 live *Brugia malayi* mf. After 24 h, the corneas were removed and stained with rat monoclonal antibody clone 7/4 (Serotec), which reacts with a polymorphic 40 kDa surface component of murine neutrophils (A), or haematoxylin/eosin (B). A higher (400×) magnification of a section of cornea showing an intact microfilaria is shown in (C) (haematoxylin and eosin stained). Bar equals 100 μm.
1, ICAM-1 and VCAM-1 in the recruitment of neutrophils and eosinophils to the cornea in response to *O. volvulus* antigens (Kaifi *et al.*, 2001). The recruitment of cells into sites of inflammation occurs in part through the production of a chemokine gradient. Most chemokines fall into one of two broad groups: CC chemokines with two adjacent cysteine residues and the CXC chemokines in which the cysteine residues are connected by another amino acid (Mantovani, 1999). In the mouse model of onchocerciasis, recruitment of eosinophils is mediated by eotaxin, a CC chemokine (Forssmann *et al.*, 1997; Pearlman *et al.*, 1999b). Other CC chemokines (MIP-1α, MIP-1β and RANTES) use the receptor CCR1; however, this is not necessary for neutrophil recruitment in *O. volvulus*-induced keratitis. Instead, the CXC chemokine receptor 2 (CXCR2) was found to be essential (Hall *et al.*, 2001b). In humans, CXCR2 is the receptor for IL-8; however, mice do not produce this chemokine but have two homologues, KC and MIP-2, that can bind to the murine CXCR2 receptor. Both KC and MIP-2 are produced in murine models of filarial disease (Al-Qaoud *et al.*, 2000; Hall *et al.*, 2001b).

In humans, soluble extracts of *Brugia* and *Onchocerca* adult and microfilarial worms have been used to stimulate peripheral mononuclear cells (monocytes) *in vitro*, resulting in the production of TNF-α, IL-1, granulocyte–macrophage colony-stimulating factor (GM-CSF) and IL-10 (Raman *et al.*, 1999; Brattig *et al.*, 2000). Extracts of *O. volvulus* adult worms exposed *in vivo* to doxycycline resulted in reduced IL-8 and TNF-α responses from human peripheral monocytes as well as impaired neutrophil chemotaxis, whereas *A. vitaeae*, which does not have *Wolbachia*, induced negligible inflammatory responses from human monocytes (Brattig *et al.*, 2001).

**Summary and conclusions**

Although the studies described indicate that *Wolbachia* has an important role in the pathogenesis of filarial disease, there are clearly non-*Wolbachia*-related factors that are also involved. Studies of filaria that do not harbour *Wolbachia*, such as *Loa loa* and *A. vitaeae*, will be important in identifying these mechanisms. However, for filarial species that do harbour *Wolbachia*, the early immune response is consistent with a sequence of events that is initiated with death and degeneration of the parasites in host tissues. Filarial and *Wolbachia* antigens trigger release of proinflammatory and chemotactic cytokines by resident cells, which induce cellular infiltration and amplification of the inflammatory response. TLR4 appears to play a major role in this process. In lymphatic filariasis, macrophages are important in antigen presentation and lymphocyte activation, eventually leading to the Th2 and B-cell responses that have been documented previously (Mahanty *et al.*, 1994; 1997; King *et al.*, 2001). In contrast, neutrophils, eosinophils in addition to macrophages are important in the skin and corneal manifestations of onchocerciasis. Repeated and long-term exposure to filarial and *Wolbachia* antigens results in scarring and fibrosis, contributing to clinical manifestations such as lymphatic dysfunction and hydrocele formation in lymphatic filariasis, and skin thickening and corneal scarring in onchocerciasis. Future studies on the host–parasite interactions are likely to involve recombinant and purified *Wolbachia* and filarial products rather than total parasite extracts, and are likely to yield additional mechanistic information on receptor usage and inflammatory pathways.

Novel treatment strategies targeting filarial nematodes need to be developed to control and eradicate filariasis. Current onchocerciasis and filariasis control programmes have been successful, but may not meet target goals of eradication (WHO, 1992). The use of anti-*Wolbachia* antibiotic therapy also holds promise as a method to enhance the success of traditional mass treatment programmes. *Wolbachia*, like other Rickettsial bacteria, are susceptible to the tetracycline family of antibiotics (Bandi *et al.*, 1999). Clinical trials of anti-*Wolbachia* antibiotics in humans infected with onchocerciasis have shown a sterilizing effect on adult nematodes, with sustained reduction in microfilaraemia after a 6 week daily regimen of doxycycline (Hoerauf *et al.*, 2001; 2003b). *Wolbachia*-targeting antibiotics such as doxycycline may improve compliance with mass treatment programmes by reducing side-effects that may result from the release of intact bacteria and *Wolbachia* products from dying nematodes (Cross *et al.*, 2001). Pretreatment targeting of *Wolbachia* may abrogate inflammatory responses and allow a wider range of antifilarial drugs to be used. In conclusion, the discovery of endosymbiotic bacteria infecting most species of filarial nematodes that are pathogenic to humans has opened exciting new avenues of research into the pathogenesis and immunology of filarial infections. Further characterization of the *Wolbachia*–nematode relationship will allow the development of new therapeutic approaches to these devastating parasitic diseases.

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