CHAPTER I

INTRODUCTION

Background and Rationale

Lymphatic filariasis is mainly caused by the filarial nematodes, *Wuchereria bancrofti* and *Brugia malayi* (Ottesen *et al.*, 1997). Over 120 million people in 83 endemic countries, including Thailand are infected, with more than 40 million incapacitated or disfigured by the disease (WHO, 2003; Triteeraprapab and Songtrus, 1999; Triteeraprapab *et al.*, 2001; Nuchprayoon *et al.*, 2001; 2003a; 2003b). It is ranked by the World Health Organization (WHO) as the world's second leading cause of permanent and long-term disability, and targeted by the WHO to be eliminated as a public health problem by the year 2020 (WHO, 1995; Behbehani, 1998). In terms of disability-adjusted life years (DALYs: the number of healthy years of life lost due to premature death and disability), lymphatic filariasis is responsible for 5.8 million DALYs lost annually, ranking third among the TDR diseases, after malaria and TB (WHO, 2004). This vector-borne disease is transmitted by mosquito vectors. Infection is established with adult worms that can dwell in the human lymphatic system, where offsprings of female adults, microfilariae, are released into the host's blood circulation. There are a broad range of clinical and subclinical symptoms of longstanding infection with these filarial parasites, including recurrent adenolymphangitis, hydrocele, lymphedema, elephantiasis, and tropical pulmonary eosinophilia.

The pathogenesis of lymphatic filariasis is thought to be caused by adult worms, host immune responses, and secondary bacterial infection (Ottesen, 1992). The pathology in examined lymphatic vessels consists of distinct histological features

related to the existence of both alive and dead parasite (Jungmann *et al.*, 1991; 1992). In asymptomatic patients living in endemic areas, lymphangiectasia is the most common change observed (Dreyer *et al.*, 2000). The lymphatic vessels are dilated with the presence of intact worms that do not provoke any inflammatory responses. In contrast, an inflammatory pathology is usually detected with the presence of dead worms from natural occurring, or antifilarial-drug (diethylcarbamazine; DEC, or ivermectin) treatment reaction (Figueredo-Silva *et al.*, 1996; Dreyer *et al.*, 1999; 2000). The underlying pathology may be associated without any clinical symptoms, or with the acute lymphagitis. This acute manifestation is characterized by local inflammatory reactions around dead worms, and systemic febrile responses with significantly elevated levels of tumor necrosis factor alpha (TNF-α) (Dreyer *et al.*, 2000; Das *et al.*, 1996). Therefore, lymphatic dysfunction can be caused by lymphangiectasia (lymphangiectasia: a term of pathology that describes dilation of the lymphatic vessels; lymphangiectasia may be congenital or acquired), and persistent attacks of acute lymphangitis. These factors together with recurrent opportunistic bacterial infections are major risk for development of the chronic manifestations of lymphatic filariasis (Ottesen, 1992; Dreyer *et al.*, 2000).

The drug-induced adverse reactions are also described in lymphatic filariasis as a pathological event of inflammatory responses to the death of worms (Ottessen, 1985). Following antifilarial treatment of microfilaremic patients, adverse reactions commonly occur in which the severity are correlated with pre-treatment microfilarial density (Ismail *et al.*, 1998; Richards *et al.*, 1991; Addiss *et al.*, 1997; Haarbrink *et al.*, 2000). This also suggests that it is less likely to be a result of direct drug toxicity. Treated patients may experience systemic reactions, including headache, fever, chills, and body aches and/or localized reactions, which cause painful inflammatory granulomas (Jayakody *et al.*, 1993; Dreyer *et al.*, 1994; Figueredo-Silva *et al.*, 1996). Symptomatic treatment of these post-treatment reactions with analgesics, antipyretics,

antihypotensive agents etc. has been successful, but their prevention has been achieved only with the broadly anti-inflammatory corticosteroids (Ottessen, 1987). This observation is related to the findings that adverse reactions are associated with the increased post-treatment concentrations of proinflammatory cytokines and inflammatory mediators, including tumor necrosis factor (TNF), interleukin (IL)-6, IL-10, lipopolysaccharide-binding protein (LBP), and soluble TNF receptors (sTNF-Rs) (Turner *et al.*, 1994; Haarbrink *et al.*, 1999; 2000). However, IL-6 and LBP plasma levels show the strongest association with the severity of adverse reactions (Haarbrink *et al.*, 2000).

It has been discovered that filarial nematodes themselves harbor the intracellular bacteria *Wolbachia* (McLaren *et al*, 1975; Kozek, 1977; Kozek and Manoquin, 1977). *Wolbachia* is a genus of the class Alphaproteobacteria belonging to the order Rickettsiales, and family Anaplasmataceae (Dumler *et al.*, 2001). These obligate intracellular gram-negative bacteria are widespread in arthropods and filarial nematodes, including *B. malayi, W. bancrofti*, and *Onchocerca volvulus*, the major human parasitic filarial nematodes, and *Dirofilaria immitis*, the pathogenic filarial nematode of dog heartworm disease (Sironi *et al.*, 1995; Bandi *et al.*, 1998; Henkle-Duhrsen *et al.*, 1998). Two supergroups of the genus *Wolbachia* are found in filarial nematodes: supergroup D *Wolbachia* are present in most species of the genus *Onchocerca* and *Dirofilaria*, including *O. volvulus* and *D. immitis*, while supergroup C *Wolbachia* are found in the causative agents of lymphatic filariasis, *W. bancrofti* and *B. malayi* (Bandi *et al.*, 1998; Casiraghi *et al.*, 2001). *Wolbachia* are found in all developmental stages of filarial nematodes, in which the bacteria are restricted in the lateral hypodermal cords of filarial nematodes, and in the reproductive tissues of the females (i.e. in the oogonia, oocytes, embryos and microfilariae) (Kozek, 1977; Kozek and Marroquin, 1977; Fenn and Blaxter, 2004a; McGarry *et al.*, 2004). This suggests that *Wolbachia* are vertically transmitted through the cytoplasm of the egg. While the

arthropod *Wolbachia* can be characterized as parasitic, there is evidence to suggest that the association between *Wolbachia* and filarial nematodes is mutualistic (Fenn and Blaxter, 2004b). The phylogeny of the filarial nematode *Wolbachia* is congruent with that of their hosts. The evolutionary aspect suggests long-term co-evolution and co-adaptation, which is usually seen in mutualistic relationships (Baumann *et al.*, 1995).

In addition, experimental studies have provided evidence that filarial nematodes require *Wolbachia* for successful reproduction and development (Bosshardt *et al*, 1993; Hoerauf *et al*, 1999; Bandi *et al*, 1999). Treatment of *Litomosoides sigmodontis*-infected rodents with tetracycline, an antibiotic with antirickettsial activity, results in inhibition of embryogenesis and infertility, inhibition of L3 larvae development, and stunting of adult worm growth, coincident with the depletion of *Wolbachia* (Hoerauf *et al*, 1999). In addition to the rodent filariae, effects of tetracycline in blocking embryo development have been observed in two filarial nematodes, *B. pahangi* (a feline filaria) and *D. immitis* (a dog heartworm) (Bandi *et al*, 1999). Notably, the study in cattle infected with the African bovine parasite *O. ochengi*, a nodule-dwelling filarial nematode closely related to *O. volvulus*, demonstrated the death of the adult filariae, following 6 months of oxytetracycline therapy (Langworthy *et al.*, 2000). The anti-filarial effects would be mediated by the antibiotic effects on *Wolbachia*, since there is neither an effect of penicillin, an antibiotic known to be ineffective against rickettsiae, on the filariae with *Wolbachia*, nor an effect of tetracycline on *Acanthocheilonema viteae*, a filarial nematode without *Wolbachia* (Hoerauf *et al.*, 1999).

Wolbachia-derived molecules, therefore, probably serve worms for some significant biological functions. Although one might speculate that the bacteria are essential for nutrition and metabolism, or protecting the nematodes against the host immune responses (Bandi *et al.*, 2001), a molecular basis of the *Wolbachia*-filarial

nematode relationship is unknown. The data also suggest that tetracycline and their derivatives are likely to be a novel microfilaricidal drug target, as well as macrofilaricidal (adulticidal) therapy against filarial infections. So far, the results obtained from human trials have clearly revealed the superior efficacy of doxycycline, both for onchocerciasis and lymphatic filariasis to clear the filarial adults, and microfilaraemia (Hoerauf *et al.*, 2000; 2001; 2003a; 2003b; Taylor *et al.*, 2005). However, at present, the major difficulty is the required long length of treatment for 8 weeks at 200 mg/day (Taylor *et al.*, 2005). Novel drug targets to *Wolbachia* that are used for the shorter length of treatment are thus needed to find and test.

The presence of large numbers of *Wolbachia* in filarial nematodes raises questions whether *Wolbachia* and their molecules are released, and trigger filarial host immune responses. Recent studies on the pathogenesis of filariasis have detected antibody responses to *Wolbachia* surface protein (WSP) in cats infected with *D. immitis* (Bazzocchi *et al.*, 2000), and rhesus monkeys infected with *B. malayi* (Punkosdy *et al.*, 2001). Subsequently, the specific antibody responses to WSP have been detected in patient sera, and the levels are correlated with the chronic manifestations of lymphatic filariasis (Punkosdy *et al.*, 2003). In addition to the antibody responses, *Wolbachia*-derived molecules likely induce host inflammatory responses that are thought to be associated with pathogenesis of human filarial diseases and the chemotherapy-induced adverse reactions.

In murine model, *B. malayi* extracts could induce macrophages to produce proinflammatory molecules, interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and nitric oxide (NO) (Taylor *et al.*, 2000). These inflammatory responses are dependent on molecules with lipopolysaccharide (LPS)-like activity. However, LPS of *Wolbachia* has never been characterized. Additionally, the relevant biosynthetic machinery is absent from the genomes of *Wolbachia* (Foster *et al.*, 2005). Therefore,

the other *Wolbachia* molecules responsible for the inflammatory responses are needed to be clarified. An additional study on human monocytes stimulated with adult worm *O. volvulus* extracts has provided evidence that the filarial nematode *Wolbachia* are major components for the monocytic inflammatory responses (Brattig *et al.*, 2001). The activations of monocytes to produce TNF-α and IL-8 by *O. volvulus* extracts were significantly reduced by *Wolbachia*-depleted *O. volvulus* extracts. Although *Wolbachia* are probably released following worm dead, and induce inflammatory responses, neither purified *Wolbachia* nor recombinant *Wolbachia* proteins are tested, which are likely to yield additional mechanism information on receptor usages, and inflammatory signaling pathways.

The recent publication of the *Wolbachia* Genome of *B. malayi* provides the opportunity to understand the molecular basis for the *Wolbachia*-filarial nematode relationship, and to examine pathways for new gene targets necessary for *Wolbachia*, as well as to select candidate molecules for investigation into the pathogenesis of filarial diseases (Foster *et al.*, 2005). However, DNA on its own provides limited data. The structure, function, and abundance of proteins in organisms, including *Wolbachia*, cannot yet be predicted from the DNA sequences alone (Anderson and Anderson, 1996; Washburn *et al.*, 2003; Foster *et al.*, 2005). Furthermore, a selection process is still needed to be verified at the protein expression levels (Petricoin *et al.*, 2002). The proteomic approach by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) coupled with high-throughput matrix-assisted laser desorption/ ionization time of flight mass spectrometry (MALDI-TOF-MS) is one of the technologies which can further our understanding of the molecular basis for the *Wolbachia*-filarial nematode relationship. Besides, the proteomic approach has been proved to be useful for identification and validation of drug targets (Cordwell *et al.*, 2001; Bandow *et al.*, 2003a; 2003b).

Nevertheless, proteomic studies of *Wolbachia* have been impeded by the obligatory nature of *Wolbachia*. Until recently, there has been neither cell nor cell-free culturing system for growing filarial nematode *Wolbachia* that places limitations in obtaining sufficient quantity as well as quality of *Wolbachia* materials. In this study, we developed a *Wolbachia* isolation method from filarial nematodes for protein analysis, and characterize proteins derived from *Wolbachia* of *B. malayi* by 2D-PAGE coupled with MALDI-TOF-MS. In addition, the role of a candidate *Wolbachia* protein in proinflammatory cytokine responses by the murine macrophage RAW 264.7 cell line was examined.

Hypothesis

1. Proteins of *Wolbachia* of *B. malayi* could be characterized by the developed isolation protocol for filarial nematode *Wolbachia*, and the high-resolution 2D-PAGE coupled with MALDI-TOF-MS.

2. Candidate *Wolbachia* proteins could induce macrophage cell line to produce proinflammatory cytokine responses.

Objectives

1. To develop a *Wolbachia* isolation method from filarial nematodes, and characterize proteins of *Wolbachia* of *B. malayi* by 2D-PAGE coupled with MALDI-TOF-MS.

2. To characterize the role of a protein candidate in induction of proinflammatory cytokine responses by the murine macrophage RAW 264.7 cell line.

Keywords

B. malayi

Wolbachia

WSP

2D-PAGE

Proinflammatory responses

Expected Benefits & Application

1. The purification method for *Wolbachia* from filarial nematodes will be developed for protein analysis.

2. The proteome map of *Wolbachia* of *B. malayi* will provide protein expression profile, which is useful for further studies in a molecular basic of the *Wolbachia*-filarial nematode relationship, in drug target identification, as well as in target proteins for diagnosis.

3. The study of *Wolbachia* surface protein (WSP) as a candidate proinflammatory molecule will provide understanding of the molecular and immunological basis of the proinflammatory cytokine responses in the murine macrophage RAW 264.7 cell line for further studies in inflammatory responses associated with the pathogenesis during human filarial infection.

Research Methodology

1. *Wolbachia* extraction from *D. immitis* with 0.85% NaCl supplemented with various concentrations of non-ionic nonidet P-40 (NP-40) detergent.

2. Immunofluorescence of purified *Wolbachia* by anti-recombinant *Wolbachia* surface protein (rWSP) antibodies.
3. Western blot of *Wolbachia* extracts by different homogenization buffers with anti-rWSP antibodies.
4. 2D-gel electrophoresis of *Wolbachia* of *B. malayi.*
5. Western blot of *Wolbachia* and *Acanthocheilonema viteae*[*] extracts with anti-insect *Wolbachia* antibodies.
6. 2D-image analysis.
7. Identification of spots specific for *Wolbachia* by in-gel digestion, MALDI-TOF-MS, and peptide-mass fingerprint searching.
8. Selection of candidate *Wolbachia* proteins.
9. Cloning, expression, purification and refolding of rWSP of *B. malayi Wolbachia.*
10. Incubation of the rWSP with the murine macrophage RAW 264.7 cell line at various concentrations, and different time points.
11. Determination of proinflammatory cytokine mRNA expression by real-time reverse transcriptase-polymerase chain reaction (real-time RT-PCR).
12. Data analysis by using the PD-QUEST imaging software, and the Sequence Detector 7000 software for quantification by real-time PCR.

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[*] *Acanthocheilonema viteae* is a filarial nematode species without harboring of *Wolbachia.*