Materials and Methods

1. Studied population

This study was approved by the Research Ethics Committee of Faculty of Medicine of Chiang Mai University. Seventy-two fecal specimens were kindly provided by Associate Prof. Dr. Kom Sukontasan. They were from school children aged 12-19 years of Wat Vivak Wanaram school in Thumbon Nong-han, Amphoe Sansai, Chiang Mai Province. Another 144 samples were feces specimens from patients of Maharaj Nakorn Chiang Mai Hospital sent to Parasitology Laboratory, Department of Parasitology, Faculty of Medicine for routine parasitological examinations. All samples were frozen at –70 °C until used.

2. Examinations of fecal specimens

2.1. Formalin-ether concentration method

Fecal specimens were routinely examined by formalin-ether concentration method by Mr. Somsak Piangjai and Mr. Yongyut Muangyimpong of the Parasitology Laboratory, Department of Parasitology.

2.2. Agar plate culture method

Fecal specimens were cultured on agar plates (See Appendix) as described (Koga *et al.*, 1991). Briefly, approximately 1-3 g of feces was placed at the center of a small- or medium-sized agar plate, respectively. For the screening experiment, the plate was placed in a plastic bag and left at room temperature for 5 days. After which,

the plates were washed with 10% formalin. The larval suspension was centrifuged at 1,000*g* for 5 min and the larvae in the sediment were identified microscopically.

3. Standard direct smear

This method was applied to determine the density of *S. stercoralis* larvae in fecal specimens (Beaver *et al.*, 1984). The principle of this method was to stir a fecal sample in a drop (0.05 ml) of normal saline solution (NSS) such that its turbidity measured by a photoelectric light meter equals that of a standard barium sulfate solution. The photoelectric light meter used to measure the turbidity of fecal suspensions was calibrated with a barium sulfate solution (1N $BaCl_2$: 2N Na_2SO_4 = 3:2). A drop of fecal suspensions with the same meter reading as this standard barium sulfate solution will contain 5 mg of feces. The number of all larvae present in the fecal suspension was determined microscopically and averaged from two examinations. The number of larvae per gram of feces was then calculated.

4. Collection of L3

4.1. Agar plate culture method

The agar plate culture method was employed to collect a large number of L3 for further antigen preparation. The dishes used in this experiment were double-walled plastic dished (outer wall diameter, 9 cm; inner wall diameter, 8 cm). The agar was poured into the inner wall. One to three grams of *Strongyloides*-positive feces was placed on the surface of the agar. After two days at room temperature, when adult worms and L1 were observed under an inverted microscope, distilled water was added to the outer well. A strip of filter paper was used to form a bridge between the

surface of agar in the inner well and the water in the outer well. The dish was left one day at room temperature to allow migration of L3 to the outer well. The L3 were transferred to a 15-ml centrifuge tube and washed twice with NSS by centrifugation at 3,000*g*. The larvae were finally incubated with 1% HCl solution for 10 min to rid of contaminating bacteria, washed once with NSS and stored at –70°C for later use.

4.2. Filter paper slant culture method

This method was modified from a method previously reported by Little (1966). A strip of filter paper with 1 g of feces was placed at one end of a microscope slide that was then inclined in a double-walled plastic Petri dish containing distilled water so that the tip of the filter paper was in the water. The dish was incubated at room temperature for 4-5 days. The L3 were collected from the water and processed as described above.

5. Collection of L1

L1 were obtained from free-living female worms. Free-living females were manually isolated from the agar plate culture on day 2. The adult worms were manually aspirated to the NSS for washing eight times. Adult female worms were transferred to a 24-well plate, each well containing 20 worms in 1 ml NSS. The plate was left overnight at room temperature to allow deposition of eggs and hatching of the L1. The L1 were collected by centrifugation in a 15-ml centrifuge tube and stored at –70°C until use.

6. Preparation of antigens

6.1. L1

L1 were obtained as described above. Since the number of L1 was very small, crude antigens could not be effectively prepared by homogenization and sonication. They were therefore prepared by freeze-thawing. Approximately 17,000 larvae were frozen in liquid nitrogen and thawed. This was repeated 4 times and the suspension was then frozen at -70°C until use.

6.2. L3 and adult worms

Approximately 2-7 X 10^5 L3 or 4,490 adult worms were pulverized in liquid nitrogen and suspended in phosphate buffer saline (PBS) with protease inhibitors (consisting of 0.5 mM EDTA, 1 mM PMSF and 0.01 mg/ml of antipain-leupeptin). The suspensions were sonicated on ice by ultrasonic disintegration (Branson) 5X10 seconds at 50% duty cycle. The homogenate was stirred overnight at 4°C and centrifuged 12,000g for 30 min at 4°C. The supernatant fluid was collected and stored at -70°C until use. The protein concentration was determined by the BCA protein assay kit (Pierce).

7. Partial purification of the 41-kDa protein of L3

The 41-kDa protein was partially purified from L3 by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and electroelution. Approximately 1.6 mg of crude somatic extract of L3 was mixed with the sample buffer containing 5% 2-mercaptoethanol and boiled for 5 min. The sample was loaded in a 12-cm wide well of a SDS-polyacrylamide gel (4% stacking gel; 8-20% gradient resolving gel) and subjected to electrophoresis at 30-40 V. Molecular weight

standards used to calibrate the gel were broad range SDS-PAGE standards from BioRad (myosin, 200 kDa; β-galactosidase, 116.2 kDa; phosphorylase b, 97.4 kDa; serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa; aprotinin, 6.5 kDa). After electrophoresis, a 4-mm strip of gel was cut and stained with Coomassie brilliant blue R-250. The 41-kDa protein of L3 was located using the molecular weight standards. The stained gel strip was placed along side with the remaining portion of the gel. A 4-mm strip was cut across the gel at the 41 kDa region and sliced into small pieces. The 41-kDa protein was eluted from the gel slices into elution buffer (see Appendix) using an Electro-eluter (BioRad) at 8-10 mA per electroelution tube for 4 hr.

8. Removal of SDS from the protein sample

SDS-out™ (Pierce) was used to remove excess SDS from the protein solution. Twenty-five microliters of SDS-Out™ sodium dodecyl sulfate precipitation reagent were mixed with 500 μl of partially purified 41-kDa protein solution. After incubation in an ice bath for 20 min, the tube was centrifuged at 10,000*g* for 10 min. The supernatant was transferred to a KwikSpin™ microfilter and centrifuged for 1 min at 10,000*g* to clarify the supernatant. The sample was stored at –20°C until use.

9. Protein concentrations

Protein concentrations were determined spectrophotometrically with the BCA protein assay kit (Pierce), using bovine serum albumin (BSA) as a protein standard. Various concentrations of BSA standard or the protein sample (0.05 ml each) were mixed with 1 ml of the working reagent (50 parts of BCA reagent A with 1 part of

BCA reagent B) and incubated at 37°C for 30 min. After cooling to room temperature, the absorbances of the standards and the sample were measured at 562 nm. The protein concentration of the sample was determined from the standard curve of BSA standard.

10. Induction of antibodies against crude somatic antigens or 41-kDa protein of *S. stercoralis*

10.1. Animals

Mice and rabbit: Balb/c mice and a rabbit were obtained from the Animal Care Unit, Faculty of Medicine, Chiang Mai University. The mice and rabbit were housed at the Animal Care Unit throughout the study.

10.2. Immunization

Mice: Each mouse was injected by the intraperitoneal route with 50 μg of the crude somatic extract or the partially purified 41-kDa protein of *S. stercoralis* in Freund's complete adjuvant (Sigma-Aldrich). The injection was repeated 2 weeks later with the same amount of the antigens in Freund's incomplete adjuvant until a high antibody level was detected by indirect ELISA.

Rabbit: The rabbit was immunized with crude somatic extract of L3. The priming dose was given as a subcutaneous inoculation of 500 μg mixed with an equal volume of Freund's complete adjuvant (Sigma). The rabbit was boosted at 2-week intervals with Freund's incomplete adjuvant until a high antibody level was detected by indirect ELISA.

11. Hybridoma production (Mishell and Shiigi, 1980; Zola, 1987)

11.1. Preparation of myeloma cells

The myeloma cell line used in this study was X63-Ag 8.653. The myeloma cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM, Gibco BRL) supplemented with 10% fetal calf serum (Gibco BRL), 40 μg/ml gentamicin and 2.5 μg/ml amphotericin B. Two days before fusion, myeloma cells were grown to log phase at the initial density of 3-5 X 10^5 cells/ml. On the day of fusion, the number and % viability of the cells were determined using hemocytometer and 0.2% trypan blue exclusion technique.

11.2. Preparation of immunized spleen cells

The immunized mouse was deeply anesthesized with ether. The dead mouse was soaked in 70% ethanol for disinfection about 2 min. The heart blood was collected and used as a positive control. Upon exposing the abdominal cavity, the spleen was removed and placed in a sterile Petri dish containing 5 ml of IMDM without fetal calf serum (incomplete medium). After removing the fatty tissue adhering to the spleen, the splenocytes were obtained by gently grinding the spleen on a sterile brass sieve with a plastic syringe plunger. The spleen cell suspension was pipetted to a 15 ml-sterile centrifuge tube. After allowing the remaining clumps to settle, the cell suspension was transferred to a new 15-ml sterile centrifuge tube and centrifuged at 1,000*g* for 10 min at room temperature. The pelleted cells were loosened by tapping the bottom of the tube and 5 ml of red cell lysis buffer (see Appendix) was added and thoroughly mixed. After standing at room temperature for exactly 5 min, 5 ml of incomplete medium was added to dilute the red cell lysis buffer. The cell suspension was then centrifuged at 1,000*g* for 10 min. The spleen

cells were suspended in 10 ml of incomplete medium and the number and % viability of the cells were determined using hemocytometer and 0.2% Trypan blue exclusion technique.

11.3. Preparation of feeder cells

Spleen cells from a normal unimmunized Balb/c mouse were prepared as described above and used as feeder cells for hybridomas. The blood was obtained for negative control serum. The feeder cells were suspended in HAT (hypoxanthine, aminopterin, and thymidine) medium (see Appendix) at the cell density of 4 X 10^5 cells/well. Five hundred microliters of the cell suspension were plated out into each well of 24-well plates. All plates were left at 37°C in a humidified with 5% CO_2 incubator overnight before the fusion day. In one experiment, HAT medium containing 10% BM Condimed H1 medium (Roche, see Appendix) was used instead of the feeder cells.

11.4. Fusion procedure

Hybridomas were produced by the standard method (Zola, 1987) with some modifications. Spleen cells and myeloma cells at the ratio of 10:1 were mixed in a 50-ml conical tube and centrifuged at 1,000*g* for 10 min. The cell pellet was resuspended and washed once with 20 ml incomplete medium. The supernatant was carefully poured off to leave the cell pellet as dry as possible. The cell pellet was loosened by flicking the bottom of tube. The cells were fused by adding 1 ml of prewarmed 50% PEG (polyethylene glycol) over 1 min with gentle stirring. After standing for 1 min, 5 ml of incomplete medium was slowly added over the next 2 min followed by another 5 ml of the same medium immediately. The suspension was centrifuged at 1,000*g* for 10 min. The supernatant was carefully discarded and the

cell pellet was gently resuspended in 50 ml of HAT medium. Five hundred microliters of the cell suspension were dispensed into each well of the 24-well plates for 2 plates. The plates were returned to the CO_2 incubator and left undisturbed. In one fusion, hybridomas were produced according to Moonsom *et al.*, (2001). Spleen cells and myeloma cells at the ratio of 2:1 were mixed in a 50-ml conical tube and centrifuged at 1,000*g* for 5 min. After discarding the supernatant, the pelleted cells were loosened by flicking the bottom of tube and warmed at 37°C for 5 min. Prewarmed 50% PEG (1.5 ml) was added to the pellet drop by drop over a period of 1 min with continuously gentle stirring for 1 min. Prewarmed incomplete medium was then added as follows: 1 ml for 30-60 seconds, 2 ml for 30-60 seconds and the final 16 ml for 60-120 seconds. The cell suspension was immediately centrifuged at 1,000*g* for 5 min and incubated for 5 min at 37°C. The cells were subsequently suspended in 100 ml of HAT medium containing BM Condimed H1 and 100 µl of the cell suspension was plated out into each well of ten 96-well plates. After one week, 1 ml of HT (hypoxanthine and thymidine selection) medium (see Appendix) was dispensed into each well of the 24-well plates or 150 µl of HT medium containing BM Condimed H1 was plated out into each well of 96-well plates. The plates were checked daily for the presence of colonies of hybridomas. When the colonies were large enough, the culture supernatants were collected for screening for specific antibodies by ELISA or Western blotting.

11.5. Limiting dilution

A small volume of hybridoma suspension from each positive well was harvested and counted for viability with 0.2% trypan blue. In most fusions, the cells were prepared in HT medium at the concentrations of 100, 30, 10 and 3 cells/ml. One

hundred microliters of the cell suspensions were plated out into each well of 96-well plates that already contained 5 X 10^4 feeder cells in 100 μl of HT medium. The numbers of plates for each concentration were 1, 2, 2 and 3, respectively. For the final fusion, the limiting dilution was carried out such that each well of 96-well plates contained 4, 2 or 1 cells in 150 μl of complete medium containing 10% BM Condimed H1. After 1 week, the cloned hybridomas were ready to be screened for the presence of antibodies to crude somatic antigens or 41-kDa protein of *S. stercoralis* L3 by ELISA and Western blotting methods. The mAbs producing clones were expanded and their supernatants were collected for further characterization.

12. Screening of immunized sera and hybridoma culture supernatants

12.1. Indirect enzyme-linked immunosorbent assay (indirect ELISA)

The ELISA was performed as described by Morakote *et al.* (1991) using peroxidase system. Each well of the microtiter plate (MicroWell Module, Nunc) was coated with 100 μl of 5 μg/ml of the crude somatic antigens in coating buffer (sodium carbonate-bicarbonate buffer, see Appendix). After incubation at 4°C overnight, the plate was washed with washing buffer (PBST, see Appendix) 5 times. One hundred microliters of hybridoma supernatant or 100 μl of optimal dilution of mouse serum were added to each well. After incubation for 30 min at 37°C, the plate was washed 5 times with PBS-T to get rid of unbound antibodies. The horseradish peroxidase conjugated goat anti-mouse immunoglobulin A+G+M diluted 1:2,000 in 100 μl of PBST was applied to each well and allowed to react with antigen-antibody complexes at 37°C for 30 min. The plate was washed 5 times with PBST. Finally, 100 μl of freshly prepared *o*-phenylenediamine (OPD) substrate solution (see Appendix) were

added to each well and incubated at room temperature in dark for 30 min. The reaction was terminated by adding 50 µl of 8 N sulfuric acid (H_2SO_4) and the absorbance of each well was monitored by a Titertek Multiscan® MCC/340 at optical densities (OD) 492 nm. In the final fusion, 50 µl was added in each step and 60 µl of 5% BSA was used in the blocking step. The incubation time in each step was 1 hour except for that of the substrate solution, which was only 10 min. All samples were done in duplicate. Positive control sera were immunized mouse sera (IMS) of the same mice whose spleen cells were used for fusions. Negative control serum was normal mouse serum (NMS) or culture supernatant of normal hybridoma (NH).

12.2. Western blotting

The SDS-PAGE was carried out on 10% polyacrylamide minigel (10 X 7.3 cm) with 4% stacking gel according to the method of Laemmli (1970). One hundred and fifty micrograms of protein sample were loaded per gel. Electrophoresis was performed at a constant voltage of 80-100 V. After electrophoresis, an immunoelectrotransfer blot (Western blotting) technique (Towbin *et al.,* 1979) was carried out to detect antigen with antibody. The proteins in the gel were fixed in Tris-glycine buffer containing 20% methanol (transfer buffer, see Appendix) for 30 min then proteins were electroblotted onto nitrocellulose (NC) membranes at 25V, 7°C overnight using an electrophoretic transfer cell. The blotted NC was then removed and blocked by soaking in blocking buffer (see Appendix) for 1 hr at room temperature on a rocking platform, then frozen at -20°C until used. The NC was cut into 4 mm-wide strips. The NC strips were incubated with 1:40 diluted human sera or 1:100 or 1:200 diluted mouse sera or undiluted hybridoma culture supernatant at 4 °C overnight. After 3 washes with PBST, the strips were reacted with peroxidase

conjugated goat anti-human IgG or peroxidase conjugated goat anti-mouse IgA+G+M (diluted 1:1,000 in blocking buffer for chromogenic substrate or 1:20,000 for chemiluminescence substrate) for 1 hr at room temperature. After washing, the strips were immersed in 3,3'diaminobenzidine (DAB) substrate (see Appendix) until brownish reaction bands were observed then the reaction was stopped by rinsing with distilled water. For the chemiluminescence substrate the strips were immersed in luminol enhancer (Pierce) for 10 min and immediately exposed to an X-ray film for 30 sec before developing.

13. Isotyping of mAbs

The isotypes of the mouse mAb were identified by using a commercial ELISA kit (Sigma). The experiment was performed according to the protocol provided. Each well of the ELISA plate was coated with 50 µl of goat anti-mouse immunoglobulin (Ig) isotypes (IgG1, IgG2a, IgG2b, IgG3, IgM, IgA) diluted to 1:1,000 in carbonate bicarbonate coating buffer (pH 9.6). After incubation at 4°C overnight, the plate was washed with PBS-T and diluted mouse Ig isotype controls or undiluted hybridoma culture supernatants were applied and incubated at 37°C for 30 min. The reaction complexes were monitored by adding 50 µl of peroxidase conjugated goat anti-mouse IgG+IgA+IgM or goat anti-mouse IgM or goat anti-mouse IgG (1:2,000 diluted) and incubated at 37°C for 30 min. After 5 washes with PBST, 50 µl of OPD substrate was added to each well and incubated at room temperature, protected from light for 30 min. The reaction was stopped by adding 50 µl of 8 N H_2SO_4 and the absorbance was read with an ELISA reader at 492 nm.

14. Purification of IgG from rabbit antiserum to L3

Serum Igs were prepared by ammonium sulfate precipitation. Antiserum (2.7 ml) from rabbit immunized with L3 antigens was added with solid ammonium sulfate to 40% saturation. After incubation at 4°C overnight, Ig precipitates were collected by centrifugation at 10,000 rpm, 4°C for 30 min. The precipitated Igs were resuspended in 1/20 volume of the starting volume in PBS. The solution was dialysed against PBS overnight at 4°C. Rabbit IgG against L3 antigens (rIgGαL3) was purified from Ig solution using HiTrap Protein G column (Pharmacia). After washing the column with 10 ml of 20 mM sodium phosphate, pH 7.0 (washing buffer) and the Ig solution was applied to the column. The column was washed by 10 ml of washing buffer. The rIgGαL3 was eluted from the column by applying 0.1 M glycine-HCl, pH 2.7. The eluted rIgGαL3 solution was neutralized with 1M Tris-HCl (pH 8.0) followed by dialysis against PBS. The rIgGαL3 concentration was determined by measuring the absorbance at 280 nm (1.35 OD at 280 nm = 1 mg/ml).

15. Purification of mAbs from culture supernatants

The purification of IgG monoclonal antibody was essentially the same as that of rIgGαL3 described above. The purification of IgM monoclonal antibody was also the same except that a column of goat anti-mouse IgM (μ-chain specific) coupled to agarose (Sigma) was used instead of HiTrap Protein G.

16. Biotinylation of Igs

The purified IgM or rIgGαL3 was biotinylated by the method described by Kongtawelert and Ghosh (1990) with slight modification. One milligram of Ig was

dialysed against 50 mM $NaHCO_3$ (pH 8.5) at 4°C overnight. After this, *N*-hydroxysuccinimidobiotin in dimethylsulfoxide (DMSO) was added to Ig solution such that the ratio of *N*-hydroxysuccinimidobiotin to Ig was 3:1 (w:w). The mixture was incubated at 4°C overnight followed by dialysis against PBS to separate biotinylated-IgM or rIgGαL3 from free biotin.

17. Labeling of Igs with peroxidase

The EZ-Link™ Plus Activated Peroxidase kit was used to label Igs. One mg of Ig was dialysed against 0.2 M carbonate/bicarbonate buffer (pH 9.4) at 4°C overnight. Then the solution was added with the 100 µl of EZ-Link™ Plus Activated Peroxidase and incubated for 1 hr at room temperature. The solution was incubated at room temperature for 15 min after the addition of 10 µl of reductant buffer and another 15 min after the addition of Quench buffer. The conjugate was finally dialysed against PBS at 4°C overnight.

18. Absorption of biotinylated rIgGαL3

Biotinylated rIgGαL3 (rIgGαL3-B) was absorbed with normal fecal samples to remove antibodies to nonspecific substances in the feces (Allan *et al.*, 1992; Nageswaran *et al.*, 1994). Forty normal fecal samples (negative for all parasites, examined by formalin-ether concentration and agar plate culture methods), 0.5 gm each, were pooled and mixed with equal volume of PBS containing 0.3% Tween20. The fecal suspension was stored at -40°C. To absorb the rIgGαL3-B, 200 µl of fecal suspension was boiled for 30 min and centrifuged at 10,000 rpm for 10 min. After the supernatant was decanted, the packed feces was suspended in 100 µl of rIgGαL3-B

and mixed on a rocking platform for 1 hr at room temperature. The absorbed rIgGα L3-B was separated from the fecal pellet by centrifugation at 10,000 rpm for 10 min and stored at -20°C for later use.

19. Fecal supernatant preparation

Frozen fecal samples were thawed and PBS containing 0.3% Tween 20 was added in 1:1 ratio (v/v). The suspension was well shaken in a centrifuge tube until a uniform mixture was obtained. It was then centrifuged at 10,000*g* for 10 min or boiled for 10 min at 80 °C before the centrifugation and the clear supernatant fraction was used for coproantigen analysis.

20. Preparation of pooled positive and negative fecal samples

Pooled positive fecal sample was prepared by combining 42 *S. stercoralis* positive fecal specimens, 0.5 g each, and resuspended in equal volume of 0.3% Tween 20 in PBS. The suspension was vortexed until a uniform mixture was obtained and stored at –40°C until use. Forty negative fecal samples were pooled and similarly processed.

21. Coproantigen sandwich ELISA (modified from Espino and Finlay, 1994)

Optimal concentrations of rIgGαL3 or Ig from mouse mAb to L3 used to coat microtiter plates were determined to be 5-20 μg/ml. Polystyrene microtiter plates (Maxisorp; Nunc) were sensitized with 100 μl of rIgGαL3 or mAb diluted in coating buffer (see Appendix) overnight at 4°C. The plates were washed four times with PBST. The fecal supernatants (100 μl) prepared as above were added to the wells and

the plates were incubated for 90 min at 37°C. After four washes with PBST, each well was incubated with 100 μl of rIgGαL3-B or biotinylated mAb (IgM-B) (10 μ g/ml) for 30 min at 37°C. The plates were again washed and each well was incubated with 100 μl of peroxidase conjugated mouse monoclonal antibody to biotin (PO-anti-biotin mAb) (1:2000 dilution) for 30 min at 37°C. After another washing step, the OPD substrate (see Appendix) was added and the plates were incubated in dark at room temperature for 30 min. The enzyme reaction was stopped by the addition of 50 μl of 8N H_2SO_4 to each well. The absorbance at 492 nm was read using a Titertek Multiskan ELISA reader. The cut-off point for the positive value of individual fecal sample was taken as the mean OD of negative samples + 2 SD. For the pilot study, the sample was considered positive when its OD was more than or equal to two times of the OD of the negative control.

22. Statistical analyses

The difference between agar plate and formalin-ether concentration methods was determined by McNemar chi-square test. Independent *t*-test was used to test the difference between individual *S. stercoralis*-infected and uninfected fecal samples.