CHAPTER III

MATERIALS AND METHODS

1. Study population

Blood samples were collected from infected individuals, infected with *Wuchereria bancrofti*, from the endemic area in 5 districts (Mae Sot, Umphang, Phop Phra, Tha Song Yang, and Mae Ramat) of Tak province at the Thai-Myanmar border, Thailand, were selected for the study, in cooperation with Filariasis Division, and The Center of Vector Borne Disease Control (VDC) 18 (Mae Sot district), Department of Communicable Disease Control (CDC), Ministry of Public Health, Thailand. The Center of VDC 18 is located in Mae Sot district, about 500 km northwest of Bangkok. Signs and symptoms of lymphatic filariasis were evaluated from interview and physical examination. The clinical symptoms observed in those individuals include lymphadenopathy, scrotal hydrocele and lymphedema of the legs. Infection status of each volunteer was determined by the presence of microfilaraemia and/or filarial antigenemia (The NOW® ICT Filariasis Test, Binax). Serum samples were collected before the initiation of the ongoing annual mass treatment (a single dose of diethylcarbamazine (DEC) at 6mg/kg). The serum samples were classified according to the clinical and parasitological status of each individual. Informed consent was obtained from each individual in the presence of two witnesses. All participants were given an explanation of the purpose, methods, merits, and risks of the study. Inform consent was obtained from all study subjects (and from parents of minors) for participation in this study. This study was approved by the Ethics Committee of the Faculty of Medicine at Chulalongkorn University, Bangkok, Thailand.

2. Blood specimens

Two to five milliliters of venous blood was collected from each individual under sterile technique and universal precaution. A whole blood sample aliquoted from the blood specimen was preserved in an EDTA tube. The rest of blood specimen was left for clotting, and serum was then separated. The samples were frozen at –70°C for later use. Plasma from endemic normals (antigen-negative and amicrofilaraemics from endemic areas with no apparent clinical symptoms) and non-endemic normals (healthy blood donors) were used for negative controls.

3. Parasitological study

Approximately 60 microliters of finger-prick blood specimen was collected between 8.00 p.m. and midnight. Thick-blood films were prepared in duplicate as described previously (Triteeraprapab and Songtrus, 1999; Triteeraprapab et al. 2000a; 2001; Nuchprayoon et al., 2001). After Giemsa's staining, microfilariae were examined independently under microscope by 2 technicians with consistent results. Identification of the parasite species was established. All of the microfilaria-positive specimens were *W. bancrofti.*

4. ICT Filariasis card test

The whole blood version of the ICT test kit (NOW ICT Filariasis Test; Binax, Portland, ME) for the qualitative detection of *W. bancrofti* antigen was performed according to the manufacturer's instruction. Briefly, approximately 50 µl whole blood was added to the sample pad. Thereafter, the card was closed and the result was read after 2 minutes through the viewing window of the test card. All negative tests were reassessed after 15 minutes by two independent individuals.

5. Enzyme-linked immunosorbent assay (ELISA) for anti-rWSP subclasses

Antigen concentrations and serum and conjugate dilutions were optimized by checkerboard titration using representative sera from healthy control, patients with lymphatic filariasis (chronic pathology, microfilaria+ ICT +, microfilaria- ICT+). Anti-WSP IgG subclass antibodies were determined as above with the exception of the antigen and the conjugate concentration. In brief, 96-well plates were coated with Wolbachia surface protein (WSP) 1 μg/ml. After three washes with 0.01 M phosphate buffered saline/0.05% Tween 20 (PBS/T20) pH7.4, each well was blocked with 100μl of 3% non-fat dried milk (Carnation) in PBS /T20 at 37°C for 30 minute. Serum samples diluted in PBS/T20 (1:100) were added and incubated overnight at 4 °C. After five washing, anti-human IgG1-, IgG2- IgG3- and IgG4- horseradish peroxidase conjugates (Zymed, South San Francisco, Calif.) diluted in PBS/T20 (1:1000) were added to each well and incubated at 37°C for 30 min. After another five washes, 100μl of a substrate mixture 3,3',5,5',-tetramethylbenzidine (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added to each well and kept in the dark at room temperature for 30 min. The reactions were stopped by adding 50μl of 4N H_2SO_4. The optical density (OD) was read at 450 nm. The cut-off values for anti-WSP IgG1, IgG2, IgG3 and IgG4 were obtained as OD arithmetical means + 3 standard deviations of sera of healthy volunteers.

6. Statistic analysis

The data were analyzed by using Excel 6.0® software program. Student's t-tests were used to evaluate statistically significant differences of mean antibody level in clinical manifestations and anti-WSP IgG subclass antibodies.