รายงานฉบับสมบูรณ์โครงการวิจัย

เรื่อง

การตอบสนองทางภูมิคุ้มกันระดับสารน้ำและระดับเซลล์ในสัตว์ทดลองที่ติดเชื้อพยาธิตัวจี๊ด

(Humoral and Cell-mediated immune responses of experimental Gnathostomiasis)

ทุนวิจัยจากเงินงบประมาณแผ่นดิน
ประจำปี 2548
จุฬาลงกรณ์มหาวิทยาลัย

โดย

รองศาสตราจารย์ วิไล ศักดิ์ศิริสัมพันธ์

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Wilai Saksirisampant

Wilai Saksirisampant

INTRODUCTIONS

Gnathostoma spinigerum is the major causative agent of human gnathostomiasis in Thailand. The parasite life cycle, which has cat and dog as the definitive hosts, involves two intermediate hosts. The first intermediate hosts are crustaceans and copepods. The second intermediate host are fresh water fishes harboring the infective third-stage larvae (L3s)(Daengsvang, 1981).

Human are accidental hosts, acquiring the infection by consumption L3s in raw or undercooked fresh-water fish, especially swamp eel, eel, catfish and snake-headed fish (Daengsvang, 1981; Rojekittikhun et al., 1989; Saksirisampant et al., 2002).

The larva can migrate to any part of the human body and cause pathological changes in the tissues and organs which it reaches. According to the similarity of the syndromes caused by other parasitic worms as well as the absence of eggs in human excreta, immunological techniques have until now become important as a diagnostic tool. In the past many test had been tried including detecting the skin reaction and antibody with precipitation, radio-immunoassay, enzyme-linked immunosorbent assays and Western blot (Yamaguchi, 1952; Ando, 1957; Kasemsuth, 1981; Suntharasamai, 1985; Anantaphruti et al, 1986; Dharmkrong-at, 1986; Morakote, 1987; Maleewong, 1988; Tapchaisri et al., 1991). However, the results have demonstrated the false positive reaction with some other helminthic infections. Moreover, an evaluation of the effectiveness of chemotherapy as measures by ELISA changes, conversion of skin test, disappearance of swelling and return to normal of eosinophilia have shown limited potential use (Nontasut, 2000; Kraivichian et al., 2004). The host-parasite relationship in term of immune responses is still poorly understood. Much less information is currently available on both humoral and cellular immune status of this tissue nematode. On the other hand, the information of immune regulation of infections indicate immune mechanism, pathogenesis and can be applied to immunodiagnosis. When infection was established, both Th-1 and Th-2 response perform antagonistly to regulate the infection. In general the principle immune response to protozoa that survive within macrophage is cell-mediated response, particularly macrophage activation by Th-1 cell-derived cytokines. Whereas in many helminthic infections, the responses are mediated by the activation of Th-2 subset of $CD4^+$ helper T cells. The activated Th-2

cells secrete IL-4 and IL-5 which IL-4 stimulates the production of the neutralizing antibodies (IgG1, IgG4 and IgE), while IL-5 stimulates the development and activation of eosinophils (Abbas and Lichtman, 2003). Limited evidences previously showed that human gnathostomiasis had an elevation of serum total IgE and specific IgE-Ab levels, and blood eosinophil (Soestayo et al., 1987; Saksirisampant et al., 2001; Kraivichian et al., 2004). A study on the immune responses of antibodies, lymphocyte proliferation and CD4 and CD8 T- cell subset and those intra- interferon gamma of this mouse-gnathostomiasis are much of interesting. Antibodies level of IgG subclass and IgE-Ab can offer informations indicating the immuno-regulation and leading to an improvement of serodiagnosis and an evaluation of the effectiveness of chemotherapy. In addition, an in vitro detection of lymphocyte proliferation by stimulating with mitogen such as concanavalin A (Con A) and phytohemagglutinin (PHA) can confer the effector T cells function. Because in host-parasite interactions, parasites try to resist to immune effector mechanism or inhibit host immune response by multiple mechanisms. The defect of T cell function or T-cell anergy to parasite antigen and to the homologous antigens has been observed in many infections included *Trichinella spiralis* (Faubert, 1982), *Onchocerca volvulus* (Soboslay et al., 1999), *Toxocara canis* (Allen et al., 1996) and *Taenia crassiceps* (Sciutto et al., 1995). This generalized immunodepression is possibly related to Th-2 responses, which lead to down-regulation of host-immune responses, by the induction of IL-4 (Allen et al., 1996).

In this research, mice were infected orally with 10 and 20 larvae of L3s. At different time interval,an ELISA and lymphoproliferative stimulation by Con A and specific antigen had been study to compare the antibody responses and the cellular responses between infected and uninfected animal. The ELISA antibody responses of IgG, and IgE and the immunodepression of infected and drug treated mice could be shown in this study.

LITERATURE REVIEWS

1. Biology of *Gnathostoma spinigerum*

1.1 Geographical distribution

Infection caused by *Gnathostoma spinigerum* is common in many Asian countries including Thailand, China, Japan, Vietnam, Myanmar, India, Bangladesh, Malaysia, Indonesia, Israel and Philippines (Rojekittikhun et al., 1989; Rusnak and Lucey, 1993; Edward et al., 1999) with a few reported cases in Australia (Moorhouse et al., 1970) Africa (Chhuon et al., 1976), Mexico and Ecuador (Ollague et al., 1984; Ogata et al., 1998).

1.2 Classification

Gnathostomiasis is a zoonotic disease caused by roundworms of the genus Gnathostoma. There are 23 species have been reported (Almeyda, 1991). Only five species of mature adult Gnathostoma were natually recovered from the stomachs and urinary system of animals in Southeast Asia. These are *G.spinigerum, G.hispidum, G.doloresi, G.vietnamicum* and *G.malaysiae.*

Phylum Nematoda
Subclass Secernentea
Order Spirurida
Suborder Spirurina
Superfamily Gnathostomatoidea (Thelaziodea)
Family Gnathostomatidea
Subfamily Gnathostomatinea
Genus Gnathostoma

1.3 Life cycle

G.spinigerum is the only causative agent for human infection in Thailand (Daengsvang, 1980; Radomyos, 1987; Kamiya, 1987; Rojekittikhun, 2002). Adult *G. spinigerum* was first discovered by Richard Owen in 1836 from gastric tumours of a young tiger that died in the London Zoological Garden (Daengsvang, 1980). The first case of human gnathostomiasis was reported when the worm was recovered from a swelling in the breast of a young Thai women living in Bangkok (Daengsvang, 1983). Thereafter increasing number of cases with removed

G.spinigerum were reported from many countries (Morishita, 1924; Rojekittikhun et al. 1989; Akahane et al., 1994).

Prommas and Daengsvang firstly described the life cycle of *G.spinigerum* in 1933. Cats, dogs, tigers, lions and leopards can act as the definitive host. Fresh water cyclops (*Mesocyclops leickarti, Eucyclops agilis, Cyclops varicans* and *Thermocyclops sp.*) are the first intermediate host. A wide variety of animals including fishes, birds, reptiles, amphibians and some mammals for example the domestic poultry, frog, snake are the second intermediate hosts. Adult male and female worms inhabit a stomach of the definitive host which they produce non-embryonated eggs. The eggs are released through the opening of the stomach tumor and pass out the lumen in the faeces. First-stage larvae (L1s) develop and hatch from the eggs within 12 days in fresh water at 25-31 °C. The actively swimming first-stage larvae are then ingested by species of first intermediate host, in which they penetrate the gut to the hemocoel to become complete second-stage larvae. Further developments occur inside the body cavity, and the larvae finally transform into early third stage (L3s) within 6-7 days (Daengsvang, 1986).

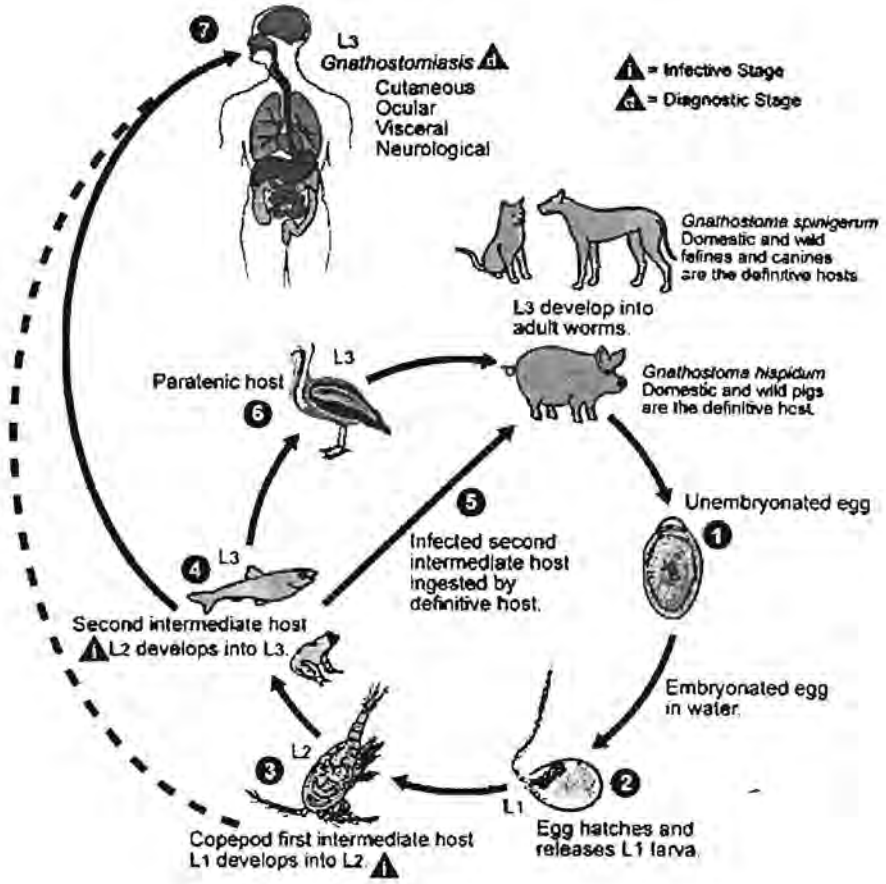
When the infected cyclops are eaten by fish or other second intermediate hosts, the larvae develop into advanced L3s in the flesh of these animals. Forty-eight species of vertebrates have been reported to serve naturally as the second intermediate or paratenic hosts. These are fish (20 species), amphibians (2 species), reptiles (11 species), avian (11 species) and mammals (4 species). Among these animals, swamp eels (*Monopterus albus,* previously *Fluta alba*) was the best second intermediate of *G.spinigerum* on the basis of having the highest prevalence rate and the greatest infection intensity (Daengsvang, 1980; Rojekittikhun et al., 2002; Saksirisampant et al., 2002). The maximum number of gnathostome larvae collected as ever been recorded was 2,582 larvae from one eel in Nakhon Nayok Province (Setasuban et al., 1991). Almost all of the gnathostome larvae recovered from eels have been in their encysted form. About half (43%-52%) of the total number of larvae were concentrated in the liver, whereas the remaining half were in the whole muscles (Rojekittikhun, 2002). The definitive hosts get infection by eating these infected fleshes. The taken up larvae will penetrate the stomach wall, enter the liver, and migrate to muscle and connective tissues where they molt to immature worms. The worms return to the stomach by penetrating the stomach wall from the serosal side and become mature adults in the cavity of a tumor-like mass which

communicates with the stomach lumen through a small opening. It took about 6-7 months for the worm to become sexually mature.

Man is an accidental host, because after the L3s being ingested, the parasite cannot fully develop. The larvae then could move to various following organs: namely respiratory tract, gastrointestinal tract, genito-urinary tract, mucous membrane of the soft palate, central nervous system and eyeball (Kittiponghansa, 1987; Prijyamonda, 1955; Bodhidat, 1956).

From a total of 23 worms obtained from patients attending various hospitals and were sent to the Faculty of Tropical Medicine for identification (Radomyos, 1987), 5 were advanced L3s (21.7%), 14 were immature worms (60.9%) and 4 were mature worms (17.4%). The size of these advanced L3s varied from 2.20-3.50 mm x 0.40-0.63 mm. The immature worms were those of 8 males and 6 females with the average size of 4.63-9.35 mm x 0.60-1.05 mm and 3.8-16.2 mm x 0.83-1.0 mm respectively. The mature worms were all male whose size varied from 9.9-12.5 mm x 1.0-1.25 mm (Daengsvang, 1980).

There are thirty species of vertebrates that are experimentally susceptible to be accidental hosts or second intermediate hosts. Those are 2 species of fresh-water fish, 5 species of amphibian, 5 species of reptile, 3 species of avian, 2 species of crustacean and 13 species of mammal (Norway rat, white rat, black rat, little house rat, white mouse, hamster, guinea pig, tree shrew, squirrel, domestic pig, Macaque monkeys and leaf monkey)(Daengsvang, 1980; Rojekittikhun, 2002).



Life cycle of *Gnathostoma spinigerum*

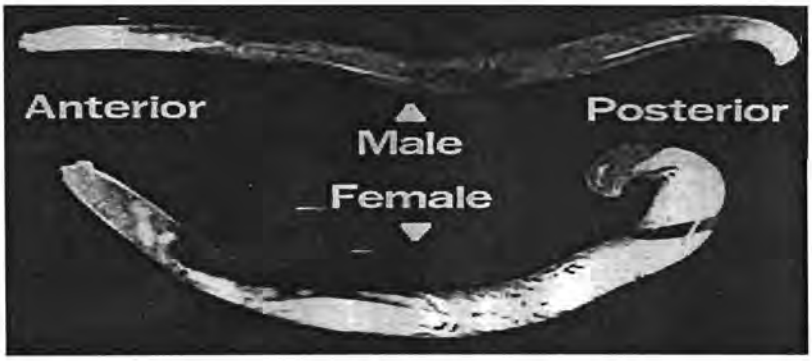
1.4 Morphology

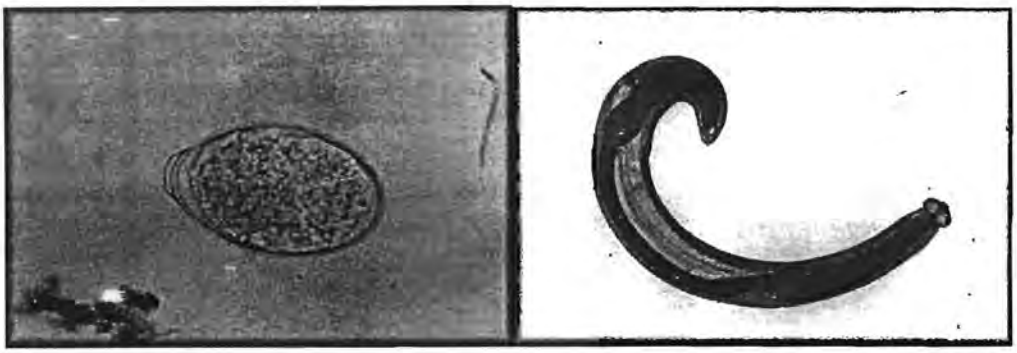
The parasite has a characteristic head-bulb providing with cephalic hooklets. The number of hooklets generally varies from one row to another. The morphology and the arrangement of hooklets are used for the identification of species (Daengsvang, 1980). The anterior half of the body is covered with transverse rows of flat, toothed spines, followed by a bare portion. The posterior tip of the body has numerous tiny cuticular spines.

Adults worm, males average measures are 12-40 mm x 1.0-3.0 mm and have a bluntly rounded posterior end. Female average measures are 25-54 mm x 1.0-3.0 mm and also have a blunt posterior end.

The egg is 65-70 μm x 38-40 μm and colorless. Its polar cap is at only one end.

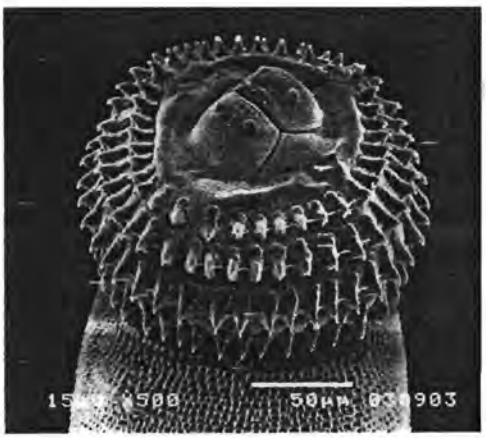
Advanced L3s possess a typical head-bulb armed with 4 rows of cephalic hooklets and a pair of trilobed lips. Each row has about 40 or more in number, which increase posteriorly. The average size is 5.0 mm long, but can up to 12.5 mm.



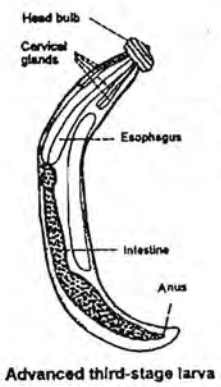


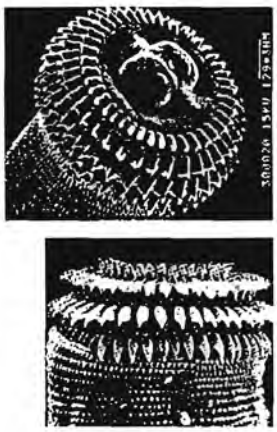
Egg

Advanced L3s



Head-bulb of advanced L3s





cephalic hooklets characteristic of L3s

Morphology of eggs, advanced L3s (Head-bulk, hooklets) and adult *G.spinigerum*

1.5 Modes of transmission

Experimentally, there are 3 following methods for spreading the infection:

1.5.1 Oral transmission

The major route of infection is ingestion of uncooked larvae presented in fresh water fishes that harbor the infective stage.

1.5.2 Skin penetration

The infective stage or the advanced L3s is capable of penetrating the skin of the host (Prommas and Daengsvang, 1933).

1.5.3 Prenatal infection

Prenatal transmission of advanced L3s from mother to offspring can occur in experimental mice. In man, two cases of prenatal gnathostomiasis were reported. It was the three-day old baby, which had creeping eruption with immature male on the skin of the chest. Another was one month old baby with immature worm removed from the skin of the umbilical area (Daengsvang, 1986).

2. Clinical manifestations and pathology

During 1961 and 1963, about 900 patients with gnathostomiasis were clinically diagnosed each year in Thailand. There are about 10 deaths reported between 1967-1981 (Daengsvang, 1980; Daengsvang, 1986). In each year from 1985 to 1988, between 300-600 suspected gnathostomiasis patients came to the gnathostomiasis clinic at the hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University, Bangkok. Although in the recent past, people have received better health education, the disease still prevalent, possibly because of their social customs and dietary habits (Tuntipopipat, 1989). From 1989 until now, about 100-400 new cases of suspected gnathostomiasis have been seen each year at this hospital (Rojekittikhun, 2002). At Chulalongkorn Memorial Hospital in Bangkok, approximately 100-150 patients of the new suspected cases come to the Parasitology Clinic each year (Kraivichian, 2004).

2.1 Symptoms and pathogenesis

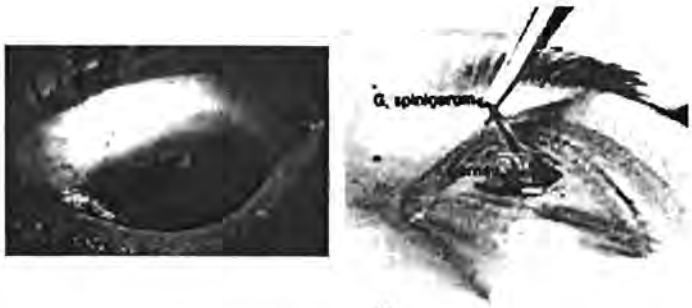
A few days after advanced L3s are ingested, these larvae will migrate through the intestinal wall and into the abdominal cavity and then through several tissues causing lesion in many organs and subcutaneous tissues. Mechanical action associated with migrating worm and host responses may also contribute to two

classified pathological changes: namely cutaneous and visceral gnathostomiasis (Miyazaki, 1960).

For cutaneous gnathostomiasis, it appears as intermittent migratory swelling of various circumscribed sizes lasting one to two weeks. The edema is mainly due to the inflammatory reaction of the tissue with mononuclear cell, mainly eosinophil infiltration (Boongird et al., 1977; Rusnak et al., 1993; Kraivichian et al., 2004). Patients can have allergic reaction with itching or irritation with pain at the swelling areas.

For visceral gnathostomiasis, L3s may migrate to the central nervous system (CNS) (Chitanondh and Rosen, 1967; Punyagupta, 1968). When cerebral gnathostomiasis occurs, the disease is known as eosinophilic myeloencephalitis with long-term sequel or even death. The CSF pressure in these patients is high with a marked increase of white blood cells, especially eosinophils. The clinical course of CNS gnathostomiasis that involves only the spinal cord leads to paralysis of extremities (Bunnag, 1970). A severe agonizing pain over the trunk and lower extremities commonly appears before paralysis occurs (Punyagupta, 1968). The mortality of CNS gnathostomiasis was reported to be as high as 25 percent (Punyagupta, 1978). Gnathostomiasis is one of the three most common parasitic infection that involve the CNS in Thailand (Vejjajira, 1978; Tuntipopipat et al., 1989).

a



b

Symptoms of human gnathostomiasis

a: *G.spinigerum* from eyeball

b: creeping eruption

2.2 Diagnosis

The definitive diagnosis of human gnathostomiasis must be made by recovery of the worm. However, if the parasite is not recovered the following clinical and laboratory findings of the suspected cases may be useful in bringing closer diagnosis to the infection:

1) Presumptive diagnosis may be made on the basic of clinical symptoms and signs for cutaneous and visceral gnathostomiasis.
2) Previous history of eating raw or improperly cooked fish.
3) History of living in or visiting the endemic or near-by area of the infection.
4) Blood picture

Gnathostomiasis should be suspected when a localized edema is accompanied by leucocytosis with a high percentage of eosinophils. Leucocytosis of

over 10,000 cells/mm^3 is common and between 10 and 96 percent of the cells are usually eosinophils (Daengsvang, 1980; Nuchprayoon et al., 2003; Kraivichian et al., 2004).

5) Immunodiagnosis

Since, there is similarity between the syndromes caused by other parasitic infections such as angiostrongyliasis and many ectopic parasitic infection and that of gnathostomiasis; the miss diagnosis may be made (Tuntipopipat et al., 1989; Tapchaisri et al 1991). Following were the immunological test for detecting antibodies.

a) Skin test

The skin test was firstly used to diagnose human gnathostomiasis, it gave 100% sensitivity but poor specificity (Egashira, 1953 and Yamaguchi, 1951; Chanthachume et al., 1985). The extract of the esophagus was more antigenic than that of the other parts of the worm (Miyazaki, 1960; Miyazaki, 1966). In experimental animal, this skin reaction could be demonstrated at the first month of infected mice (Morakote et al., 1987).

b) Precipitin test

A precipitation reaction appeared prominently in the anterior tip of the adult worm within 2 weeks and reached a peak at the 6^{th} week and declined within 10 to 18 weeks (Furuno, 1959).

Microprecipitation reaction of larva with infected human and experimental animals sera showed membranous precipitates around esophageal region and filamentous precipitates randomly on the surface of the worm. This indicated that the epitopes are located exteriorly on the advanced L3s. (Rojekittkhun, 1989).

c) Indirect hemagglutination antibody test (IHA)

Haemagglutinating (HA) antibodies reactive with advanced L3s somatic extract could be detected as early as one week after infection with titer 1:40 (Priwan, 1985). The titer gradually increased to 1:1,280 within eight weeks.

d) Enzyme Linked Immunosorbent Assay (ELISA)

By using advanced L3s *G.spinigerum* as an antigen, IgG ELISA has been trialled to diagnose human gnathostomiasis. The test showed 100% positive in all parasitological confirmed cases and all eosinophilic meningo-myeloencephalitis cases. But not all of the sera from cutaneous migratory swelling cases (CMS) were positive (Suntharasamai et al., 1985; Dharmkrong-at et al., 1986).

Similar results were obtained when excretory and secretory (ES) antigen which released in the culture medium with the highest amount at day 4 was used (Rattanasiriwilai et al., 1985). However, these ELISA showed superior results in diagnosis than that of indirect hemagglutination test and counter immunoelectrophoresis (Maleewong et al., 1988).

In addition, the total IgE and specific IgE in gnathostomiasis sera were markedly higher than healthy controls. This indicated that *G.spinigerum* potentiates IgE production (Soesatyo et al., 1987 and Saksirisampant et al., 2001).

Isotype of both IgG and IgE cross-react with following infections namely: angiostrongyliasis, opisthorchiasis, filariasis, paragonimiasis, strongyliasis, enterobiasis and hook worm infection (Suntharasamai et al., 1985; Soesatyo et al., 1987; Maleewong et al., 1988; Saksirisampant et al., 2001).

The use of IgG subclass antibodies could improve the overall sensitivity and specificity of the anti-*G.spinigerun* L3s ELISA. The detection of IgG2 antibody against *G.spinigerum* L3s reduced cross reactivity to most parasites, particularly *A.cantonensis*. The ELISA IgG1 antibody was suggested a reliable laboratory screening test, while anti-*G.spinigerum* L3s IgG2 antibody could be used to confirm the diagnosis (Nuchprayoon et al., 2003).

Since IgG-Ab could persist more than six months following drug treatment (Kraivichian et al., 1992 and Nontasut et al., 2000); thus the evaluation of the effectiveness of chemotherapy by detecting this IgG-Ab was still not satisfactory. However, suitable immunodiagnosis for such evaluation has not been reported.

By using a two-site enzyme-linked immunosorbent assay, the circulating antigen was detected at the first week, and increased steadily up to week 3, with the peak at week 4 of infection. There was no significant amount of the circulating antigen which was detected after week 4 of infection (Maleewong et al., 1992).

e) Western blot analysis

About 20 protein components of advanced L3s *G.spinigerum* has been shown by SDS-PAGE with relative molecular weight ranging from 10 to 99 kDa (Priwan, 1985; Saksirisampant, 1986). This purified 24 kDa protein demonstrated 100% specificity and sensitivity in ELISA when tested with parasitologically and clinically diagnosed gnathostomiasis patients (Nopparatana et al., 1991). However, from many study, it has been shown that specific antigen of *G.spinigerum* is a protein with molecular weight of 24 kDa, while bands of 43 and 49 kDa showed

reaction with IgE-Ab (Tapchaisri et al., 1991; Saksirisampant, unpublished observation). The protein band of approximately 50 kDa showed specificity to mice infected sera as well (Priwan, 1985).

3. Immunological studies on Gnathostomiasis

3.1 Antigenicity of *Gnathostoma spinigerum*

Nematode has a wide variety of antigens capable of stimulating immune responses in mammalian hosts, namely: surface or cuticular, metabolic or excretory-secretory (ES) and somatic antigens. Moreover, all of these antigens can be found in all developmental stages, starting from larval to mature adult. The surface components can be prepared by solubilizing the parasites with either ionic or non-ionic detergents. Excretory-secretory products can be obtained from an in vitro maintenance or cultivation of living parasites in protein-free culture medium. Generally, the most common source is the whole worm or somatic extract.

The larvae stage antigen of *G.spinigerum* which migrant in host is more immunogenic than that of adult worm. The sensitivity of larvae somatic antigen by ELISA was 100% while that of adult female and male somatic antigens were 70%, 60% respectively (Morakote et al., 1991).

Western blot analysis has been shown that a protein with a relative molecular weight of 24 KD prepared from the advanced L3s is highly specific to this infection (Tapchaisri et al., 1991). Among the three preparation of advanced L3s somatic extract from distilled water, Triton X-100 and sodium deoxycholate (NaDOC), the distilled water preparation yielded the greatest amount of diagnostic 24 kDa antigen. Little difference in protein and antigenic patterns was found between the extracts of larvae collected from naturally infected eels and from experimentally infected mice (Nopparatana et al., 1992). This 24 kDa component was found predominantly in the esophagus and intestine. By in direct fluorescent antibody assays the target antigens were anterior part of the esophagus, surface of the cuticle and in the cytoplasmic granules of the intestine (Morakote et al., 1989).

The ES products may be present more potential components than those of the somatic extract for evaluation of the effectiveness of chemotherapy. Because the antibody responses to secreted products are more closely related to active infection than the anti-whole worm antibody that may persist following the death of the parasites (Saksirisampant et al., 2001).

3.2 Antibody responses

IgG and IgE antibodies response were induced in gnathostoma infection. The sensitivity of ELISA for these anti-*G.spinigerum* antibodies ranged from 59% to 95%, while the specificity ranged from 79% to 96% (Suntharasamai et al., 1985; Maleewong et al 1988; Anantaphruti, 1989; Saksirisampant et al., 2001).

In addition, the IgG antibody had quite persisting titers in post-treatment condition of many parasitic infections including gnathostomiasis (Nontasut et al., 2000). IgG antibody persist for several months after successful treatment. These facts should be taken into consideration in the interpretation of serological results, since positive IgG antibody level may reflect a previous rather than a present infection. In many helminthic infections, the activated Th-2 cells secrete IL-4 and IL-5, which stimulates the production of IgG1, IgG4, IgE and eosinophils. In *G.spinigerum*, evidences previously showed that gnathostomiasis sera had an elevation of IgG1, IgG4 and IgE.

3.3 Cellular immune responses

In contrast to the antibody response, cell-mediated immune response to this nematode infection has not been reported.

It is well recognized that various types of infections, including protozoa and helminthes may be associated with alteration of immune response to specific antigens and mitogens. Both immunoenhancement and immunodepression have been reported in many host-parasite systems. And most nematode infections are chronic in nature because of their ability to evade or resist elimination by adaptive immunity (Terry and Hunson, 1982).

Parasites evade protective immunity by reducing their immunogenicity and by inhibiting host immune responses. Different parasites have developed remarkably multiple mechanisms of resisting immunity such as antigenic variation, inhibition of host immune responses and antigen shedding. T-cell anergy to parasite antigens has been observed in severe schistosomiasis involving the liver and spleen and in filarial infections. In lymphatic filariasis, infection of lymph nodes with subsequent architectural T-cell disruption may contribute to deficient immunity. In malaria and African trypanosomiasis generalized immunosuppression is caused by the production of immunosupressive cytokines from activated macrophages and T cells (Abbas and Lichman, 2003).

The immunodepression which has been demonstrated in several helminth infections were more obvious in those of heavy and chronic infection. For example, hamsters experimentally infected with *Opisthorchis viverrini* had significant depression of lymphoproliferative response to mitogen, homologous and heterlogous antigens. These immune-alteration was abolished by anthelmintic treatment. It was suggest that defective T cell functions could interfere immune surveillance system against neoplastic transformation. Since, chlolangiocarcinoma was the severe complication of this liver fluke infection (Sirisinha et al., 1983). Furthermore, in schistosomiasis the defective T-cell function demonstrated by lymphocyte proliferation resulted in drastically decreased of egg granulomatous reaction. T-cell defect may be considered as a host evasion of the parasite and as a modulation of the host immune reaction. Since adaptive T-cell responses to parasites can contribute to tissue injury. The severity of the immune reaction or the hypersensitivity can be depressed (Abbas and Lichman, 2003).

Study of T-cell function or adaptive T-cell response in animal infected with this parasite may lead to the understanding the host-parasite relationship in term of immunity and pathophysiology.

MATERIALS AND METHODS

1. Experimental infection of mouse with *Gnathostoma spinigerum*

1.1 Animals

Eight weeks old male outbred ICR strain mice, weighed 34±2 grams, were purchased by the National Laboratory Animal Center, Salaya campus, Mahidol University. The mouse was highly susceptible to and easily infected with *G.spinigerum*. They were reared strict hygienic conventional in polycarbonate shoe-box, in the air conditioned room with a constant temperature of approximately 22-25 °C in the animal house, Faculty of Science, Mahidol University and were fed ad libitum with food pellet and dechlorinated tap water. The behavior and physiological changes of mice such as social interactions, feeding, drinking, sleeping, wool and tears were observed twice a week.

1.2 Collection of advanced third stage larvae (L3s)

The visceral organs of the infected swamp eels (*Monopterus albus*) were purchased from Klong Toey and Tevej markets in Bangkok. The livers were separated, washed 2-3 times with tap water and were then cut into small pieces. Approximately, 1 part of the small cut livers was digested in 3-4 parts of 1.5% (w/v) pepsin solution (Sigma, U.S.A.) pH 2.0 for 4 hours at 37 °C. Thereafter the digested tissue was put into a conical flask. The settle sediment was washed by resuspending in normal saline solution (0.85% NaCl). The washing and sedimentation was repeated twice more and then the final sediment was examined under a dissecting microscope. The L3s were individually picked from the digested liver. The larvae were identified to species as described by Daengsvang, 1980.

1.3 Preparation of somatic L3s antigen and Excretory-secretory products

The L3s were homogenized in a ground glass tissue grinder with a small volume of sterile distilled water. The ground-up worm was then sonicated under an Ultrasonic Disintegrator (Soniprep 150, MSE Scientific Inc., England) at an amplitude of 6 microns at 1 min intervals. The sonication step was carried out until most cells were broken judging from microscopic examination. Thereafter it was centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant fluid was obtained. Protein content of the extract was determined by the Lowry's method using bovine serum albumin (BSA, crystalline form, Sigma, U.S.A.) as a standard. The crude somatic L3s extract was kept in small aliquots at -70°C.

The Excretory- secretory (ES) antigen of L3s was prepared from spent culture medium (BME, Gibco, USA).After incubation, the worms were removed and the collected spent medium was centrifuged at 10,000 rpm for 30 min. at 4_o C. The medium was concentrated by ultrafiltration using Amicon PM-10 membrane filter (Grace CO, USA).Protein content was determined by the Lowry method.

1.4 Infection of mouse with advanced third stage larvae of *G. spinigerum*

Each animal was infected with L3s *G.spinigerum* through 5 ml syringe attached to polyethylene gastric tube. The animal was held tightly without anesthesia and the tube was gently passed through the oral cavity into the esophagus until reaching a stomach. Food and water were withheld for another 3-6 hours. Thereafter, pellet diet and water were given ad libitum.

The first and the second group of mice were infected with 10 larvae and 20 larvae of L3s.

2. Eosinophil counts and source of normal sera and anti-*G.spinigerum* sera

To examine the blood eosinophil counts and antibody responses, blood was regularly taken from the ophthalmic vein by using microhematocrit tube as described by Goligen and Shevach, 1994. Eosinophils were counted by staining with Field's stain solution as dercribed (Collier and Lomgmore, 1983). Sera were separated and kept at -20 °C.

3. Antibody detections

3.1 Determination by Enzyme Linked Immunosorbent Assay(ELISA)

The indirect technique of enzyme-linked immunosorbent assay (ELISA) of Tuntipopipat et al. (Tuntipopipat et al., 1993) was followed in this study.

3.1.1 Optimizing condition for ELISA

The optimal concentration of antigen for coating the wells of microtiter plates was determined by performing checkerboard titrations with two-fold dilution of antigen starting from 0.625 to 20 μg/ml and five-fold serial dilutions of both positive and negative sera starting from 1:5 to 1:3125. The optimal concentration was 5 μg/ml.

3.1.2 Determination of serum antibody specific for *G.spinigerum*

The antigen was diluted with coating buffer (0.05 M Carbonate) pH 9.6, to a concentration of 5 μg/ml. Fifty microliters of the antigen solution was added to

each well of 96-well flat-bottomed microplate (Maxisorp-Nunc, U.S.A.). After an overnight incubation at 4°C, the plates were washed three times by using immuno washer (Nunc, U.S.A.). After tapping dry the plates were then added with blocking buffer [1%Bovine serum albumin (BSA), Sigma, U.S.A.] for 60 min at 30°C. The plates were washed again and tapped dry. Fifty microliters of serum were added to each well.

For determination of specific IgG antibody, each serum sample was diluted at 1:125 in 1% BSA buffer (buffer 10 ml +Tween 20 5 µl +0.1 gm BSA). The samples were then incubated for 60 min at 30°C. After one more washing, fifty microlitres of 1:2,000 dilution of horseraddish peroxidase (HRP)-conjugated rat anti-mouse immunoglobulin G was in PBST-1%BSA was added to each well. After three times washing, one hundred microlitres of substrate 3,3′,5,5′-tetramethyl benzidine (TMB, 0.1 mg/ml) was added. The plates were then incubated at the dark at room temperature for 10 min, and the reaction was terminated by the addition of 25 µl of 5N sulfuric acid.

For determination of specific IgE antibody, the procedures are as same as above protocol of IgG subclass 1. Differences of the procedure are dilution of serum, and conjugate and incubation time. Each serum sample was diluted at 1:25 and samples were incubated for 120 min at 30°C. The dilution used for rat anti-mouse immunoglobulin E was 1:400 and HRP-rabbit anti-rat immunoglobulin G was 1:2 00 in PBST-1%BSA.

The absorbance of the yellow color developed with substrate 3,3′,5,5′-tetramethyl benzidine (TMB) was read at 450 nm with a Minireader II Photometer (Dynatech Laboratories, U.S.A.).

3.2 Determination by SDS-PAGE and immunoblotting technique

Sera of normal and infected mice were analysed by SDS-PAGE and western blotting in order to detect the specific protein band(s) of L3s somatic antigen and ES antigen which reacted with IgG-Ab and IgE-Ab. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using discontinuous buffer system, with a 12.5 % separating gel and a 4.5% stacking gel. After the proteins were trasferred onto the nitrocellulose membrane (NC), each membrane was immersed in blocking solution (1% skim milk and 0.1% Tween 20 in 100 mM phosphate-buffer saline, pH 7.4, PBS-T) for 30 minutes at room temperature and cut vertically to strips (0.5x5.5cm).Each strip was then incubated with each serum sample (diluted 1:50 for

IgG-Ab and diluted 1:2 for IgE-Ab in blocking solution) for 2 hours with gentle rocking, then washed 5 times. Each serum of normal and infected mice on the NC paper was then incubated with either of two secondary antisera; rabbit anti-mouse IgG or rabbit anti-mouse IgE, both diluted 1:250 in PBS-T and 1% gelatin. Incubation was followed by exposure to either peroxidase conjugated goat anti mouse immunoglobulin G or peroxidase conjugated goat anti mouse immunoglobulin E in blocking solution.

4. Lymphoproliferation assay

Lymphopoliferative responses of spleen cells stimulated by Concanavalin A (Con A) and crude somatic antigen of L3s *G.spinigerum* were studied. The rate of DNA synthesis following mitogen and antigen stimulation was determined by the in corporation of tritiated thymidine (^{3}HT). Sterile microcultured technique was modified from one described by Kalra et al., 2002 and Sciutto et al., 1995 and was performed in a laminar airflow controlling equipment (Nuaire, Canada).

4.1 Culture medium

Roswell Park Memorial Institute medium 1640 (RPMI 1640, Gibco, U.S.A.) was used to culture the lymphocytes (see appendix).

4.1.1 Working medium I

RPMI 1640 powders were dissolved in deionized distilled water. Penicillin G (Sodium penicillin G, Merck), streptomycin (Streptomycin sulfate B.P., Glaxo) and HEPES (N-2-hydroxyethylpiperazine N-2-ethanesulfonic acid, MW 238.3, Sigma, U.S.A.) were added to final concentration of 100 units/ml, 100 μg/ml and 25mM respectively. Before used, pH of the medium was adjusted to 7.2-7.4 with 7.5% $NaHCO_3$. The working medium I solution was then sterilized by membrane filtration (Millipore, 0.22 μm pore size) and could be kept at 4°C.

4.1.2 Working medium II

The working medium I was supplemented with 10% (v/v) fetal bovine serum (Gibco, U.S.A.), 4 mM L-glutamine (Gibco, U.S.A.) and 50 μM 2-mercaptoethanol. The working culture medium II was made up freshly each time.

4.2 Preparation of Concanavalin A

Five hundred milligrams of Concanavalin A (Sigma, U.S.A.) was dissolved in 5 ml of sterile PBS, divided into small aliquots and kept frozen at -20 °C. The concentration of the stock solution was 1 mg/ml. The stock solution was thawed

only once diluted with working RPMI 1640 medium II to the required concentration of 1 μg/ml and used immediately.

4.3 L3s *Gnathostoma spinigerum* somatic antigen

The crude somatic antigen of L3s was thawed and diluted with working RPMI 1640 medium II to a concentration of 100 μg/ml and sterile by membrane filtration (millipore 0.45 μm pore size).

4.4 Splenic cell suspension

Normal and infected mice were sacrificed by anesthesia with ether in fume hood. Spleens were removed aseptically and placed in sterile petridish containing working RPMI 1640 medium I. Single cell suspension was prepared by gently pressing the spleen through a sterile stainless steel sieve. The spleen cell suspension was pipetted up and down 5 times to make a single cell suspension. The cells were washed 2 times with working RPMI 1640 medium I in a 15 ml polypropylene conical tube (Costar, U.S.A.), followed by centrifugation at 1,500 rpm for 5 minutes at room temperature. Spleen cells were resuspend with 1 ml working RPMI 1640 medium II for cell culture. After adding the trypan blue dye, spleen cells were counted in a hemocytometer. The suspension was adjusted to contain $5x10^6$ cells/ml with working RPMI 1640 medium II.

4.5 Method for cell culture

One hundred microlitres of 5×10^6 spleen cells/ml were added in triplicate wells of 96-well flat bottom plastic tissue culture plate (Costar, Corning, NY, U.S.A.). One hundred microlitres of crude somatic antigen (100 μg/ml) or Concavalin A (1 μg/ml) were added to the well with cells. The final volume of the medium in each well was 200 μl. The final concentration of Con A in the well was 0.5 μg/ml and that of antigen was 50 μg/ml. The final concentrations of serum, L-glutamine and 2-mercaptoethanol in the cell culture medium were 10%, 4mM and 50 μM respectively. The culture plate was incubated at 37 °C in a humidified incubator with 5% CO_2 atmosphere (Forma Scientific, U.S.A.).

4.6 Determination of thymidine incorporation

For mitogen stimulation, the cells were cultured 48 hours before pulsing with 50 μl of 20 μCi/ml of tritiated thymidine (1 μCi/well, M.W.242, Amersham). The cultures were incubated further under the same condition for another 18 hr before the cell were harvested. On the other hand, when the cell were stimulated with the antigen they were cultured for 5 days and pulsed with ^{3}H-Tdr as described

for the mitogen. After 18 hr incubation, and cells were harvested in the multiwell harvestor (Nunc, U.S.A.) and rinsed with distilled water on a glass fiber filter paper (Whatman, England). The dried filter discs were placed in glass scintillation vials. One milliliters of scintillation fluid containing 0.5% (W/V) PPO (2,5-Diphenyloxazole, Eastman) and 0.1%(W/V) POPOP (5-phenyloxazole, Eastman) in toluene was added to each vial. All vials were pre cooled in the dark for at least 3 hours before counting. Radioactivity was counted (5 min) in a liquid scintillation β-counter (Beckman, U.S.A.).

4.7 Data analysis

4.7.1 Change in counts per minute (H-U or Δ cpm)

The value represented the absolute difference in cpm between the stimulation cultures (H) and the corresponding unstimulated controls (U).

Δ cpm = mean cpm of stimulated culture – mean cpm of unstimulated culture

4.7.2 Stimulation index (SI)

$$S.I. = \frac{\text{mean cpm of stimulated culture}}{\text{mean cpm of unstimulated culture}}$$

4.7.3 Statistical test

One-Way ANOVA and Student's t test were used.

5. Analysis of splenic T lymphocyte subset and intracellular interferon gamma by flow cytometry

Splenic mononuclear cell was analysed CD4 and CD8 cell subsets by tecnique as described (Kojima et al.,2002, Janossy ey al., 2002)) with minor modification.Briefly, spleen homongenates were suspended in RPMI 1640 culture medium and subsequently the red cells were lysed in lysis buffer by gently inverting the tube. The red blood cell lysed spleen suspension was washed in PBS. The sediment cells were then suspened in RPMI 1640 containg 5% foetal bovine serum to prepare $1x10^8$ cells per ml and incubated 2 hours on ice with block protein (BD Biosciences, USA) These cells were then washed in PBS- tween 20 and incubated for 30 min on ice with r-phycoerythrin (R-PE) –conjugated rat anti-mouse CD_4 (L3T4) monoclonal antibody (BD Biosciences, USA) for detection of CD_4 differentiation antigen expressed on a subpopulation of mature T lymphocytes. For detection the CD_8 subpopulation, the cells were incubated on ice for 30 min with r-phycoerythrin (R-PE)-conjugated rat anti-mouse CD8a (LY-2) monoclonal antibody

(BD Biosciences, USA). After staining, the cells were fixed with cytofix cytoperm (Cat no. 555028,BD Bioscience Pharminggen, San Diego, CA, USA) for 10 min on ice and subsequently washed in wash buffer (Perm/Wash solution)The percentage of CD_4^+ T and CD_8^+ T cell population was determined by flow cytometry .

For intracellular cytokine staining of interferon-gamma (IFN-γ), the lysed red blood cell of $1x10^8$ isolated spleen cells were used for the ICCS according to the following procedure:

(a) Add $1x10^8$ spleen cells in 1 ml of culture medium into the well of 24-well tissue culture plate (costar)

(b) Stimulate with 40 ug/well of Con –A and100 ug of specific advanced 3 stage antigen compare to those of unstimulation

(c) Incubate at 37 C with 5% CO_2 for 18 hours and then wash with PBS tween20

(d) Add 1 ul/well of Golgi Plug (Cat no. 555028,BD Bioscience Pharminggen, San Diego, CA, USA) and incubate for another 2 hours

(e) Collect cells in 1.5ml Eppendorf tube and wash with 1 ml of staining buffer (3% FBS, 0.09% sodium azide in PBS)

(f) Add 4% normal mouse serum in staining buffer /tube

(g) Incubate at 4 C for 30minutes

(h) Wash cell with 1 ml of staing buffer , 2times

(i) Add 1ul of PE-labelled anti-mouse CD4a or CD8a (Bioscience Pharmingen)

(j) Incubate at 4C for 30 minutes

(k) Wash cells with 1 ml of staining buffer, 2 times

(l) Add 250ul Cytotox/ Cytoperm solution (Cat no. 555028,BD Bioscience Pharminggen, San Diego, CA, USA)

(m)Incubate at 4C for 30minutes

(n) Wash with 1ml of Perm/ Wash solution for 2 times

(o) Add 1ul FITC-labelled anti-mouse interferon-gamma (IFN-γ, Cat no 11-7311-82, BD Bioscience Pharmingen)

(p) Incubate at 4C for 30 minutes

(q) Wash with 1ml of Perm/Wash solution for 2 times

(r) Add 700ul staining buffer

(s) Analyse by Flow cytometry

6. Analysis of splenic T lymphocyte subset and intracellular interferon gamma

The sample will be considered positive if the %positive is more than or equal to the cut- off value which is the mean of % positivity of the control group.

7. Histological section

Infected mouse with 5, 10 and 20 larvae were sacrified and examined for the invaded larvae in various organs at different time points under dissecting microscope. The detected cyst were cut out and fix in 10% formalin solution.The cysts from muscle and liver were made the histological section and stained with hematoxylin-eosin stain.

8. Statistical analysis

Statistical significance was determined by ANOVA for one way classification or by student's test, depending on the experimental designs. A value of $P<0.05$ was considered to be significant.

RESULTS

1. Blood eosinophilia counts

The percentage of blood eosinophils taken from the ophthalmic venous plexus of mice infected with 5,10 and 20 larvae were counted. Mice infected with 5 and 10 larvae, blood eosinophil reached a maximun on day 13 PI with significant different (P = 0.000) between 9.25% and 22.61% respectively. When the degree of infection increased to 20 larvae, the peak of eosinophil count was day 15 PI. This 20 larvae infected mice showed peak with 19.00% on day 15 PI. The percentage of both 10 and 20 larvae group was not significantly different.

After reached a plateu on day 13 PI and day 15 PI, it gradually decreased. Some infected mice had the same percentage of eosinophil as the non-infected animal on 6 months PI.

Table 1.1 Change of eosinophils in mice infected with 5 AdL3 of *G. spinigerum*.

Dav PI	No. of mice	% Eosinophil		P-value
		Range	X	
0	12	0-9	1.58	
3	8	2-6	3.75	1.000
6	8	1-5	2.75	1.000
8	8	2-8	4.88	0.980
10	9	1-8	4.67	0.986
13	8	1-22	9.25	0.006
20	8	0-5	3.00	1.000
30	8	0-4	3.00	1.000
31	4	3-4	3.50	1.000
37	4	1-6	4.00	1.000
51	4	0-9	3.00	1.000
66	8	0-10	4.13	0.999
79	8	1-5	1.88	1.000
80	8	0-8	3.50	1.000
90	8	0-4	1.63	1.000
99	8	1-9	4.25	0.998
113	8	0-5	2.00	1.000
125	8	1-5	2.63	1.000

Table 1.2 Change of eosinophils in mice infected with 10 AdL3 of *G. spinigerum*.

Day PI	No. of mice	% Eosinophil		P-value
		Range	X	
0	52	0-11	1.79	
3	24	0-6	1.92	1.000
5	16	0-4	1.00	1.000
7	34	0-13	2.85	1.000
9	9	1-6	4.11	1.000
10	12	1-11	6.50	0.999
12	28	4-26	12.61	0.000
13	33	5-47	22.61	0.000
14	10	12-33	20.40	0.000
18	7	0-20	9.43	0.904
19	10	4-23	11.70	0.018
20	4	7-15	9.75	0.997
21	11	4-12	7.64	0.966
23	12	1-14	5.42	1.000
30	6	3-14	7.67	1.000
33	15	1-12	4.00	1.000
40	6	2-9	4.33	1.000
41	36	0-18	8.67	0.001
48	20	3-23	7.25	0.739
50	42	0-12	5.24	0.987
59	22	0-6	3.50	1.000
62	48	0-17	5.33	0.964
69	36	1-14	5.06	0.998
70	25	0-8	2.68	1.000
79	36	1-11	5.25	0.994
90	38	0-13	4.89	0.999
99	15	1-8	4.47	1.000
112	28	0-12	4.32	1.000
119	19	1-14	7.37	0.732
129	9	0-4	1.56	1.000
140	8	0-5	2.25	1.000
149	12	1-6	2.58	1.000
160	23	0-8	2.78	1.000
168	24	0-6	1.46	1.000
184	20	0-5	1.25	1.000

Table 1. 3 Change of eosinophils in mice infected with 20 AdL3 of *G. spinigerum*

Day PI	No. of mice	% Eosinophil		P-value
		Range	X	
0	8	0-5	1.25	
5	4	2-10	5.50	0.976
6	4	3-10	5.25	0.986
8	6	0-6	3.83	1.000
9	6	1-6	3.17	1.000
10	4	1-11	4.75	0.997
11	3	9-14	11.67	0.045
13	2	10-17	13.50	0.042
15	2	19-19	19.00	0.000
20	4	4-10	6.75	0.817
24	4	1-10	5.25	0.986
25	2	5-6	5.50	0.998
29	6	2-11	7.00	0.554
52	4	1-5	2.75	1.000
66	4	1-3	1.75	1.000
70	2	4-11	7.50	0.926

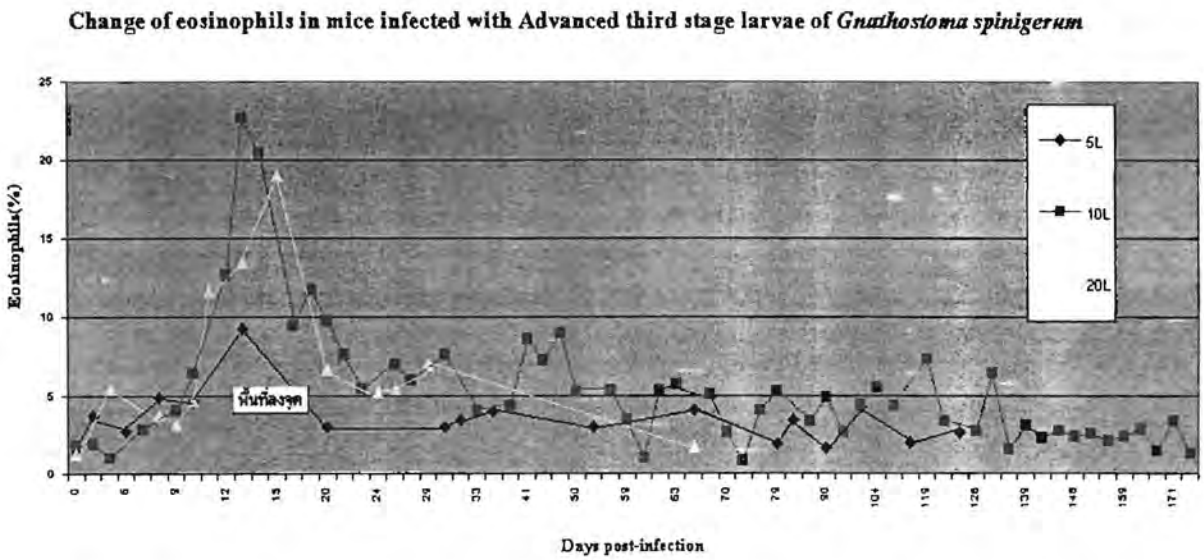


Figure 1.1 Kinetic response of blood eosin Phil in mice infected with 5, 10 and 20 AdL3 *G. spinigerum.*

Table 1.4 Comparison of the highest percentage of blood eosinophil in three different infected groups of 5, 10 and 20 AdL3 *G. spinigerum*.

Dose of infection (larvae)	% Eosinophil at day 0	Peak of eosinophil		P-value
		Day PI	Mean (%)	
5	1.58	13	9.25	0.001
10	1.79	13	22.61	
20	1.25	15	19.00	0.612

2. Antibody response of mice infected with *G.spinigerum*

2.1 Standardization of ELISA

2.1.1 Optimization of specific IgG antibody

Pooled sera obtained from mice infected with 20 larvae of *G.spinigerum* at day 50 and pooled normal sera collected from normal healthy mice were used for the standardization assay. The optimal concentration of advanced L3s antigen was determined by performing a checkerboard titration with two-fold dilution of antigen starting from 1.25 to 5 μg/ml. The normal control serum samples serial diluted five-fold from 1:25 to 1:625. The most optimal binding of specific IgG antibody was obtained with an antigen concentration at 5 μg/ml. It was therefore decided that, advanced L3s at a concentration of 5 μg/ml and dilution of serum samples at 1:125 was used for IgG determinations.

The optimal condition for the horseradish peroxidase (HRP)-conjugated rat anti-mouse IgG was determined by testing the conjugate diluted at 1:1,000, 1:1,500 and 1:2,000. The optimal dilution for this conjugate was 1:2,000. Enzymatic reaction with substrate of 3,3′,5,5′-tetramethyl benzidine (TMB) was allowed to take place for 10 min (Fig 1).

2.1.2 Optimization of specific IgE antibody

The antigen was diluted to a concentration at 5 μg/ml and five-fold serial dilutions of both positive and negative sera starting from 1:5 to 1:25. The most optimal concentration of serum samples was 1:25.

The secondary anti-immunoglobulin E of rat anti-mouse IgE was tested with dilution of two-fold from 1:100 to 1:400. The enzyme conjugated of HRP-rabbit anti-rat immunoglobulin G was then added. The rat anti-mouse immunoglobulin E and HRP-rabbit anti-rat immunoglobulin G were used at dilution of 1:400 and 1:2,000 respectively, the enzymatic reaction was allowed to take place for 10 min (Fig 2).

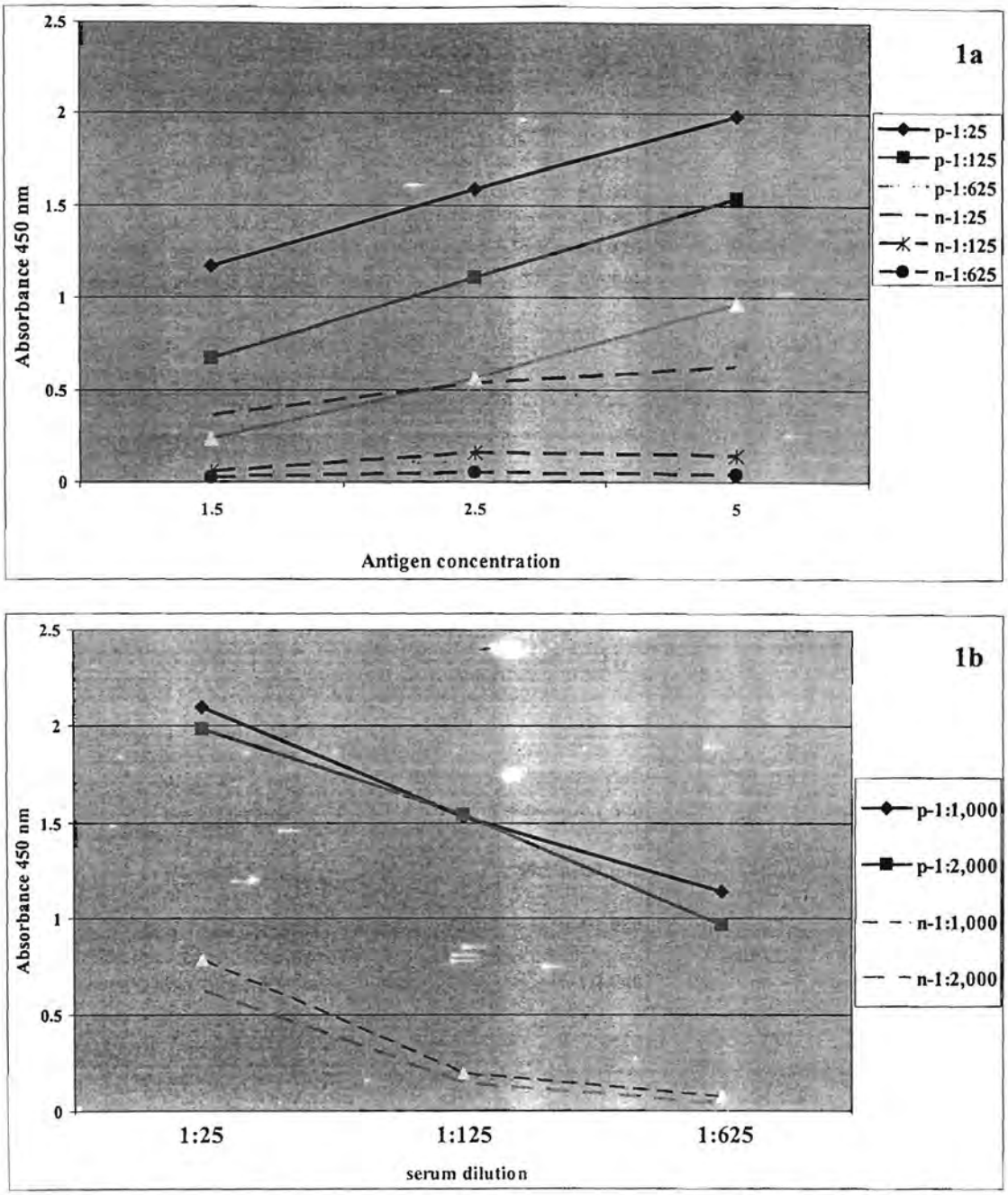


Fig2.1 Checkerboard titration for IgG antibody against advanced L3s *G.spinigerum* antigen using pooled infected (P) (___) and negative control (n) (_ _ _) sera.

1a). Plates were coated with 50 µl of antigen at concentration 1.25, 2.5 and 5 µg/ml. Serum dilutions used were 1:25, 1:125 and 1:625. Dilution of HRP-conjugated rat anti-mouse IgG was 1:2,000.

2b). Plates were coated with 50 µl of antigen at concentration 5 µg/ml. Serum dilutions used were 1:25, 1:125 and 1:625. The dilutions of HRP-conjugated rat anti-mouse IgG were 1:1,000 and 1:2,000.

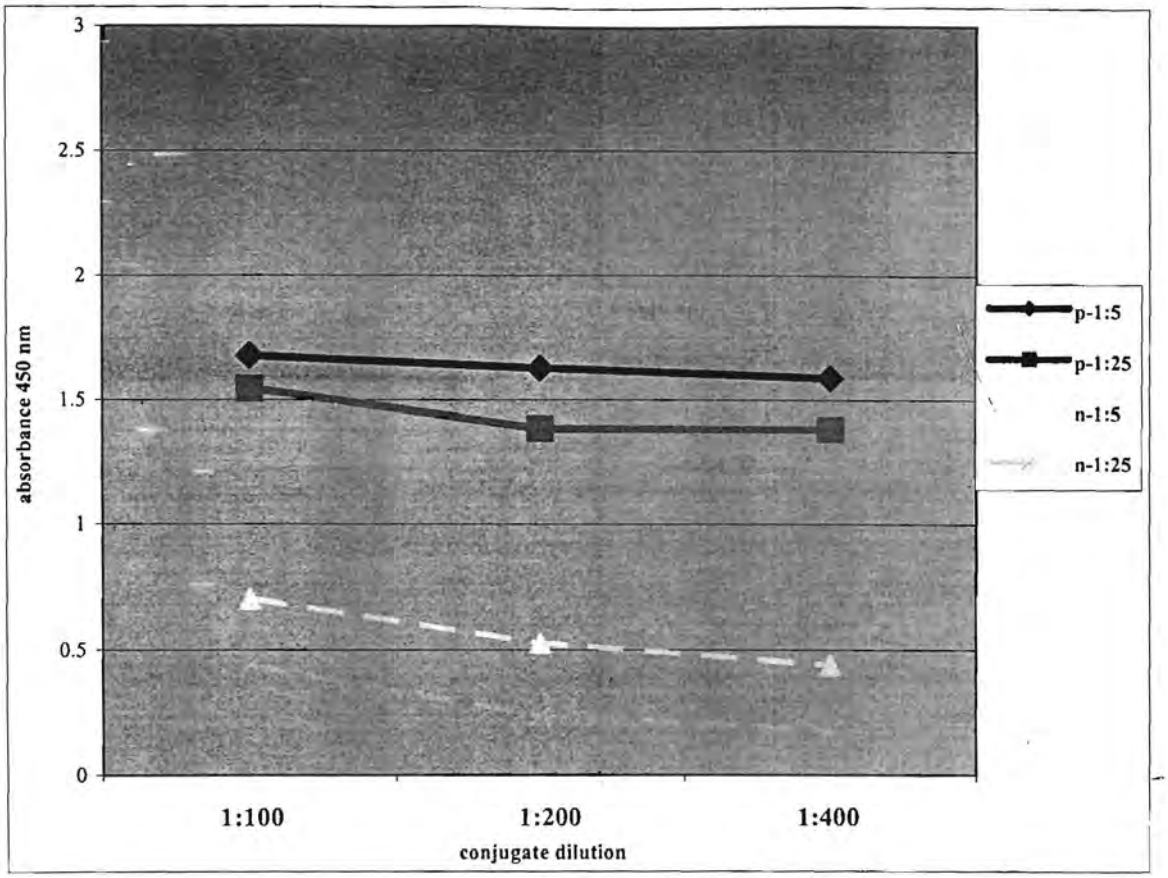


Fig 2.2 Checkerboard titration for IgE antibody against advanced L3s *G.spinigerum* antigen using pooled infected (p) (_____) and negative control (n) (_ _ _) sera. Plates were coated with 50 µl of antigen at concentration 5 µg/ml. Dilutions of sera used were 1:5 and 1:25. The dilutions of conjugate rat anti-mouse immunoglobulin E were 1:100, 1:200 and 1:400 and HRP-rabbit anti-rat immunoglobulin G was 1:2,000.

Table 2.1 Optimal conditions of ELISA test for the detection of IgG and IgE antibody

Step of ELISA test	IgG		IgE	
	Concentration or dilution	Reaction time	Concentration or dilution	Reaction time
Coating of L3s antigen	5 μg/ml	18 hr	5 μg/ml	18 hr
Reacting with tested sera	1:125	1 hr	1:25	2 hr
Reacting with conjugate 1	1:2,000	1 hr	1:400	1 hr
Reacting with conjugate 2	–	–	1:2,000	1 hr
Color development	TMB	10 min	TMB	10 min

2.2 ELISA antibody level of mice infected with *G.spinigerum*

In this experiment, mice were infected with different degree of infection (10 and 20 larvae) and blood was collected at different time intervals (day 10, 30, 60, 90, 120 and 180 post infection, PI). To detect the IgG-Ab and IgE-Ab levels to advanced L3s somatic antigen, individual serum from both group of mice which was infected at different dose and different time interval were performed. The standard sera, both pooled infected and normal control sera, were included in every plate to permit adjustment of the results obtained from different plates and different occasion.

2.2.1 Kinetic IgG antibody responses of *Gnathostoma spinigerum* infected mice

After potimal conditions of ELISA have been standardized; blood was taken from opthalmic venous plexus of mice infected with 10 and 20 larvae. Sera were investigated for kinetic study. These mice were continued kept in animal house.

The results presented in Figure 2.3 and Table 2.2 showed that the ELISA values of IgG antibody in the serum of infected mice were considerably significant higher than those of healthy controls. The levels of IgG Ab could be early detected within Day 10 PI and gradually increased thereafter. Among the time points of post infection, Day 120 after being infected with both 10 larvae and 20 larvae, had the highest optical density (OD = 1.755 and OD = 2.088 respectively) as shown in Fig 3. After Day 120 PI, the responses gradually decreased to through the end of the experiment (Day 250 PI). The antibody responses to 20 larvae infected mice were higher than those of 10 larvae infected and were statistical significant difference at the some time interval of post infection (Day 10, 60, 120 and 180 PI).

2.2.2 Kinetic IgE antibody responses of *Gnathostoma spinigerum* infected mice

The levels of IgE antibody to somatic L3s antigen in infected mice both 10 larvae and 20 larvae were also significantly higher than the healthy controls (Table 2.3). The antibody in all infected mice could be detected within Day 10 PI. The IgE antibody responses in group of 10 larvae and 20 larvae infection demonstrated highest at Day 90 PI and Day 60 PI respectively (OD=1.426 and OD=1.430, Fig 2.4). After that, IgE level had gradually decrease however, it was not significant from each other at any different time points.

Table2. 2 Absorbance values of specific IgG antibody in serum of mice infected with *G.spinigerum* at different time intervals.

Group of mice		Absorbance density (450 nm)								
		Day 0	Day 10	Day 30	Day 60	Day 90	Day 120	Day 150	Day 180	Day 250
10 larvae	N	14	16	15	16	14	14	14	15	4
	X	0.151	0.517	1.262	1.400	1.692	1.755	1.736	1.535	1.233
	SD	0.069	0.220	0.240	0.495	0.228	0.193	0.316	0.301	0.417
20 larvae	N	25	9	8	9	7	6	5	5	3
	X	0.150	0.925	1.492	1.774	1.916	2.088	2.020	1.969	1.632
	SD	0.051	0.261	0.295	0.311	0.343	0.223	0.292	0.245	0.254

N = Number tested

X = Mean of Absorbance density

SD = Standard deviation of the mean

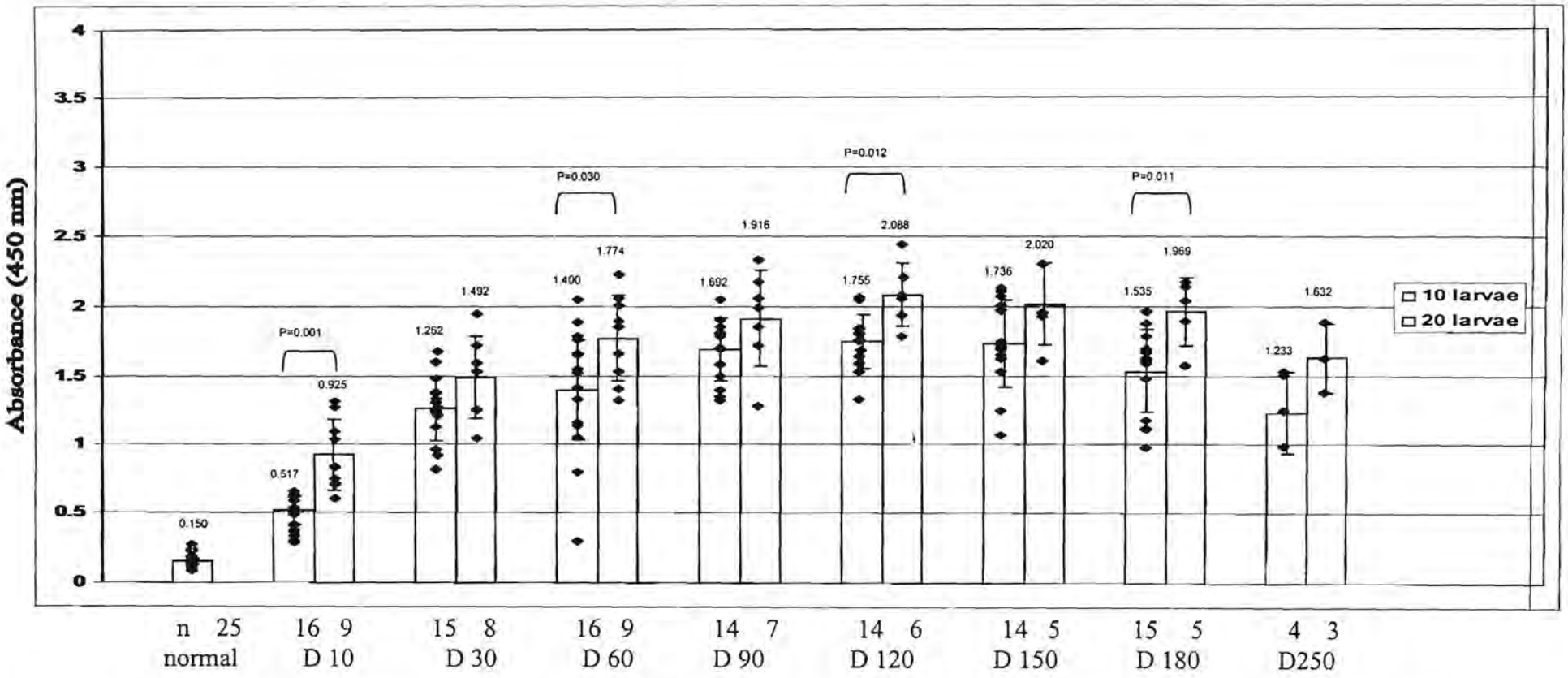


Fig 2.3 Optical density value of ELISA for IgG antibody to somatic L3s antigen in normal control and mice infected with 10 and 20 larvae of *G.spinigerum* at different time points (D 10, 30, 60, 90, 120, 150, 180 and 250). Bars and lines represent mean and SD of individual animal indicated (•); n is the number of animal .

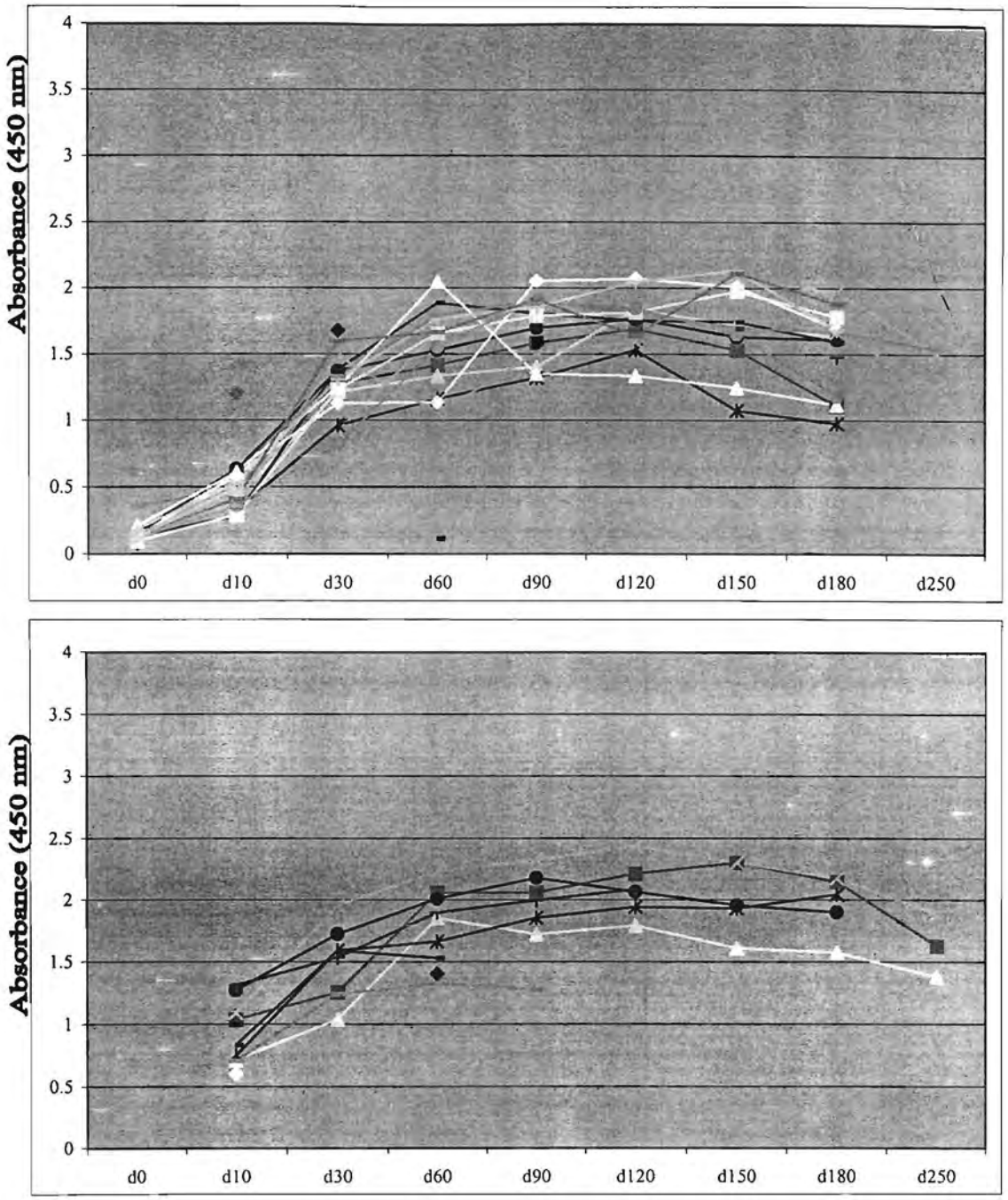


Fig 2.4 Kinetic of optical density of ELISA for IgG antibody to somatic L3s antigen in normal control and mice infected with 10 and 20 larvae of *G.spinigerum* at different time points (D 10, 30, 60, 90, 120, 150, 180 and 250). Each line represents OD values of individual animal.

Table 2.3 Absorbance values of specific IgE antibody in serum of mice infected with *G.spinigerum* at different time intervals.

Group of mice		Absorbance density (450 nm)								
		Day 0	Day 10	Day 30	Day 60	Day 90	Day 120	Day 150	Day 180	Day 250
10 larvae	N	11	15	15	17	14	14	15	15	3
	X	0.171	0.484	1.143	1.397	1.426	1.348	1.346	1.121	1.369
	SD	0.113	0.216	0.351	0.328	0.485	0.427	0.470	0.621	0.364
20 larvae	N	11	9	8	9	7	6	5	5	
	X	0.205	0.554	1.289	1.430	1.372	1.361	1.229	1.082	-
	SD	0.086	0.257	0.337	0.325	0.239	0.216	0.148	0.107	

N = Number tested

X = Mean of absorbance density

SD = Standard deviation of the mean

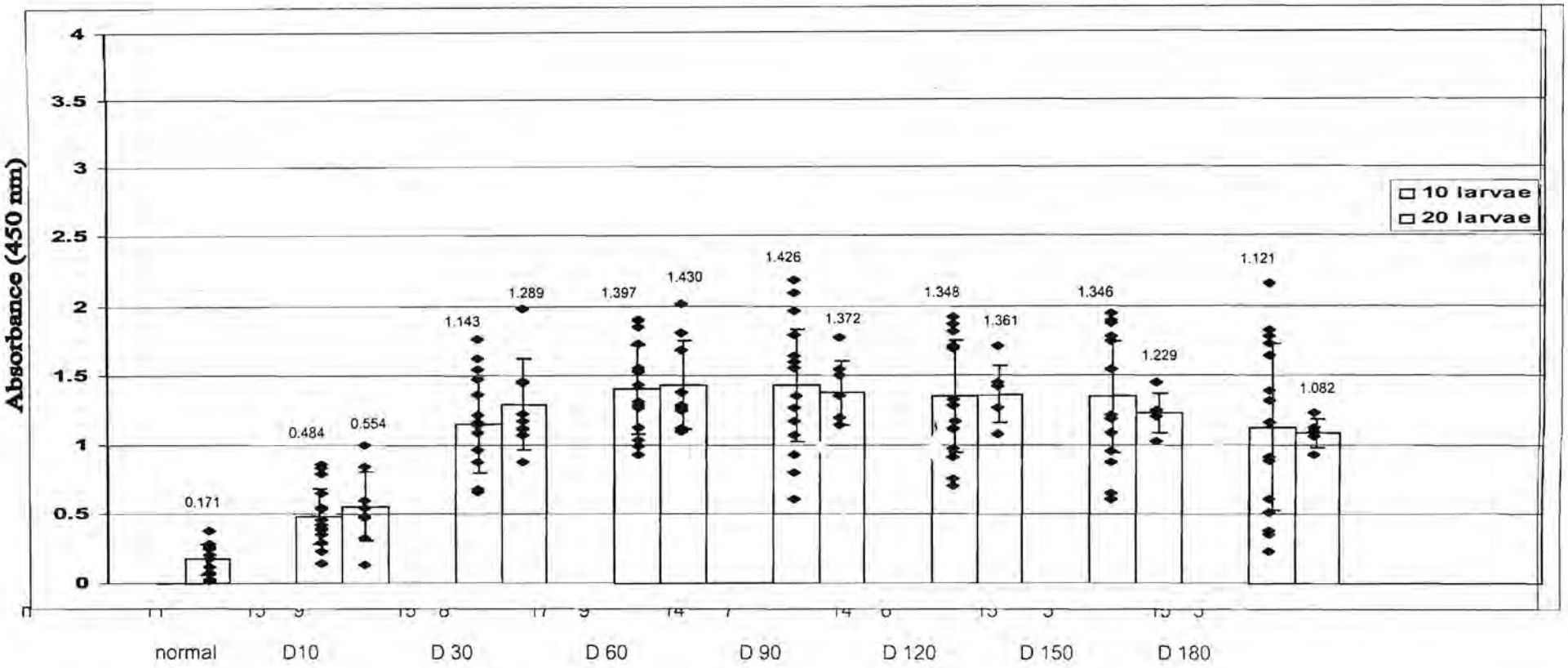


Fig 2.5 Optical density value of ELISA for IgE antibody to somatic L3s antigen in normal control and mice infected with 10 and 20 larvae of *G.spinigerum* at different time points (D 10, 30, 60, 90, 120, 150 and 180). Bars and lines represent mean and SD of individual animal indicated (•); n is the number of animal left after lymphoproliferative test.

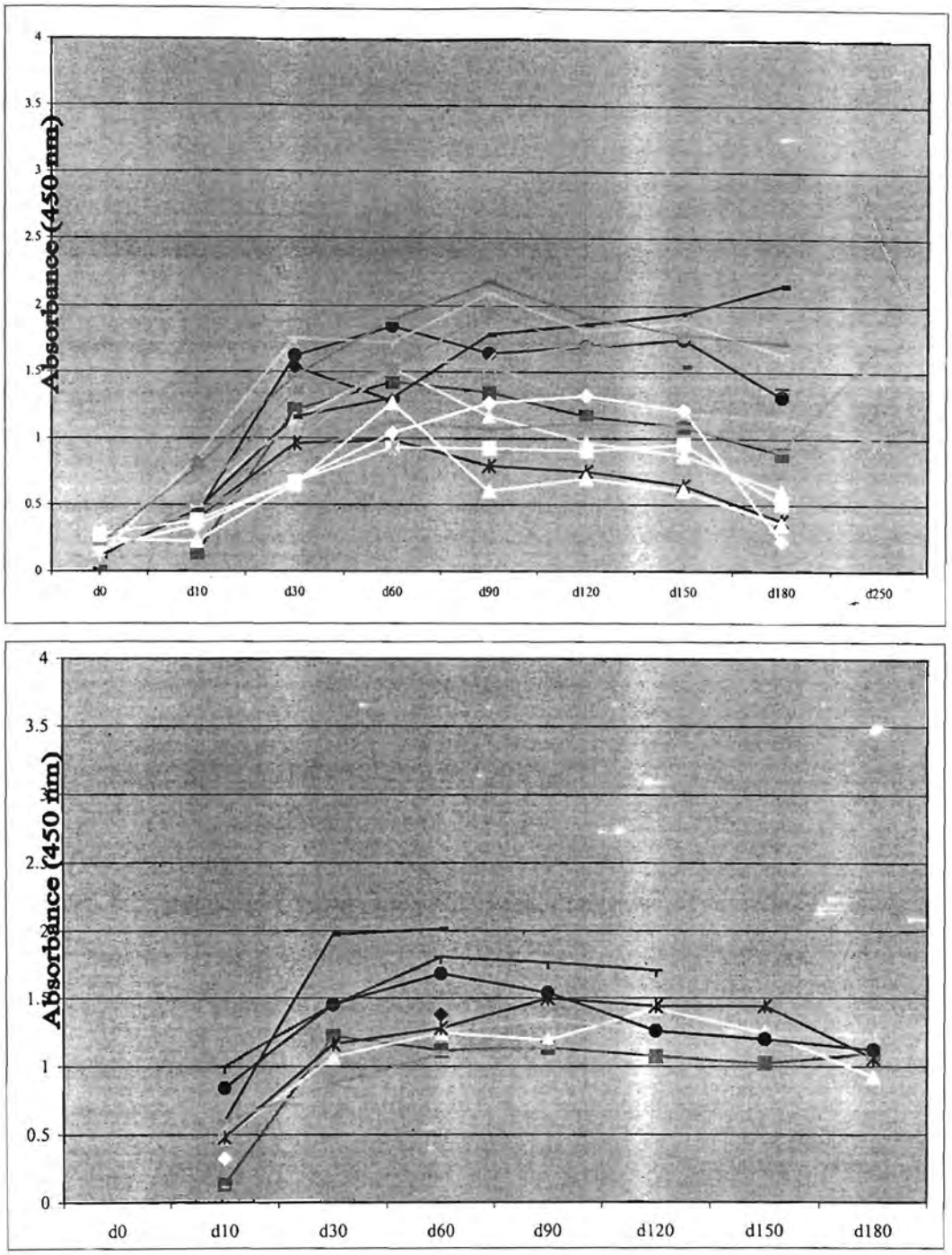


Fig 2.6 Kinetic of optical density of ELISA for IgE antibody to somatic L3s antigen in normal control and mice infected with 10 and 20 larvae of *G.spinigerum* at different time points (D 10, 30, 60, 90, 120, 150 and 180). Each line represents OD values of individual animal.

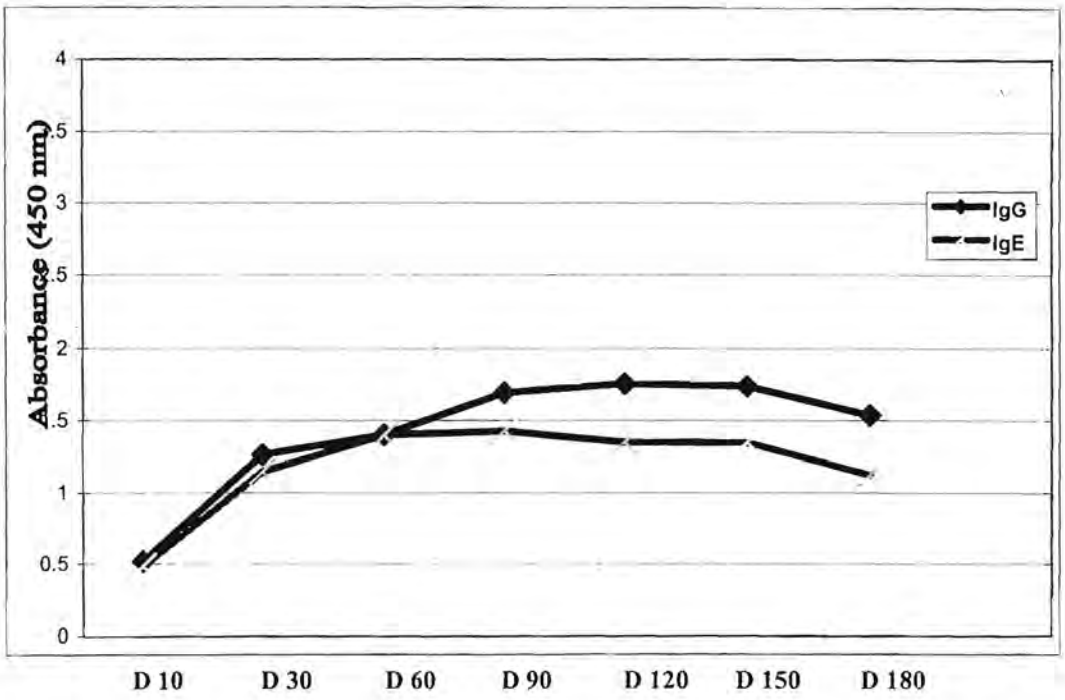


Fig 2.7 Means of IgG and IgE-ELISA of mice infected with 10 larvae advanced L3s *G.spinigerum* at different time intervals (D 10, 30, 60, 90, 120, 150 and 180 PI).

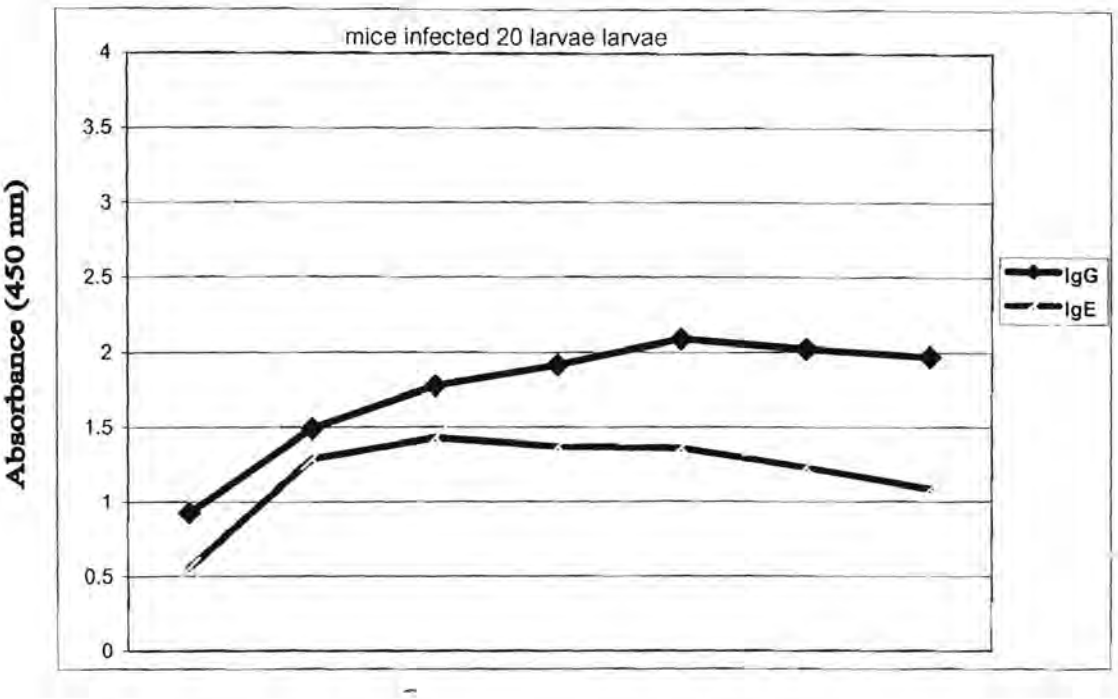


Fig2.8 Means of IgG and IgE-ELISA of mice infected with 20 larvae advanced L3s *G.spinigerum* at different time intervals (D 10, 30, 60, 90, 120, 150 and 180 PI).

2.3 Western blotting analysis

In sodium dodecyl sulfate-polyacrylamide gel electropforesis, samples containing approximately 50 ug of the L3s water extract antigen were boiled at 100 c for 3 minutes in sodium dodecyl sulfate and 2- mercaptoethanol before loading onto the gel. There were at least 29 bands could be demonstrated in this somatic antigen with the relative molecular weight (MW) ranging from 15- 150 kd.In western blots, both IgG and IgE antibodies showed specific to many protein bands. However day 10 post infection the antibody detection by this tecnique had low sensitivity since specific band could not be detected. At day 30 apecific bands with approximate molecular weight of 34, 37 39, 50, 62, 87, 125 and 140 kd were demonstrated. When the infection was prolong (day 60-120 post infection) more intensity of the specific bands were showed.The prominent bands were 20, 24, 37, 39, 50, 87 and 125 kd. Interestingly, reactivity against excretory – secretory antigen (ES) could demonstrated at day 10 post infection with MW of 50 kd especially reacted with IgE antibody.The reactivity of IgE antibody could be detected from day 10 and were stronger when the infection prolong. The most intense band were 34 and 50 kd. However, ES product showed specific to IgG only with the sera from day 30 and 60 post infection.

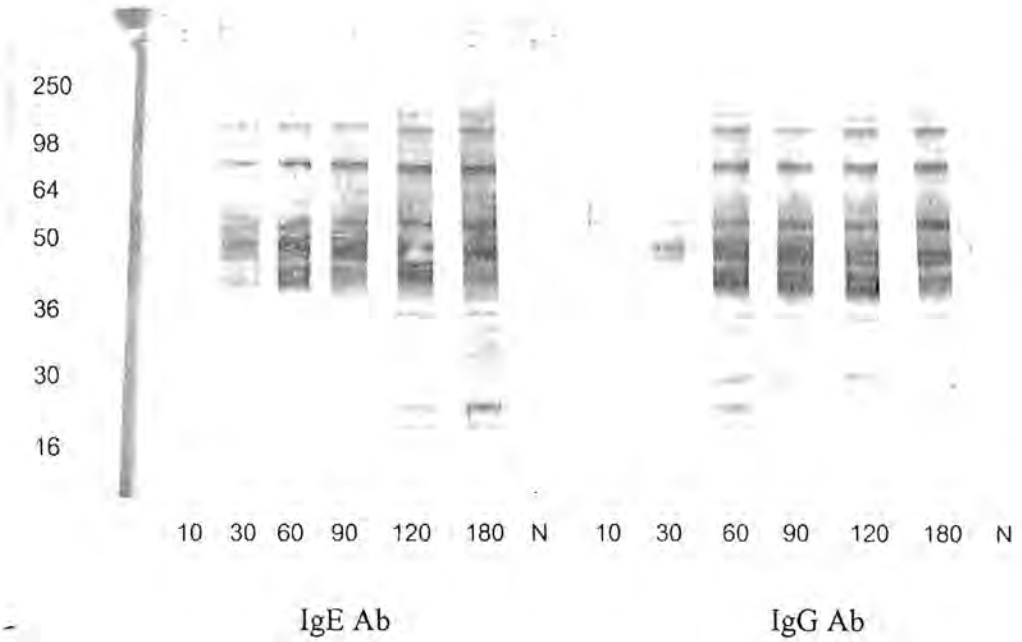


Fig 2.9 Serum reactivities of IgE and IgG antibodies with somatic antigen by SDS-PAGE and western blot analysis.Number on left indicate molecular weight in kd.

(A) Standard melecular weight
(B) Serum of mouse at day 10 post infection
(C) Serum of mouse at day 30 post infection
(D) Serum of mouse at day 60 post infection
(E) Serum of mouse at day 90 post infection
(F) Serum of mouse at day 120 post infection

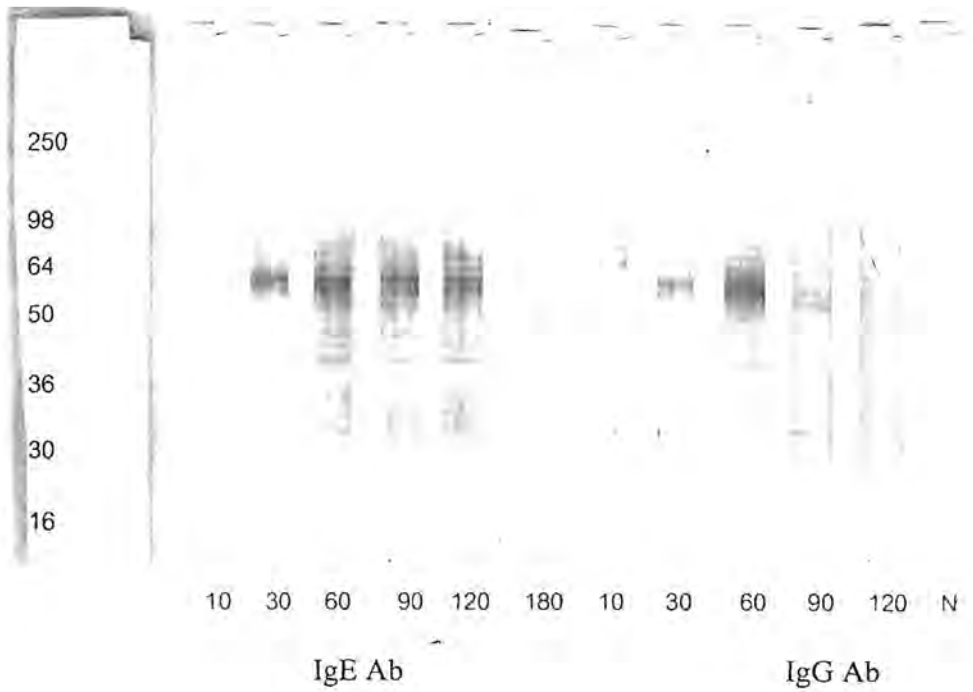


Fig 2.10 Serum reactivities of IgE and IgG antibodies with excretory secretory antigen by SDS-PAGE and western blot analysis. Number on left indicate molecular weight in kd.

(G) Standard melecular weight
(H) Serum of mouse at day 10 post infection
(I) Serum of mouse at day 30 post infection
(J) Serum of mouse at day 60 post infection
(K) Serum of mouse at day 90 post infection
(L) Serum of mouse at day 120 post infection

3. Lymphoproliferative response of mice infected with *G.spinigerum*

3.1 Determination of optimal conditions for Concanavalin A (Con A) - induced proliferative response of mouse splenic lymphocytes

Duration of stimulation and effect of Con A concentration

Lymphoproliferative response was set-up for mouse system using Con A as a mitogen. Mice splenic cells were adjusted to a concentration of $2x10^6$ and $5x10^6$ cells/ml in RPMI 1640. One hundred microlitres of these splenocyte suspensions were triplicately cultured (37 ^{O}C 5% CO_2) in microplate in the presence of Con A at concentrations of 1, 5 and 15 µg/ml in RPMI 1640 supplemented with 10% fetal bovine serum. After 48 hrs 66 hrs of cultivation, ^3H-thymidine was added. The cells were harvested 6-24 hrs after pulsing. The radioactivity counted at $5x10^6$ cells/ml with 1 µg/ml of Con A provided the high stimulation index (Table 4). The results were in the same order of magnitude as those obtained by Sciutto et al. (Sciutto et al., 1995) and kalra et al. (Kalra et al., 2002).

Table 3.1 Effect of time of incubation and Con A concentration for mouse splenic lymphoproliferative response.

Number of cells (cells/ml)	Time of incubation (hr)*,#	Number of mice	Stimulation index (SI)		
			Con A concentration (µg/ml)		
			1	5	15
$2x10^6$	48, 18	4	11.07	13.54	3.58
	48, 24	1	-	8.02	-
	66, 6	1	-	23.48	-
$5x10^6$	48, 18	2	20.16	13.18	8.19
	48, 24	1	12.21	-	-
	66, 6	1	6.65	-	-

*, Duration of incubation prior to the addition of ^3H-thymidine

#, Duration of incubation prior to cell harvesting.

3.2 Effect of infection by advanced L3s *G.spinigerum* on lymphoproliferative response

In this experiment, mice were infected with different degree of infection (10 and 20 larvae of advanced L3s *G. spinigerum*) and for different time intervals (10, 50 and 180 days post infection, PI). Splenic lymphoproliferative response to both non-specific mitogen (Con A) and specific antigen of somatic advanced L3s were investigated.

In addition, these responses were also performed in the animals which their infections were cured by oral administration of ivermectin prior to being tested in order to determine if those response or defect that might exist could be reversed.

3.2.1 Con A-stimulated lymphoproliferative response of mouse infected with *G.spinigerum*

Lymphocytes were taken from mice during the early-stage (10 and 50 days, PI) and the late-stage (180 days, PI) of infection. Their responses to Con A stimulation were compared with controls of the same age group.

The result of splenic lymphoproliferative responses of mice tested at 10, 50 and 180 days after infected with 10 and 20 larvae of advanced L3s summarised in Table 5. The splenic lymphocyte response of mice infected with 10 larvae at Day 10 was not significant from that of uninfected control (SI = 23.86 and 23.84). At day 50, the response was lower than that of Day 10, PI ($P = 0.023$). When the duration of infection with these 10 larvae prolong to the late stage (Day 180), a significant low response was also noted ($P = 0.001$) compare to the group of Day 10 PI and the normal age match control mice. As shown in Table 5 and Fig 9 both the stimulation index and the absolute count of radioactivity (Δcpm) of the splenic lymphocyte from group of Day 50 and Day 180 PI were significantly (SI = 20.05 and SI = 18.03) lower than group of Day 10 PI (SI = 23.86) and than the uninfected controls (SI = 23.84). Since, the responses of uninfected controls mice with age of about 1-3 months and those with age of 7-8 months were not different, therefore had not affected.

When the degree of infection was increase by infection with 20 larvae, lower lymphoproliferative responses of Con A were also noted as shown in Table 5 and Fig 10 When the mice were infected for 10 days, the response in this group (SI = 21.98) tended

to decrease but was not significant from that of uninfected control (SI = 23.86). At Day 50 PI, the response was lower than that of uninfected control (P = 0.009), but not significant lower than that of the groups of Day 10 PI (SI = 19.08 and 21.98). At Day 180, a marked reduction could be noted. The response of this group was lower or showed more unresponsed than those infected for a shorter duration. Comparing dose of infection, it was found that, the response of infection with 20 larvae of *G.spinigerum* (SI = 21.98, 19.08 and 17.57) tended to lower than that of infection with 10 larvae (SI = 23.86, 20.05 and 18.03) at the same time intervals of infection. However, it was not significantly different.

The above mentioned result showed significant depression of lymphoproliferative response (Day 50 and Day 180). It is therefore interestingly to fine out whether or not such degree of depression is reversible. Treating the infected mice did the test by treatment with a single oral dose of ivermectin at 0.4 mg/kg body weight.

In the experiment, mice infected with 10 and 20 larvae for 10-12 days, were treated with ivermectin orally. The first group of animal for testing the lymphoproliferative response was those sacrified at 10 day after treatment. Results presented in Table 5 and Fig 9-11.showed that when the treated infected mice with 10 larvae, the response was not significant slightly higher than that of uninfected controls in all time intervals after treatment (10, 50 and 180 days). When the severity of infection was increase by infection with 20 larvae as results showed in Fig 11. groups of mice were treated for 10 and 50 days, the response in this group was not significant from that of uninfected controls (SI = 22.24, 23.52 and 23.84). When the duration infection with these 20 larvae of advanced L3s prolong to the chronic stage (180 days), a significant high response was noted (SI = 29.14) compare to the group of 10 and 50 days post treatment and the uninfected controls (P = 0.000, 0.001 and 0.001).

As show in figure, the mean value of SI and Δcpm of group of non-treated infected mice compare to group of treated infected mice, it was found that the response was not significant for acute infection (10 days) both infected with 10 and 20 larvae. When duration of infection was prolonged to day 50 and 180, the response of infected treated mice group was higher than in those non-treated infected groups.

It should be mentioned that prior to the drug treatment, the lymphoproliferative response of these animals was already somewhat unresponsive or depressed. Data from Table 5 showed 10 days after treatment, the lymphoproliferative response to Con A stimulation of 20 larvae infected group was still depressed. Interestingly, there was an increase in response of 50 days and 180 days interval after treatment. The responses were enhanced when compared to the non-treated group. The mean stimulation indexes were 23.52 and 29.14, which were significantly higher than the non-treated correspond mice of 50 days PI (SI = 19.08) and 180 days PI (SI = 17.57) respectively

Table 3.2 Concanavalin A stimulated lymphocyte proliferative response in mice infected *G.spinigerum*

Group / Day after infection	No. of mice	Counts per minute (cpm)		SI (S/U)	Δ cpm (S-U)
		Unstimulated (U)	Stimulated (S)		
Control	18	5,654.52 ± 935.84*	133,844.49 ± 27,042.15	23.84 ± 3.78	128,123.22 ± 26,531.68
10 larvae					
10 days	4	4,520.02 ± 490.13	107,309.90 ± 6,097.93	23.86 ± 1.62	102,789.89 ± 5,666.82
50 days	6	5,787.66 ± 885.49	114,777.17 ± 14,422.80	20.05 ± 2.67	108,989.51 ± 13,949.82
180 days	12	5,951.95 ± 965.45	105,387.37 ± 14,924.57	18.03 ± 3.25	99,435.42 ± 14,769.03
20 larvae					
10 days	4	4,990.96 ± 354.48	109,511.59 ± 7,352.49	21.98 ± 1.32	104,520.63 ± 7,131.55
50 days	5	5,922.14 ± 979.04	112,519.64 ± 23,537.77	19.08 ± 2.61	106,597.51 ± 22,838.38
180 days	5	5,733.00 ± 331.67	102,692.97 ± 6,957.13	17.57 ± 1.13	94,959.98 ± 8,304.00
10 larvae + Rx					
10 days	5	4,659.36 ± 470.94	111,420.77 ± 9,070.87	23.97 ± 1.01	106,761.41 ± 8,640.62
50 days	6	5,835.69 ± 1,398.74	146,338.75 ± 29,484.72	25.36 ± 3.06	140,503.06 ± 28,250.48
180 days	6	4,434.20 ± 257.97	114,635.51 ± 5,662.60	25.59 ± 2.40	110,201.32 ± 5,752.16
20 larvae + Rx					
10 days	4	5,132.39 ± 590.33	113,738.85 ± 9,529.52	22.24 ± 1.28	108,606.47 ± 9,019.18
50 days	8	6,507.44 ± 761.09	154,057.74 ± 31,286.46	23.52 ± 2.73	147,550.29 ± 30,634.37
180 days	5	6,721.42 ± 851.71	190,581.46 ± 11,004.37	28.59 ± 2.48	183,860.04 ± 10,285.04

* Mean ± SD

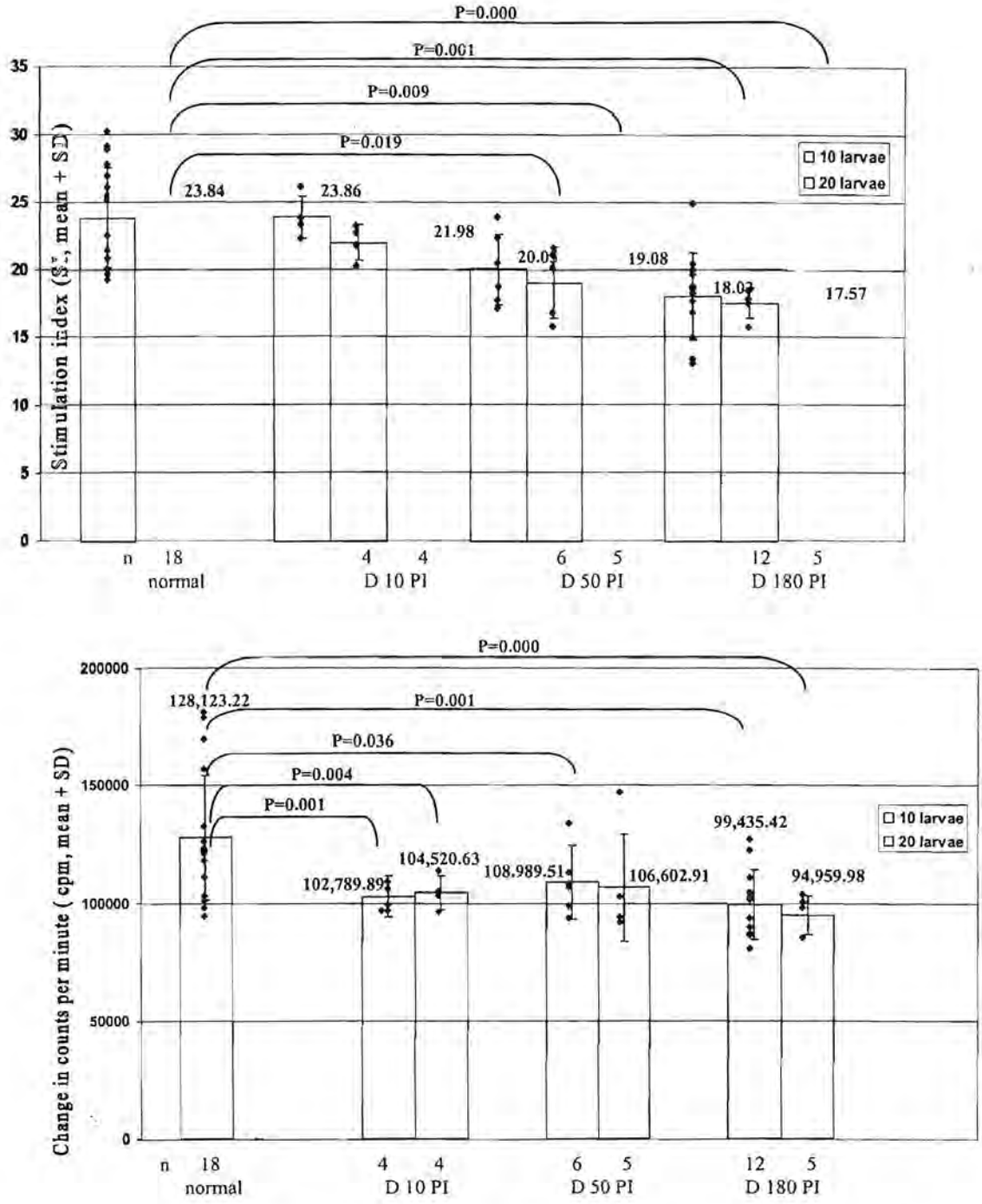


Fig 3.1. Stimulation index (SI) and change in counts per minute (Δcpm) of Con A-stimulated splenic lymphocytes of mice infected with 10 and 20 larvae of *G.spinigerum* at different time intervals (D 10, 50 and 180). Bars and lines represent mean and SD of individual animal indicated (•); n is the number of animal in each experimental group. PI is post infection.

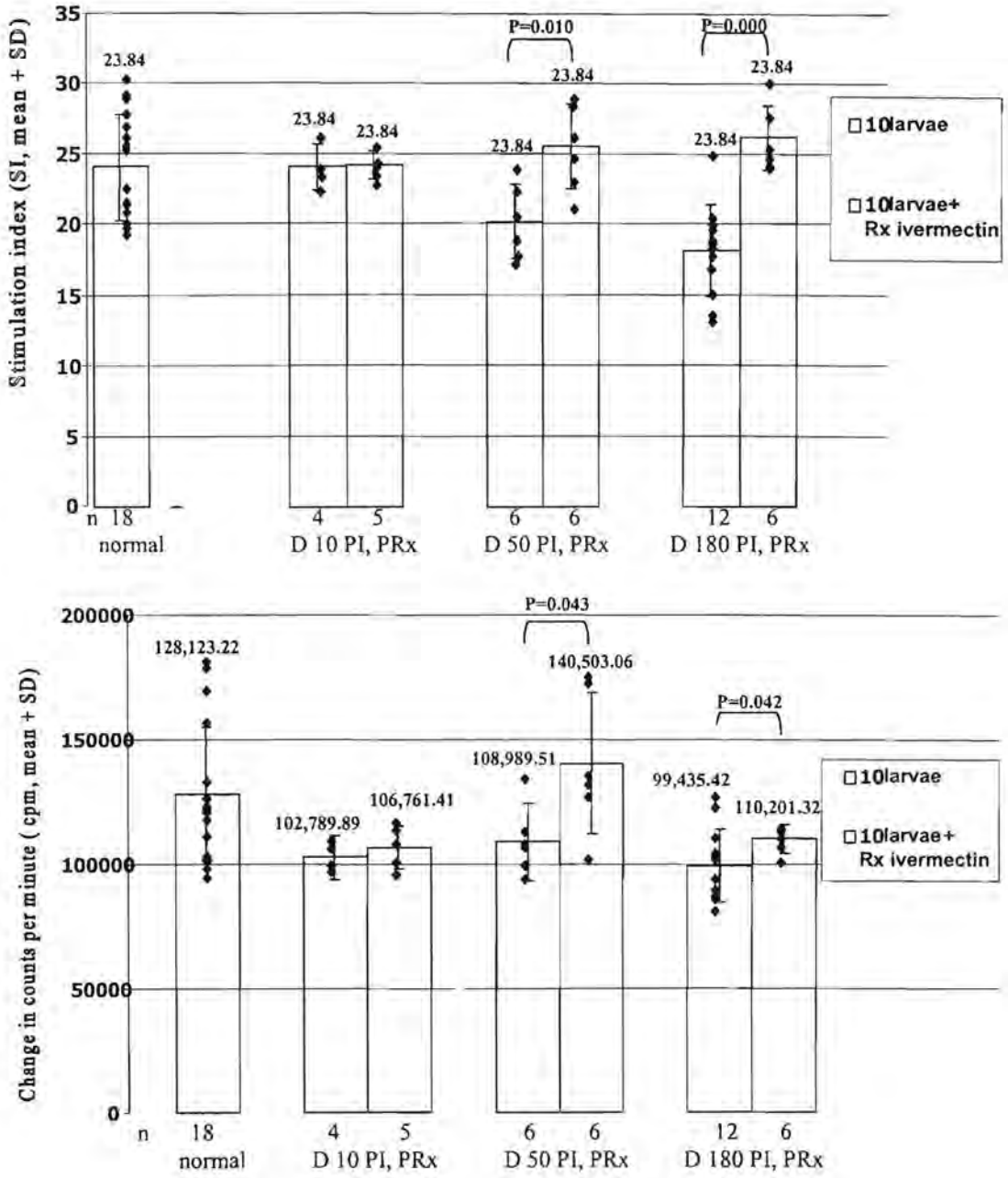


Fig 3.2 Stimulation index (SI) and change in counts per minute (Δcpm) of Con A-stimulated splenic lymphocytes of mice infected with 10 larvae and treated infected 10 larvae of *G.spinigerum* at different time intervals (D 10, 50 and 180). Bars and lines represent mean and SD of individual animal indicated (•); n is the number of animal in each experimental group. PI is post infection; PRx is post drug treatment.

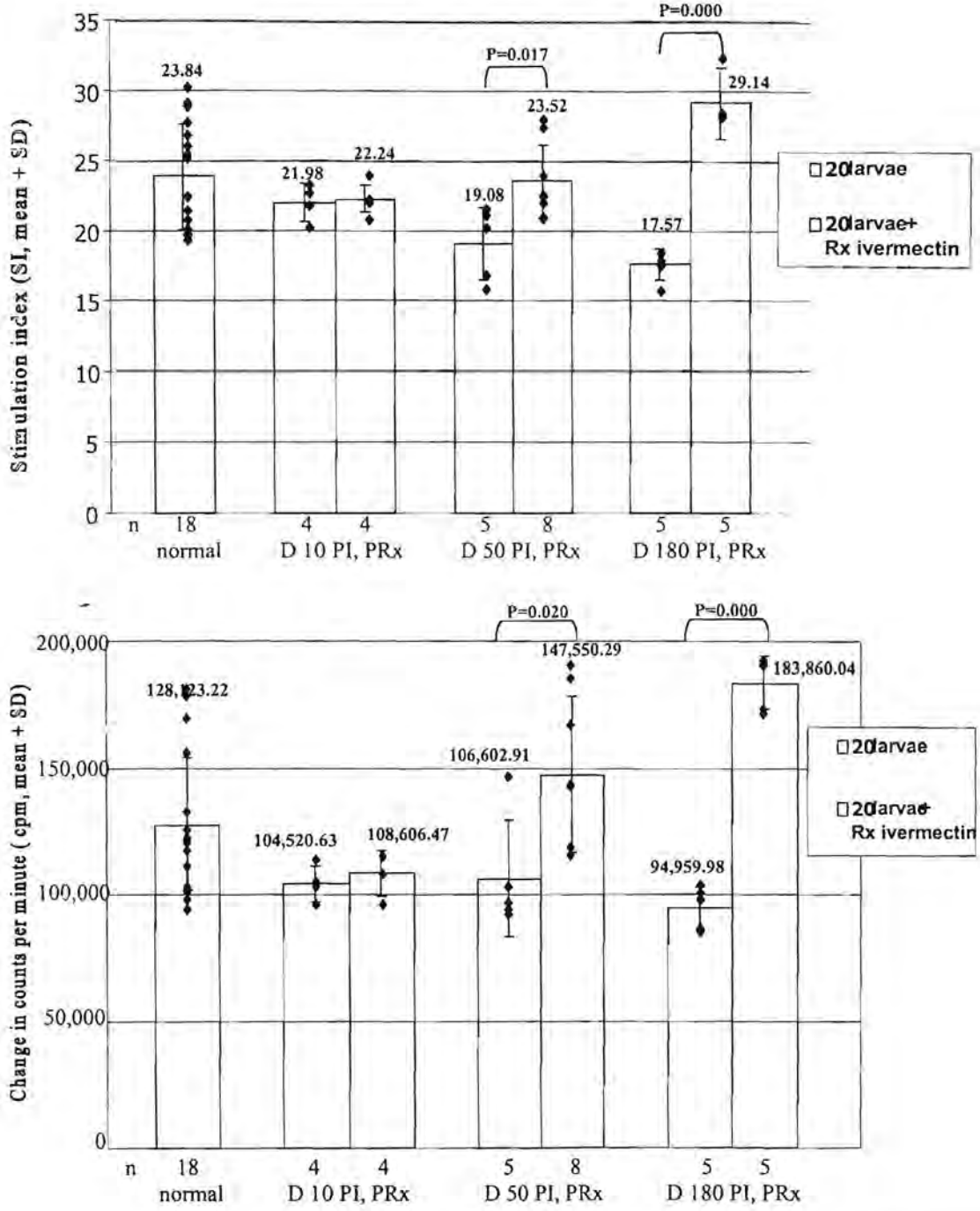


Fig 3.3 Stimulation index (SI) and change in counts per minute (Δcpm) of Con A-stimulated splenic lymphocytes of mice infected with 20 larvae and treated infected 20 larvae of *G.spinigerum* at different time intervals (D 10, 50 and 180). Bars and lines

represent mean and SD of individual animal indicated (•); n is the number of animal in each experimental group. PI is post infection; PRx is post drug treatment.

3.2.2 Antigen-stimulated Lymphoproliferative response of mouse infected with L3s *G.spinigerum*

In this experiment a specific response to somatic antigens was determined. Lymphocytes taken from mice during the early-stage (10 and 50 days) and the late-stage (180 days) of infection were tested with 100 μg/ml somatic L3s *G.spinigerum* antigen. The responses were compared with controls of the same age group. The uninfected controls groups responded slightly to antigen stimulation, with a mean stimulation index of 0.97 (Table 8). Ten days after being infected with 10 and 20 larvae, there was a slight increase in response to antigen stimulation (SI = 1.47 and SI = 1.12). It was statistically significant (P = 0.000) between the group infected with 10 larvae and the uninfected controls group. Among these experimental animals, 50 days after being infected with 10 and 20 larvae had the highest stimulation index (SI = 2.34 and SI = 2.38) as shown in Fig 49. When the infected animals of 10 and 20 larvae were prolonged to 180 days, the stimulation indices were not as high as the group of 50 days PI. However, they were still significantly higher than the group of day 10 PI and uninfected controls. Almost of the infected mice, the stimulation index of somatic L3s antigen stimulation was significantly higher than uninfected control group.

Prior to single dose of ivermectin (0.4 mg/kg body weight) giving, animals was given to animals. Results summarized in Table 8 and Fig 50 and 51 showed that the response of both infected 10 and 20 larvae was gradually increase following days post treatment ($P<0.05$). For acute infection (10 and 50 days) the response was significant higher than uninfected controls. For chronic infection (180 days) the response a significant high response was noted ($P<0.05$) compare to the group of 10 and 50 days post treatment and the uninfected controls. The interesting observation however was the finding that 180 days after treated with ivermectin, the lymphocyte responses were markedly enhanced. The mean stimulation index was more than about 8

times higher than that of the control groups. The response of this group was higher than in those treated for a shorter duration.

Comparison stimulation index between non-treated infected mice and treated infected mice showed in Fig 50-51 both the SI and Δcpm. The interesting observation was the finding that 10, 50 and 180 days after the infection were cured by ivermectin treatment; the lymphoproliferative responses were markedly enhanced. The mean stimulation index was about 2 times that of the non-treated mice in group of day 10 and day 50 after being treated with chemotherapy. The late stage of infection (180 day), the mean stimulation index was more than about 4 times higher than that of the non-treated group. Differentiation at same days post treatment showed that the response of treated infected mice significant higher than non-treated infected mice in all group of time interval of infection (10, 50 and 180 days).

The results presented in this study showed that splenic lymphocytes from mice infected with both 10 and 20 larvae of *G.spinigerum* had altered reactivity to stimulation by Con A and were responsive to specific stimulation by somatic L3s antigen.

Table 3.3 Antigen- stimulated lymphocyte proliferative response in mice infected *G.spinigerum*

Group / Day after infection	No. of mice	Counts per minute (cpm)		SI (S/U)	Δ cpm (S-U)
		Unstimulated (U)	Stimulated (S)		
Control	18	4,712.19 ± 1,896.15*	4,554.37 ± 1,958.93	0.97 ± 0.18	-157.82 ± 995.88
10 larvae					
10 days	4	4,538.81 ± 439.24	6,660.26 ± 541.50	1.47 ± 0.09	2,121.45 ± 362.03
50 days	6	4,796.92 ± 537.49	11,112.25 ± 314.60	2.34 ± 0.25	6,315.33 ± 473.87
180 days	12	4,265.67 ± 1,273.21	8,163.17 ± 1,688.47	1.99 ± 0.39	3,897.50 ± 1,310.36
20 larvae					
10 days	4	4,325.31 ± 668.33	4,796.16 ± 214.56	1.12 ± 0.14	470.86 ± 463.36
50 days	5	2,116.03 ± 254.71	4,941.3[illegible] ± 1,112.98	2.38 ± 0.70	2,825.32 ± 1,200.37
180 days	5	4,886.66 ± 1,216.66	7,845.84 ± 1,998.70	1.63 ± 0.40	2,959.17 ± 1,786.37
10 larvae + Rx					
10 days	5	5,753.05 ± 1,659.72	15,272.11 + 4,294.18	2.67 ± 0.25	9,519.06 ± 2,783.37
50 days	6	5,981.59 ± 1,507.18	30,015.56 ± 6,184.03	5.13 ± 0.68	24,033.97 ± 4,882.34
180 days	6	5,311.79 ± 642.14	45,819.41 ± 4,007.19	8.78 ± 1.71	40,507.62 ± 4,320.23
20 larvae + Rx					
10 days	4	4,79.53 ± 279.07	12,107.78 ± 224.88	2.56 ± 0.12	7,358.25 ± 212.50
50 days	8	5,507.55 ± 580.76	20,091.83 ± 3,144.66	3.62 ± 0.72	14,209.28 ± 3,459.23
180 days	5	4,282.25 ± 1,534.11	29,599.81 ± 7,778.08	7.68 ± 2.86	25,317.56 ± 6,918.11

* Mean ± SD

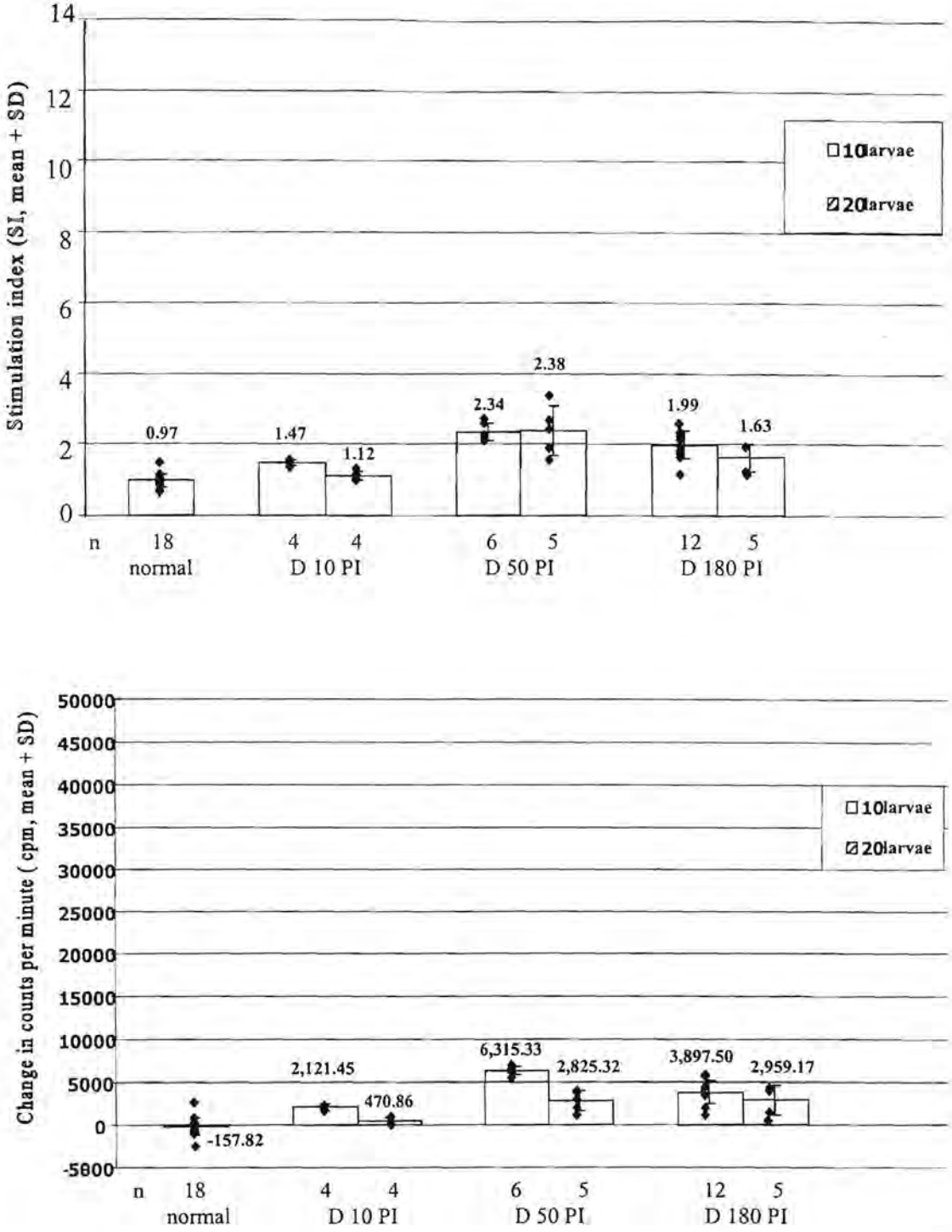


Fig 3.4 Stimulation index (SI) and change in counts per minute (Δcpm) of somatic L3s antigen stimulated splenic lymphocytes of mice infected with 10 and 20 larvae of *G.spinigerum* at different time intervals (D 10, 50 and 180). Bars and lines represent mean and SD of individual animal indicated (•); n is the number of animal in each experimental group. PI is post infection.

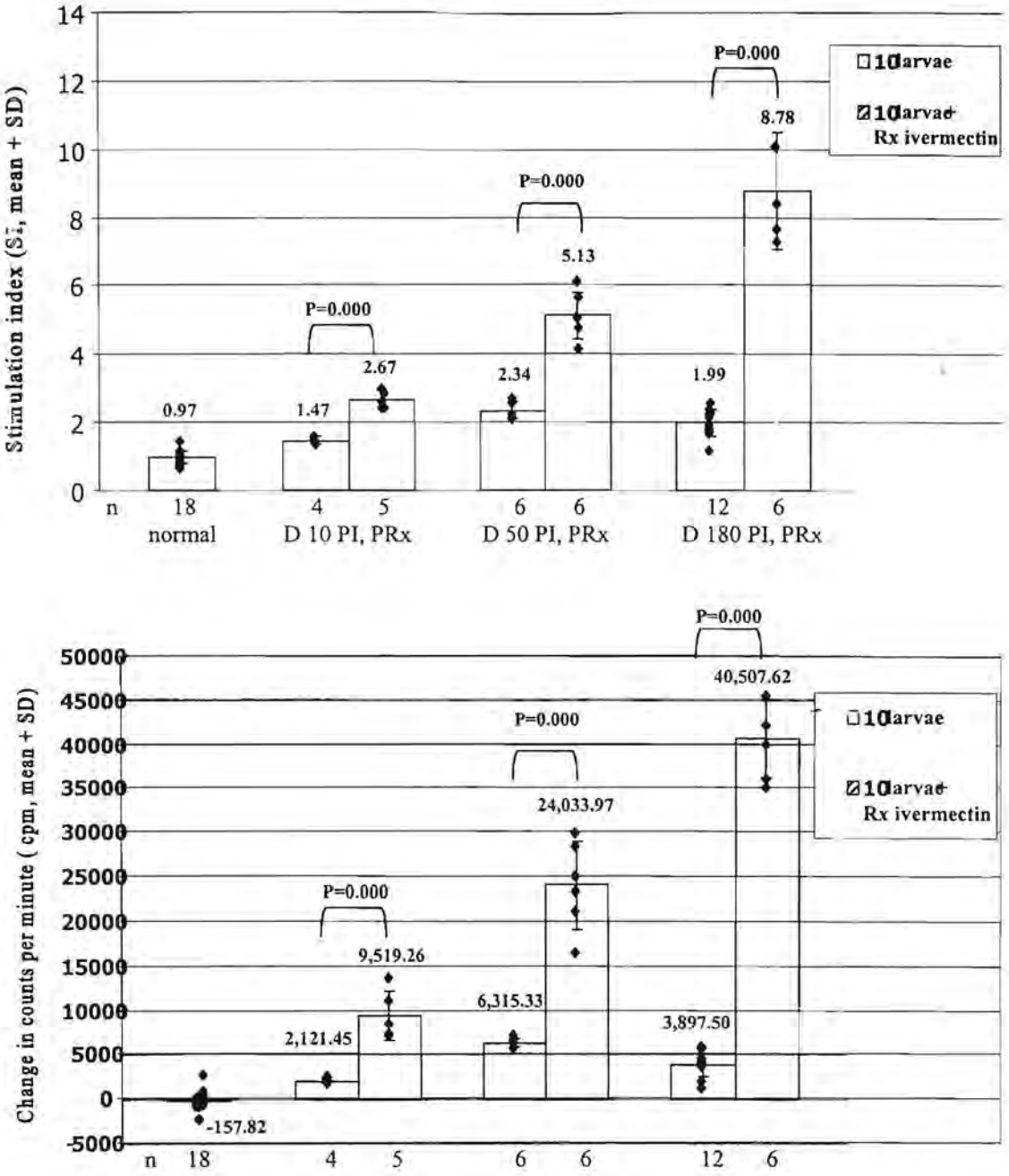


Fig 3.5 Stimulation index (SI) and change in counts per minute (Δcpm) of somatic L3s antigen stimulated splenic lymphocytes of mice infected with 10 larvae and treated infected 10 larvae of *G.spinigerum* at different time intervals (D 10, 50 and 180). Bars and lines represent mean and SD of individual animal indicated (•); n is the number of animal in each experimental group. PI is post infection; PRx is post drug treatment.

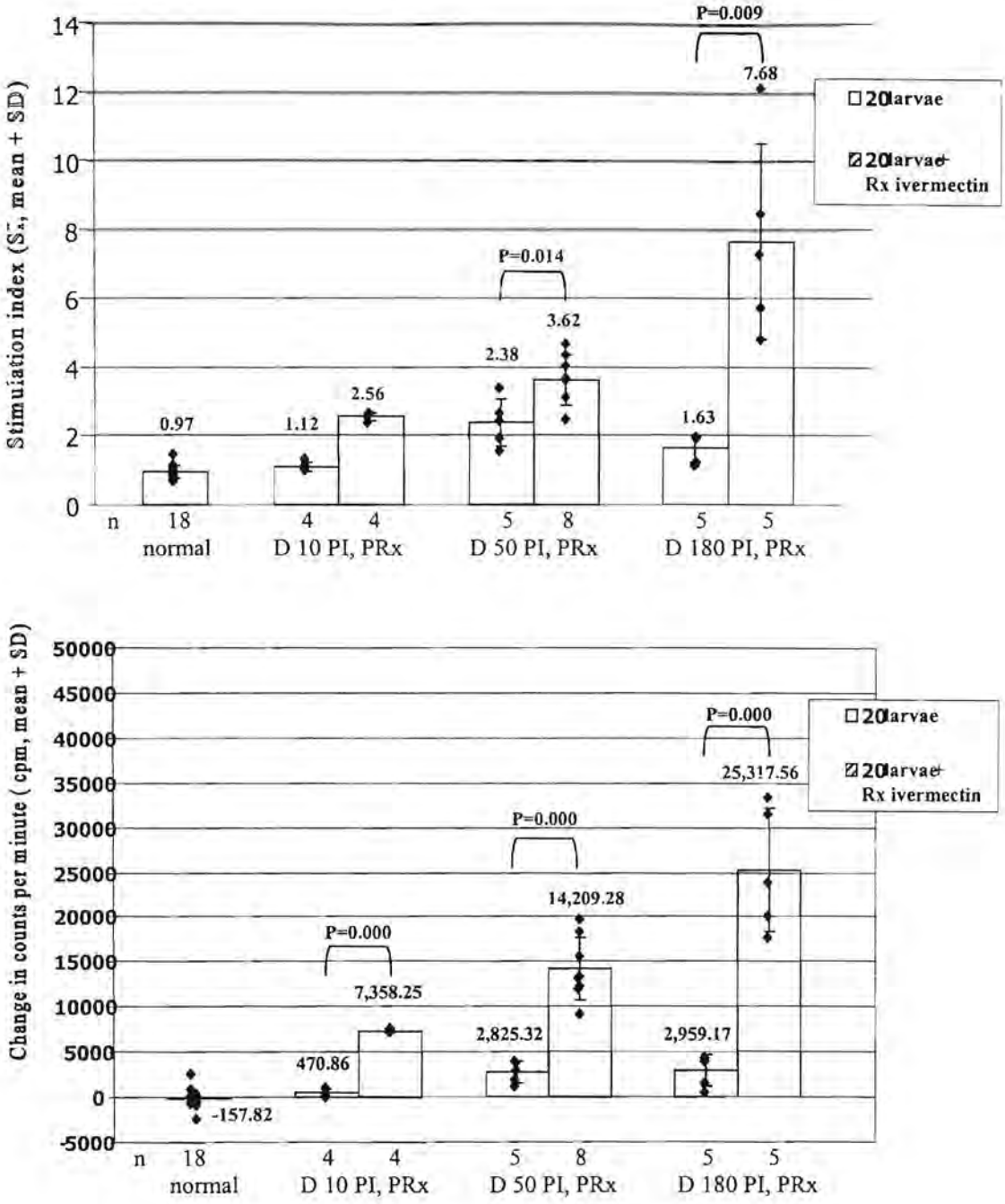


Fig 3.6. Stimulation index (SI) and change in counts per minute (Δcpm) of somatic L3s antigen stimulated splenic lymphocytes of mice infected with 20 larvae and treated infected 20 larvae of *G.spinigerum* at different time intervals (D 10, 50 and 180). Bars and lines represent mean and SD of individual animal indicated (•); n is the number of animal in each experimental group. PI is post infection; PRx is post drugtreatment.

Table 4.1 The mean percentage cell population of CD_4 and CD_8 and intracellular IFN-γ in normal and acute G. spinigerum infected mice

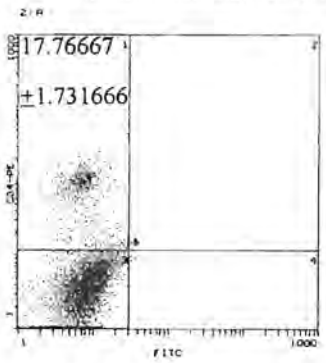
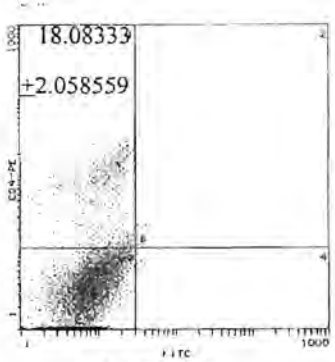
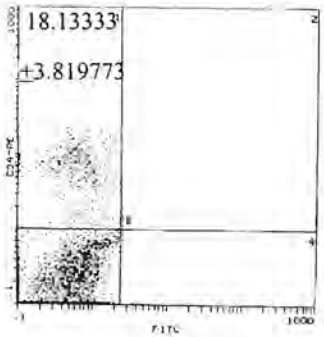
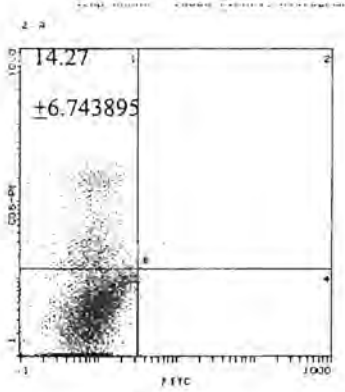
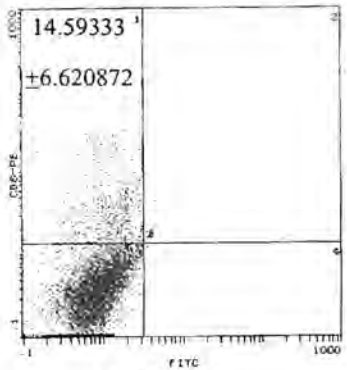
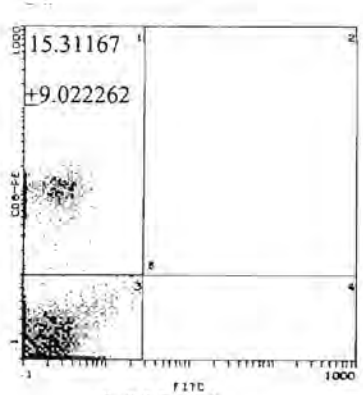
Mice		Unstimulated	Con A	Antigen
Normal mice				
CD_4				
CD4-Intracellular IFN-γ,	**Mean (%)**	0.268889	0.851111	0.307778
	S.D. (%)	0.190948	0.585778	0.184714
CD_8				
CD8-Intracellular IFN-γ,	**Mean(%)**	0.137778	0.686667	0.235556
	S.D. (%)	0.117556	0.499975	0.259572

Table 4.1 (Continue)

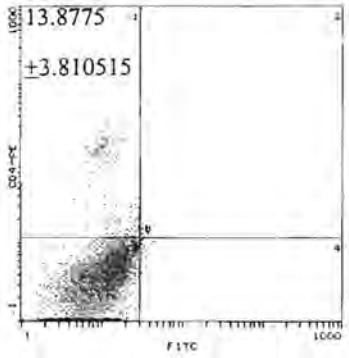
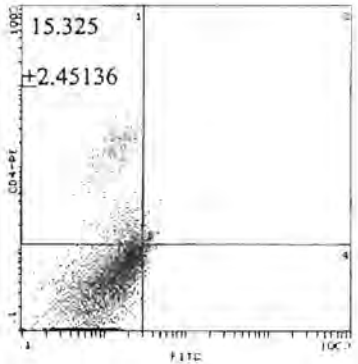
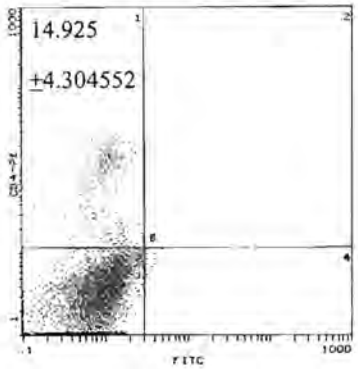
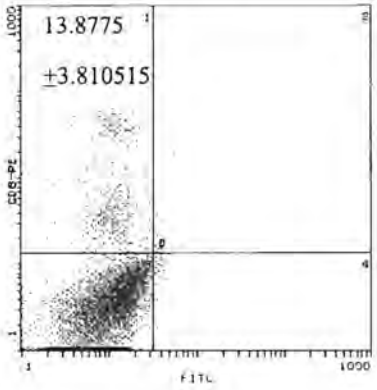
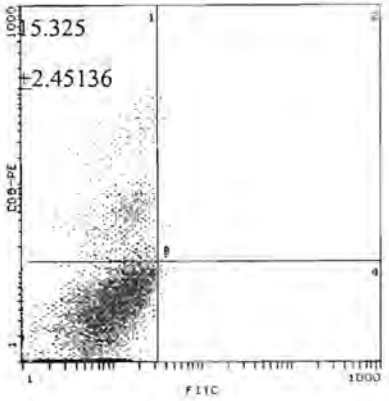
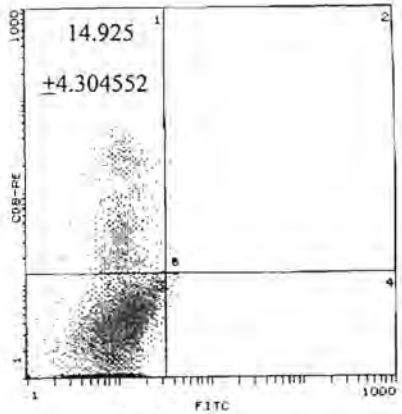
Mice		Unstimulated	Con A	Antigen
Infection Day 10 PI				
CD_4				
CD 4-Intracellular IFN-γ	**Mean(%)**	0.22	0.405	0.27
	S.D. (%)	0.19799	0.148492	0.042426
CD_8				
CD8-Intracellular IFN-γ	Mean(%)	0.325	0.455	0.18
	S.D. (%)	**0.035355**	**0.091924**	**0.014142**

Table 4.1 (Continue)

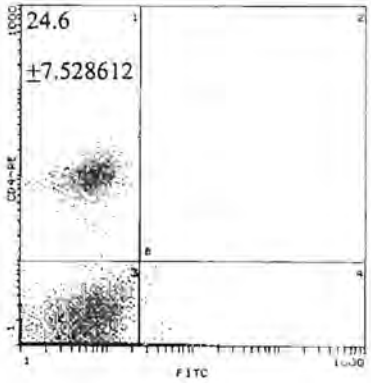
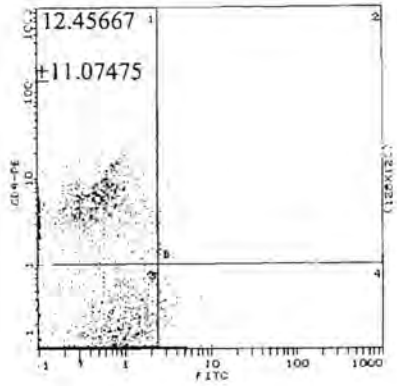
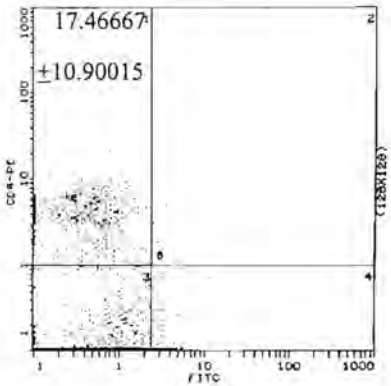
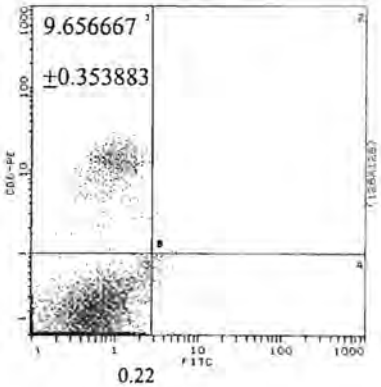
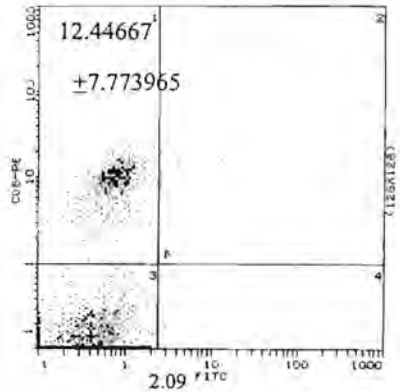
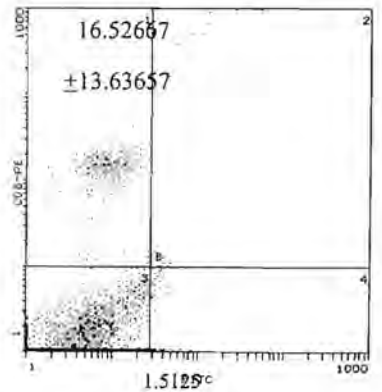
Mice		Unstimulated	Con A	Antigen
Infection Day 30 PI				
CD_4				
CD4-Intracellular IFN-γ	**Mean(%)**	0.29	1.9425	0.365
	S.D. (%)	0.321973	1.828522	0.352373
CD_8				
CD8-Intracellular IFN-γ	**Mean(%)**	0.22	2.09	1.5125
	S.D. (%)	0.202485	1.692907	0.499224

Table 4.1 (Continue)

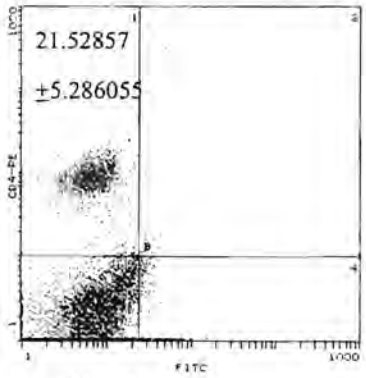
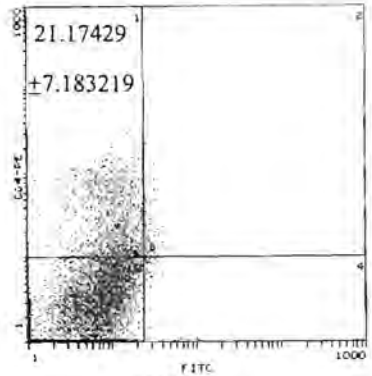
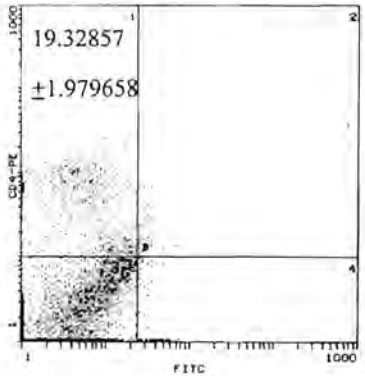
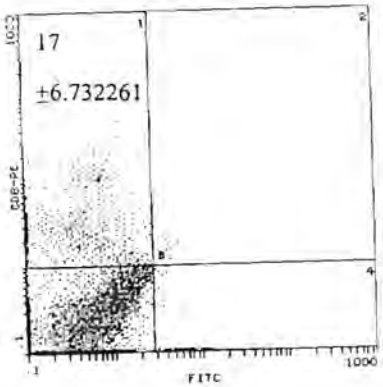
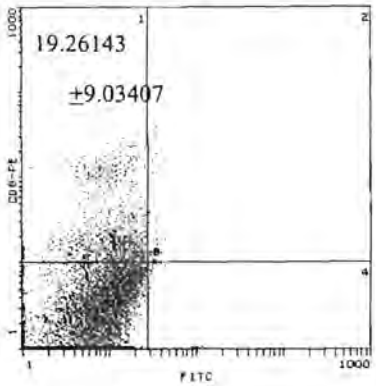
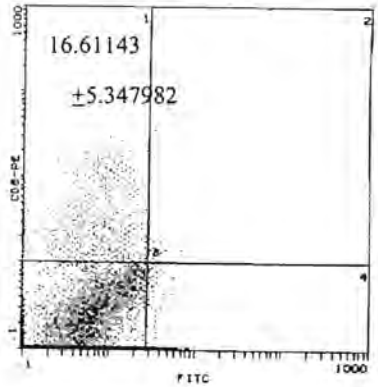
Mice		Unstimulated	Con A	Antigen
Infection Day 50 PI				
CD_4				
CD4-Intracellular IFN-γ	**Mean(%)**	1.355714	3.564286	1.222857
	S.D. (%)	1.168116	1.885567	0.515678
CD_8				
CD8-Intracellular IFN-γ	**Mean(%)**	0.788571	3.568571	0.795714
	S.D. (%)	0.658038	1.766856	0.457306

Table4.1 (Continue)

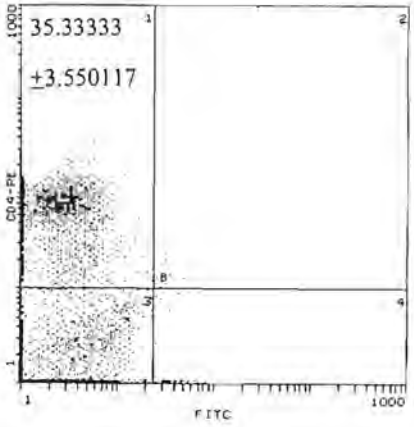
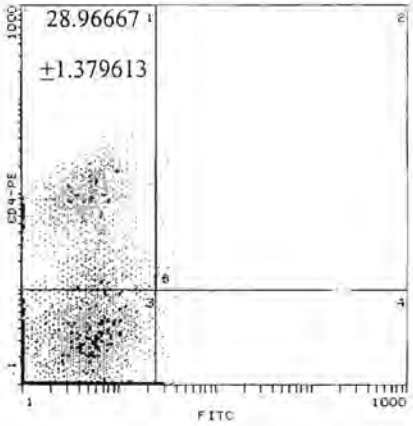
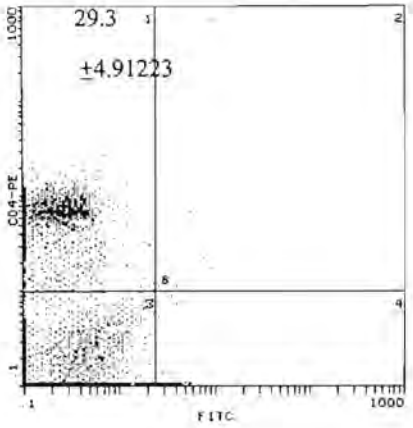
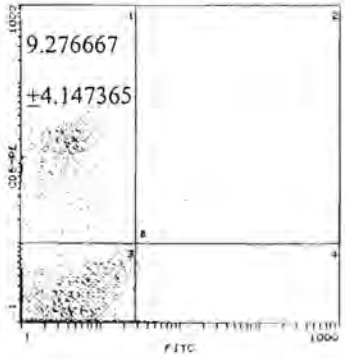
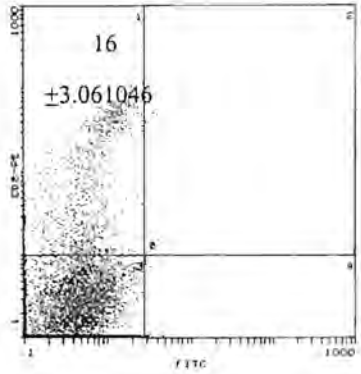
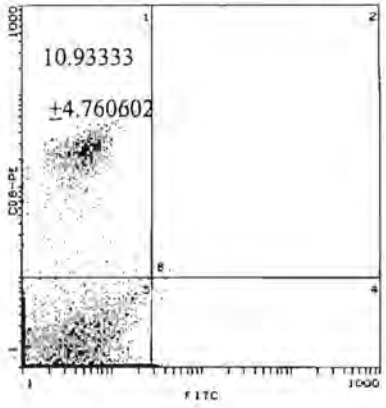
Mice		Unstimulated	Con A	Antigen
Infection Day 100 PI				
CD_4				
CD4-Intracellular IFN-γ	**Mean(%)**	0.26	0.52	0.19
	S.D. (%)	0.150997	0.180831	0.026458
CD_8				
CD8-Intracellular IFN-γ	**Mean(%)**	0.17	0.706667	0.12
	S.D. (%)	0.141067	0.375278	0.026458

Table4.1 (Continue)

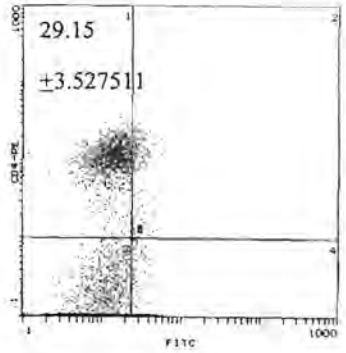
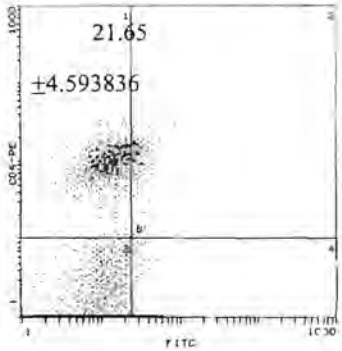
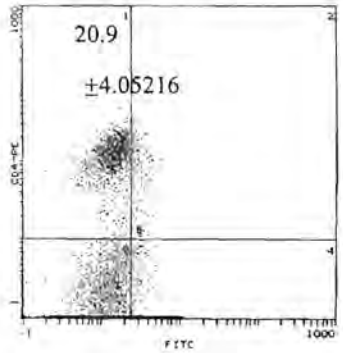
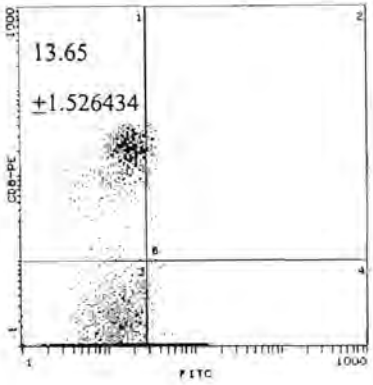
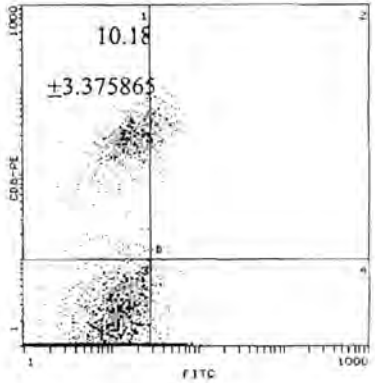
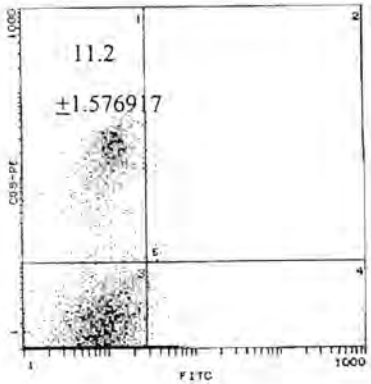
Mice		Unstimulated	Con A	Antigen
Infection Day 180 PI				
CD_4				
CD4-Intracellular IFN-γ	**Mean(%)**	1.6375	5.2325	3.8025
	S.D. (%)	**1.343388**	**2.889249**	**1.118969**
CD_8				
CD8-Intracellular IFN-γ	**Mean(%)**	1.0725	3.855	1.4775
	S.D. (%)	0.764651	1.851495	2.269881

Table4.2 The mean percentage of CD4 and CD8 subsets in splenic mice

Normal / No.	CD4+			CD8+		
	US	CONA	Ag	US	CONA	Ag
1	20.4	21.5	21.8	20	20.1	23.6
2	18.7	16.7	18.8	17.6	18.4	23.3
3	18.1	18	18.8	22.9	22.8	23.4
4	17.5	15.4	22.2	10.1	7	9.92
5	15.5	18.2	12.9	7.18	9.84	5.75
6	16.4	18.7	14.3	7.84	9.42	5.9
Mean	17.76667	18.08333	18.13333	14.27	14.59333	15.31167
S.D.	1.731666	2.058559	3.819773	6.743895	6.620872	9.022262

(a) normal mice control

10L, d10PI	CD4+			CD8+		
	US	CONA	Ag	US	CONA	Ag
1	9.51	13.4	10.2	10.5	12.6	10.8
2	12.9	13.3	12.4	6.83	14.8	13
3	14.4	16.2	18	10.1	12.4	16.9
4	18.7	18.4	19.1	15.2	19.4	19.1
Mean	13.8775	15.325	14.925	10.6575	14.8	14.95
S.D.	3.810515	2.45136	4.304552	3.445755	3.253716	3.743884

(b) mice infected whit 10 larvae at day 10 post infection

10L , d30PI	CD4+			CD8+		
	US	CONA	Ag	US	CONA	Ag
1	30	25.2	12.2	9.94	13.4	32.2
2	27.8	5.16	30	9.26	19.7	10
3	16	7.01	10.2	9.77	4.24	7.38
Mean	24.6	12.45667	17.46667	9.656667	12.44667	16.52667
S.D.	7.528612	11.07475	10.90015	0.353883	7.773965	13.63657

(c) mice infected whit 10 larvae at day 30 post infection

10L, d50PI	CD4+			CD8+		
	US	CONA	Ag	US	CONA	Ag
1	31.3	27.3	21.6	26.4	26.6	21.7
2	24.9	29.5	20.4	23.6	30.8	22.9
3	16.1	20.2	19.2	18.8	17.1	19.3
4	16.5	23.8	16.8	18.4	26.6	17.5
5	22	16.5	21.3	7.8	5.43	8.68
6	19.3	8.12	19.3	12.3	14.7	15.4
7	20.6	22.8	16.7	11.7	13.6	10.8
Mean	21.52857	21.17429	19.32857	17	19.26143	16.61143
S.D.	5.286055	7.183219	1.979658	6.732261	9.03407	5.347982

(d) mice infected whit 10 larvae at day 50 post infection

10L, d100PI	CD4+			CD8+		
	US	CONA	Ag	US	CONA	Ag
1	38.3	27.4	33.7	11.1	19.2	11.3
2	36.3	29.5	30.2	12.2	13.1	15.5
3	31.4	30	24			
Mean	35.33333	28.96667	29.3	11.65	16.15	13.4
S.D.	3.550117	1.379613	4.91223	0.777817	4.313351	2.969848
S.E.						

(e) mice infected whit 10 larvae at day 100 post infection

10L, d180PI	CD4+			CD8+		
	US	CONA	Ag	US	CONA	Ag
1	32.2	27.4	25.3	13.4	13.9	13.5
2	31.9	19.8	22.6	14.6	12	10
3	24.9	16.6	15.8	15	8.33	10.4
4	27.6	22.8	19.9	11.6	6.49	10.9
Mean	29.15	21.65	20.9	13.65	10.18	11.2
S.D.	3.527511	4.593836	4.05216	1.526434	3.375865	1.576917

(f) mice infected whit 10 larvae at day 180 post infection

5. Autopsy and histological section

The results of autopsy and histological section could demonstrated that all the advanced third stage larvae (AdL3, obtained from the natural infected eels) given orally to each mouse remained in the stomach for 1 hr post–infection (PI). At 3, 5,7,10 and 24 hr PI, the larvae transiently penetrate the stomach wall and invaded various organs including mesentery, esophagus, liver, diaphragm, lung, heart and dorsal fat. Approximately, 10%-20% of the larvae moved into the muscle and the subcutaneous tissue within 5-10 days while the remainders were still in the visceral organs. A few larvae were found in the brain (day 40 PI). Encystment began at day11 PI and after that it increased gradually up to 95% at day 21 PI.

Table 5.1 The mean percentage of worm recovery and location of larvae recovered at different time interval during 1 and 24 hr of post 10 larvae infection

PI (hr)	dose (larvae)	location of larvae recovered (number)	worm recovery (%)
1	10	Stomach(10)	10 (100%)
	10	Stomach(10)	8 (80%)
3	10	Stomach(7), Mesentery(3)	10 (100%)
	10	Stomach(8), Liver(1)	9 (90%)
5	10	Esophagus(2), Lung(3), Heart(1), Liver(2)	8 (80%)
	10	Diaphragm(2), Liver(1)	3 (30%)
7	10	Stomach(4), Mesentery(1), Lung(2)	7 (70%)
10	10	Stomach(8), Liver(1)	9 (90%)
24	10	Liver(1), Lung(1), Dorsal fat(1)	3 (30%)
	10	Esophagus(1), Stomach(4), Liver(2)	7 (70%)

Table 5.2 Percentage of worm recovery and organs involvement at different time interval of 10 larvae infected mice during day 1-7 post infection

day PI	infected dose	location of larvae recovered (number)	No. of larvae (%)
1	10	Liver(1), Lung(1), Dorsal fat(1)	3 (30%)
	10	Stomach(4), Esophagus(1), Liver(2)	7 (70%)
3	10	Liver(3), Diaphragm(1), Mesentery(2)	6 (60%)
	10	Liver(3)	3 (30%)
5	10	Liver(2), Diaphragm(1), Abdominal muscle(1), Intercostal	5 (50%)
	10	muscle(1)	5 (50%)
7	30	Liver(5)	28 (93.3%)
	10	Stomach(1), Liver(24), Abdominal fat(2), Dorsal muscle(1)	6 (60%)
	10	Liver(3), Lower limb(2), Upper limb(1)	2 (20%)
	10	Liver(1), Abdominal muscle(1)	3 (30%)
		Liver(3)	

Table 5.3 Percentage of worm recovery and organs involvement at different time interval during day 11-253 post 8, 10, 20 and 25 larvae infection

Day PI	Infected dose	location of larvae recovered (number)	No. of larvae (%)
11	20	Stomach(1), Liver(1)/(11)*, Dorsal muscle(1)/(2)*, Pectoralis muscle(1)*	18 (90%)
14	10	Liver(1), Urogenital(1), Subcutaneous(1)*, Abdominal muscle(1), Dorsal muscle(2)*	6 (60%)
20	10	Abdominal fat(1)*, Dorsal muscle(1)/(1)*, Intercostal muscle(1)*, Lower limb(1)	5 (50%)
20	10	Abdominal muscle(3)*, Dorsal muscle(1)*, Pectoralis muscle(2)*	6 (60%)
30	20	Liver(3)*, Subcutaneous(1), Abdominal fat(1)*, Abdominal muscle(1)*, Dorsal muscle(5)*, Lower limb(2)*, Upper limb(1)*	14 (70%)
30	20	Liver(3), Dorsal muscle(6)*, Intercostal muscle(1)*, Upper limb(1)*	11 (55%)
36	10	Liver(2)*, Pectoralis muscle(2)*, Lower limb(1)*, Upper limb(2)*	7 (70%)
36	10	Liver(1)*, Dorsal muscle(1)*, Pectoralis muscle(2)*, Upper limb(1)/(3)*	8 (80%)
40	8	Brain(1)*, Dorsal muscle(2)*, Intercostal muscle(1)*, Upper limb(1)*	5 (62.5%)
48	20	Head(1)*, Subcutaneous(1)*, Abdominal muscle(1)*, Dorsal muscle(1)*, Pectoralis muscle(1)*, Lower limb(2)*, Upper limb(1)*	8 (40%)
48	20	Abdominal fat(1)*, Dorsal muscle(1)/(2)*, Lower limb(2)*, Upper limb(1)*	7 (35%)
55	10	Liver(1)*, Abdominal muscle(1)*, Dorsal muscle(3)*	5 (50%)
55	10	Liver(1)*, Abdominal muscle(1)*, Dorsal muscle(2)*, Lower limb(1)/(1)*	6 (60%)
60	20	Liver(5)*, Subcutaneous(1)*, Abdominal fat(2)*, Abdominal muscle(1)*, Lower limb(2), Pectoralis muscle(1)*, Upper limb(2)*	14 (70%)
61	20	Urogenital(2)*, Subcutaneous(2)*, Abdominal fat(1), Dorsal fat(1), Dorsal muscle(1)*, Lower limb(5)*	12 (60%)
95	25	Subcutaneous(2)*, Abdominal muscle(1)*, Dorsal muscle(5)*	7 (28%)
95	20	Liver(1), Abdominal muscle(1)*, Dorsal muscle(4)*	6 (30%)
95	20	Liver(3)*, Dorsal fat(1)*, Upper limb(1)*	5 (25%)
100	10	Head(1)*, Liver(1), Dorsal muscle(2)*, Lower limb(2)*	6 (60%)
100	10	Head(1)*, Liver(1)*, Subcutaneous(1), Dorsal muscle(5)*, Lower limb(1)* Upper limb(1)*	10 (100%)
100	10	Liver(3)*, Abdominal muscle(1)*, Dorsal muscle(3)*, Intercostal muscle(1)*, Lower limb(1)*	9 (90%)
122	10	Dorsal muscle(2)*, Intercostal muscle(1)*, Lower limb(1)*	4 (40%)
127	10	Liver(1)/(2)*, Abdominal fat(1), Dorsal muscle(2)*, Upper limb(2)*	8 (80%)
253	20	Head(2)*, Liver(2)*, Dorsal muscle(6)*, Lower limb(1)*, Upper limb(4)*	15 (75%)
253	20	Head(1)*, Diaphragm(1)*, Liver(3)*, Dorsal muscle(6)*, Intercostal muscle(1)*, Pectoralis muscle(2)*, Lower limb(1)*, Upper limb(1)*	16 (80%)

Table 5.4 Percentage of encysted and non-encysted and location of larvae recovered from four different organs at different time interval of 10 post larvae infection

Table 5.4 Percentage of encysted and non-encysted and location of larvae recovered from four different organs at different time interval of 10 post larvae infection

	location of larvae recovered (....%)							
	Stomach		**Liver**		**Subcutaneous**		**Muscle**	
Time PI	encyst	Non-encyst	encyst	Non-encyst	encyst	Non-encyst	encyst	Non-encyst
0hr	0	90	0	0	0	0	0	0
1hr	0	90	0	0	0	0	0	0
3hr	0	75	0	5	0	0	0	0
5hr	0	0	0	15	0	0	0	0
7hr	0	40	0	0	0	0	0	0
10hr	0	80	0	10	0	0	0	0
1d	0	20	0	15	0	0	0	0
3d	0	0	0	30	0	0	0	0
5d	0	0	0	35	0	0	0	10
7d	0	1.67	0	51.67	0	0	0	8.35
11d	0	5	55	5	0	0	15	10
14d	0	0	0	10	10	0	20	10
20d	0	0	0	0	0	0	20	20
21d	0	0	0	0	0	0	60	0
30d	0	0	7.5	7.5	0	2.5	42.5	0
36d	0	0	15	0	0	0	55	5
40d	0	0	0	0	0	0	50	0
48d	0	0	0	0	2.5	0	27.5	2.5
55d	0	0	10	0	0	0	40	5
60d	0	0	25	0	5	0	30	0
61d	0	0	0	0	10	0	30	0
95d	0	0	4.62	1.54	1.54	0	18.48	0
100d	0	0	13.33	3.33	0	3.33	56.61	0
122d	0	0	0	0	0	0	40	0
127d	0	0	20	10	0	0	40	0
253d	0	0	12.5	0	0	0	27.5	0

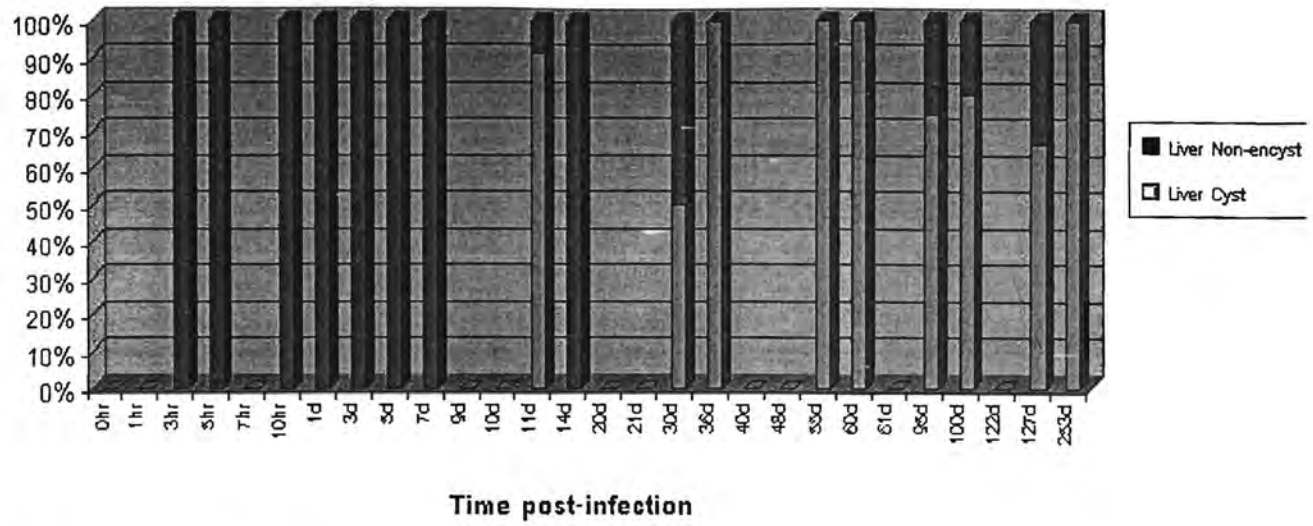


Fig 5.1 Percentage of encyst and non-encysted larvae located in liver at different time post infection

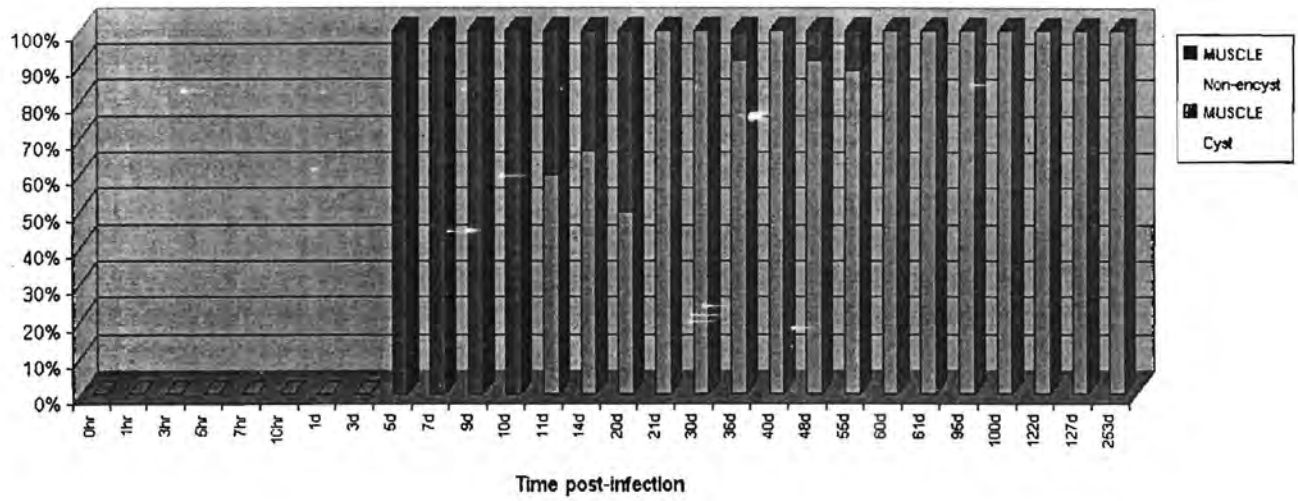


Fig 5.2 Percentage of encyst and non-encysted larvae located in muscle at different time of post infection

Fig 5.3 Picture showing mouse sacrify and organ separation

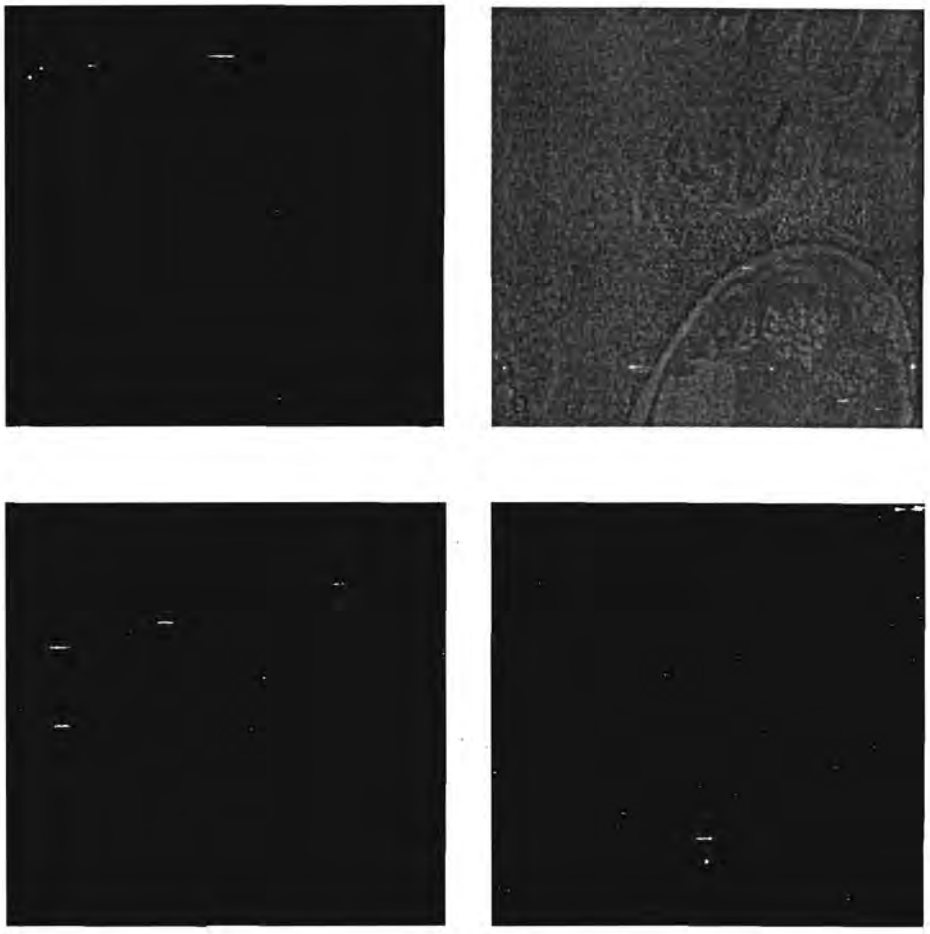


Fig 5.4 Histological section

a. muscle at Day 14 post infection

b. muscle at Day 30 post infection

c. subcutaneous tissue at Day 60 post infection

d. liver at Day 120 post infection

DISCUSSIONS

In this experiment, the high infective dose was changed from the previous assignment of 30 larvae presented in the thesis proposal to 20 larvae. Since, in our preliminary study, all three mice infected with high dose of 30 larvae died. The first animal died on Day 2; the others died on Day 3 and Day 7 post infection respectively. Autopsy photos (belowed), showed severe trauma and disruption of liver parenchyma. In addition, infection with dose of 25 larvae was too high and resulted in dead of the infected animal as well. By these reasons we designed to infect the animal with 10 and 20 larvae. In addition, conditions that affected the experiment were as following:

1) Drop out of mouse from some group

2) The inadequate numbers of worm harvested from eels prolonged the time for animal infection.

3) Death of mice infected with 25, 30 larvae (too high) made the assigned dose of infection down to 20 larvae.

4) Optimal conditions for lymphoproliferative test could not be standardized until Day over 50 post infection. Therefore the 10 larvae infected mouse was accumulated to 12 mice on Day 180 post infection.

Severe trauma and disruption of liver parenchyma of mice infected with 30 larvae *G.spinigerum*.

1. Blood eosinophil of mice infected with *G.spinigerum*

Blood eosinophil of mice infected with 5, 10 and 20 laevae reached a maximum on day 13 and day 15 post infection with9.25%,22.61% and 19.00% respectively.The percentages of both 10 and 20 larvae infected group were significantly different from the 5 larvae infected group. After reached a plateu, it gradually decreased and dropped to the normal level around day 150-180 post infection.

Eosinophil is a characteristic response of the host in helminthic infection.But Eosinophil counts can vary considerably during the course of infection, with the highest eosinophil counts occurring during the early invasive stage of the parasite life cycle, and waxing and waning of the counts during the subsequent weeks and months.
Recirculating T lymphocyte were a key component in the development of eosinophil. This due to the IL-5 and the involvement of Th-2 response of the host.

2. Humoral immune status of mice infected with *G. spinigerum*

2.1 ELISA Antibody responses

In most cases of human gnathostomiasis, the worms obtained from surgery or from their spontaneous emergences were the third-stage larvae and immature adults (Daengsvang, 1980; Akahane et al., 1994; Kraivichian et al., 2004). And the antigens generally used for antibody detection should be prepared from the same stage of parasite as that it is recovered from the host (Suntharasamai et al., 1985). Thus the third-stage larvae were selected for antigen in this study. The ELISA values of the previous study using antigen prepared from advanced L3s showed higher sensitivity and specificity when compare to those prepared from adult male and female (Morakote et al., 1991).

In this study, the advanced L3s somatic antigens could detect the antibody responses at different time intervals (Days 10, 30, 60, 90, 120 and 180 PI) of both groups of mice infected with 10 and 20 larvae. The enhancement of specific Ab (IgG-Ab and IgE-Ab) could be demonstrated within Day 10 PI. The levels gradually increased, reached a plateau during Day 60 and Day 120 PI and decreased thereafter. The Ab responses of 10 larvae infected mice were slightly lower than those of 20 larvae infected animals but they were not significantly different. In heavy infection, Ab levels are usually higher than in light infection (Abbas and Lichtman, 2003). From the overall kinetic responses, the detected Ab levels began to decrease approximately during Day 90 PI and Day 150 PI. The decreased antibody levels might be consisting with the time of larvae encystment. Since, the larvae encystment

in mice firstly appeared at Day 11 PI, and about half of the larvae encysted in muscle during Day 14 and Day 21 PI. All the larvae could completely encyst during Day 30 and Day 60 PI (Saksirisampant, unpublished obserbvations).

In human gnathostomiasis, IgG-Ab responses were used as immunodiagnosis tool or as an evidence of infection. The sensitivity varied from 56% to 100%, while the specificity ranged from 79% to 96% (Suntharasamai et al., 1985; Dharmkrong-at et al., 1986; Maleewong et al., 1988; Anantaphruti, 1989). However, for an evaluation of the effectiveness of chemotherapy, this IgG-Ab was still not satisfactory because it could persist more than six months following treatment with antihelminthic drug (Kraivichian et al., 1992; Nontasut et al., 2000).

As an antibody that involve in a defense mechanism for helminthic infection, the IgG antibody can participate in antibody-dependent cell-mediated cytotoxicity (Pier et al., 2004). Since, there are specific receptors for the Fc regions of IgG on on many cytotoxic cells including macrophages, neutrophil, eosinophil and natural killer cell. The interaction can mediate the release of mediators result in parasiticidal of the antibody-coated worms (Ronald, 2004).

Our study of splenic lymphocyte proliferation stimulated by mitogen and specific Ag, the infected mice had significant depression of the cellular response to con A and somatic Ag. The depression was more severe with prolonged and heavy infection; suggest that the Th-1 cell response is defective .These results could reinforce with the high level of above-mentioned IgE antibody.

A previous evidence of human gnathostomiasis demonstrated that involvement of Th-2 response might be existing. Since, anti-*G.spinigerum* L3s IgG1-Ab and IgG4-Ab provided the highest Ab level, while IgG2-Ab had the lowest Ab level (Nuchprayoon et al., 2003). Human IgG1 and IgG4 antibodies is in general the reflect of the induction of Th-2 responses, whereas IgG2-Ab is that of an induction of Th-1 responses (Else and Grencis, 1991).

Furthermore, our experiment could show an increase of IgE-Ab which reached a plateau on Day 90 PI. Interestingly, this ELISA level had come to the peak before the other isotypes. This can indicate that IgE-Ab response might be valuable for laboratory diagnosis and used as an evaluation of the treatment efficacy.

Since *G.spinigerum* does not reach maturity in man. After the advanced L3s being ingested, these larvae will migrate through the intestinal wall to several tissues. The significant high levels of IgE antibody in patients were reported in a number of studies

(Soesatyo et al., 1987; Saksirisampant et al., 2001). In addition, a significant decre his IgE-Ab level had been demonstrated within 3 months of post albendazole treated patients (Saksirisampant, unpublished observations). However, a study of IgE-Ab responses from post ivermectin treatment of both infected human and animals have never been reported yet.

The high level of IgE-Ab is the involvement of the Th-2 response in many parasitic infections. Since, Th2 cells can secrete IL-4, cytokines essential for IgE production (Romagnani S, 1991).

Many previous studies showed that many parasitic infections including *Trichuris trichiura*, *Enterobius vermicularis*, *Ascaris lumbricoides*, *Strongyloides stercolaris* and *Brugia malayi* could induce a concomitant increase of the IgE and IgG1 serum levels and usually had a decrease of the IgG2a level. This was due to the induction of IL-4 to the B cells to secrete both IgG1 and IgE (Romagnani, 1991; Mahant et al., 1992 and Yazdanbakhsh M et al., 1993).

We hypothesize that the immune response observed in this mouse gnathostomiasis may involve in Th-2 response, which was supported by the following reasons:

1) A higher level of IgE-Ab could be demonstrated as well. In addition, blood eosinophilia showed significantly higher in the infected group. The higher degree of infection (10 larvae) had higher percentage of blood eosinophil than the lower degree of infection (5 larvae) (Saksirisampant, personal communication).

2) The depressed lymphoproliferative response to mitogen and specifi en stimulations (Table 5 and 6) suggested that the Th-1 cell response is defective.

The antibody response in mouse gnathostomiasis of this study may lead into an application for immunodiagnosis. IgE-Ab might be used for laboratory diagnosis.

In further studies, detailed mechanism of the T-cell subpopulation, which involves in the Th-2 response of this mouse-gnathostomiasis system, should be elucidated, particularly its cytokines production such as IL-2, IL-4 and IL-5. In addition, purification of the potential protein component of the advanced L3s somatic Ag or this Ag used is another important point to study. Because, highly complicated antigenic made up of this antigen had been demonstrated with more than 20 bands in SDS-PAGE (Priwan, 1985). There was only few bands showed specific reaction with serum antibody in Western blot. Cross-reaction with other parasitic infection including angiostrongyliasis (Suntharasamai et al., 1985; Tuntipopipat et al., 1989), opisthorchiasis (Malinee, 1989), paragonimiasis, taeniasis and hook-worm infection (Welch et al., 1980; Tapchaisri et al., 1991; Saksirisampant et al., 2001) was also demonstrated. The protein band of 24 kDa showed specific reaction with

IgG-Ab, while bands of 43 and 49 kDa showed specific with IgE-Ab (Nopparatana et al, 1991; Tapchaisri et al., 1991; Saksirisampant, personal communication). Specific protein band(S) of IgE-Ab, in acute and chronic infected sera and those in post-antihelminthic treated sera should be another further study as well.

2.2 Western blotting analysis

This preliminaly study has demonstrated a munber of specific bands of L3s *G.spinigerum* somatic antigen which consistently reacted with sera from gnathostomiasis mice but not with those from normal sera. This study confirmed our report that the somatic antigen was highly complex and consisted of more antigenic make up than those of ES antigen as revealed by SDS-PAGE and Western blot analysis.When compared to infected human sera, their pattern were quite similar to the previous study in which radioiodinated protein A was used and which Western blot technique used. However, our study could clearly demonstreted more than one specific bands which were 20, 24, 37, 39, 50, 87 and 125 kd not only one band of 24 kd as previous reported by Tapchaisri et al.Our further work is study that which is the most potential diagnostic band. Since many helminthic infection has persisting antibody, but if any band could firstly decrease or disappear will be the effective tool of the evaluating the chemotherapy.Inaddition, our ES antigen or the glandular secretions products are now currently being considered.The Es products are usually simple mixtures of relatively few components. This study showed that four components were strongly recognized by IgE antisera of gnathostoma animals. Of these, two antigenic bands of 34 and 50 kd gave consistent reaction.When compared to IgG antibody, IgE may has more potential detection than IgG with ES products.However, which of those could be used for the evaluation of the effectiveness of chemotherapy should be further studied.

3. Cellular immune status of mice infected with *G.spinigerum*

With regard to the Con A stimulation response, it was found that the degree depressed lymphoproliferative response varied with both the intensity and the duration of infection. The decreased response to Con A stimulation was more obvious in animals with high dose of *G.spinigerum* and in those with longer duration of infection (Day 180 PI).

With regard to antigen stimulation as expected, positive lymphoproliferation could be demonstrated in the infected group when compared to the normal control mice. However, a trend of decreased response to antigen stimulation was observed in the chronic infected

group (Day 180 PI, both 10 and 20 larvae) compared to other groups, although it was not statistical significant.

These decreased responses were not due to aging of the animals because the data were comparable to the control animals of the same age group. In fact, the result presented in Table 5 and 6 showed that the lymphoproliferative responses of uninfected normal splenic lymphocytes taken from young (1-3 months) and old mouse (7-8 months) were not different. Thus, the data in this experiment suggest that, the immunodepression of infected mice is associated with the chronicity and heavy infection of *G.spinigerum*.

Immunosuppressions can be demonstrated in a number of parasitic infections eg. *Trichinella spiralis* (Faubert, 1982; Barriga, 1978), *Onchocerca volvulus* (Soboslay et al., 1999), *Toxocara canis* (Allen et al., 1998) and *Taenia crassiceps* (Sciutto et al., 1995) etc. These phenomenons are reversible. Therefore we are interested in studying the reversible effect in our *G.spinigerum*-mouse system. In this study, the animals were treated of infection by ivermectin. Interestingly, the lymphoproliferative response to both Con A and antigen-stimulation was significant increased, particularly at experiment on Day 180 following treatment with ivermectin. Although ivermectin is a new drug, our worm recovery study was performed to confirm the effectiveness of this drug.

In this experiment, the reversal of immunodepression to Con A and somatic antigen in *G.spinigerum*-infected mice was demonstrated after the parasites were removed. Although the direct effects of ivermectin on lymphocyte activity is not known. However, the residual effect of drug is highly unlikely, because the drug is rapidly metabolized in the liver. The apparent plasma half-life of ivermectin is approximately at least 16 hours following oral administration. Nevertheless, data of the uninfected animals receiving the same used-dose of ivermectin in the experiment tested 6 months later for lymphoprolifertive response were not altered when compared to the normal control mice (data not shown). Since, con A stimulation index of ivermectin treated mice was of the same order of magnitude as that of controls. In this experiment, it was found that worm recovery was began to decrease statistically significant at Day 180 PI (53-65%). In summary, the immunodepression in these infected animals was abolished by appropriated antihelmintic treatment, suggesting that the immunodepressive effect can be reversible and is associated with active infection.

We hypothesize that the immunodepression observed with this infection is one mechanism that the parasite used to evade host defence. For this nematode, the infected mice are normally not able to eliminate the parasites and appear to have no resistance to

reinfection. It was of interest to further of characterize the mechanism of immunodepression in *G.spinigerum*. There are various possibilities as discussed below.

The depressed lymphoproliferative response to Con A and somatic antigen stimulation suggest that the T-cell response is defective. First, it is possible that the suppression may be due to a decrease in the proportion of responsive T cell, or to a reduced response of individual cells.

Second, it is possible that some antigen from the parasite may block directly at lymphocyte receptor. It was found that the excretory and secretory products of *Fasciola hepatica* had been reported to be cytotoxic for rat lymphocytes. They may protect the parasite from it's host immune defenses (Goose, 1978). However, there are no previous evidence that any of the *G.spinigerum* antigens is cytotoxic for the mice lymphocytes or accessory cells required for efficient immune response, which should be investigated in the future.

Third, it is possible that the suppression is due to defects in interleukin-2 (IL-2) production. Decreased lymphocyte proliferation and IL-2 production have been reported in animals infected with various parasites, *Taenia crassiceps*, *Onchocerca volvulus*, *Toxoplasma gondii* and *Trypanosoma cruzi*. It was found that diminished IL-2 production during the infection with *Teania crassiceps*, may be a part for the diminished responses to Con A. The suppressed Con A response observed in Teania infected could be attributed at least in part to defects in IL-2 production. Since, the additions of recombinant IL-2 to cell cultures partially restore responsiveness. However, it remains to be explored if these defects related to IL-2 receptor, which may play a role in these processes (Sciutto et al., 1995). Similar results were seen with the suppressive effect of *Onchocerca volvulus* secretion/excretion (S/E) that can be overcome by addition of exogenous IL-2. Those results suggest that deficiency in IL-2 production rather than down-regulation of IL-2 receptor is responsible for inhibition of lymphocyte proliferative responses (M.Y.Elkhalifa et al., 1991).

Fourth, it is possible that relationship of Th2 responses and immune suppression of multicellular organisms might lead to profound down-regulation of host immune responses. Generation of suppressive cells which is dominated by a Th2-type profile, are well-established features of human filarial infection. The induction of host IL-4 is characteristic of infection which is required for the generation of suppressive cells. Peripheral blood lymphocytes from people with active infection do not proliferation in response to parasite antigen but produce antigen-specific IL-4 (Allen et al., 1996).

In conclusion, the results indicate that mice experimentally infected with *G.spinigerum* had significant depression of the cellular response to Con A and somatic L3s antigen. The depression was more severe with prolonged and heavy infection. The immunodepression in these animals was abolished by antihelmintic treatment, suggesting that the immunodepressive effect is reversible and is associated with active infection. The elimination of immunodepression resulted in a significantly lower number of encyted muscle larvae. There are many possible mechanisms, which could explain immunodepression in the parasite which should be further studied. Exploration of the mechanism by which *G.spinigerum* induces depression of lymphocyte functions could help not only to further the knowledge of immune mechanisms that participate in this host-parasite relationship but also to advance understand of the regulatory events governing lymphocyte activation.

4. Histological section

The results of autopsy and histological section could demonstrated that all the advanced third stage larvae (AdL3, obtained from the natural infected eels) given orally to each mouse remained in the stomach for 1 hr post–infection (PI). At 3, 5,7,10 and 24 hr PI, the larvae transiently penetrate the stomach wall and invaded various organs including mesentery, esophagus, liver, diaphragm, lung, heart and dorsal fat. Approximately, 10%-20% of the larvae moved into the muscle and the subcutaneous tissue within 5-10 days while the remainders were still in the visceral organs. A few larvae were found in the brain (day 40 PI). Encystment began at day11 PI and after that it increased gradually up to 95% at day 21 PI.

The larvae are unable to develop to adulthood because mouse is not the definitive host of G.*spinigerum*. Mouse is the accidental host as do in human. We may use this study of larva migration to speculate the route in patients. Yingyourd et al (1985) reported that all AdL3 of *G. spinigerum* must firstly invade liver, stay there for about 7 days and then move further into the muscle of mice. However, not all AdL3 of our experiment invade liver of the mice. After penetrating the stomach wall, some were able to migrate to other visceral organs including diaphragm, lung, heart and esophagus within 5 hr PI. The AdL3 of *G. hispidum* and *G. nipponicum* did not always invade the liver of murine animals as well as the larvae in our study did (Takakura 1988; Ando et al. 1994). Our results suggested that larvae passed through the digestive organs to the abdominal cavity, some invaded liver, some did not but could invade transiently various organs, and finally move into the muscle within 5 day PI. Encystment began at day 21 PI and completely encapsulated on day 21 PI

which was ealier than in rat model (day 100 PI) as reported by Ando et al (1992) and in rabbit (day 35 PI) as reported by Anantaphruti et al. (1986). With regard to parasite migration in human, the parasites can migrate to various organs and resulted in subcutaneous migratory swelling with about 51 % as reported by Daengsvang (1980). Recurring swelling in human developed randomly, but was reported mainly in the upper and lower extremities. (Rojas-Molina et al. 1999). Location of worm recovered in muscle of the infected mice in our study mostly were in the abdominal muscle and in those lower and upper limbs more than other organ muscle. The larvae began to be found in the subcutaneous at day 14 PI but could not increase when the infection was prolong. During 1-5 month, worm recovery in subcutaneous tissue had approximately 2.25 %. However, this location of larvae found were those harmonious with high frequency of upper and lower limbs subcutaneous migratory swelling in human gnathostomiasis (Daengsvang 1986 ; Kraivichian 1990, Rojas-Molina et al. 1999). But it still could not make a precission of how many worms could be recovered from each patient and what percentage exist in the visceral organ. Since very few (1-3 larvae) could be found each time in individual patient and re-infection within one year was infrequent reported.

This preliminary study of histological section showed that the infiltrated immune cell were mostly eosinophil in Day 14 post infection . The mononuclear cell participated on Day 30 onward.Further study should be carried on to clarify the cellular immune status by using immuno-histochemical techniques.

REFERENCES

Abbas AK and Lichtman AH. Antibodis. Cellular and Molecular Immunology. 5th edition Saunder 2003: 43-9.

Allen JE, Maizels RM. Immunology of human helminth infection. Int Arch Allergy Immunol. 1996 Jan;109(1):3-10.

Akahane H, Lamothe-Argumedo R, Martinez-Cruz JM, Osorio-Sarabia D, Garcia-Prient L. A morphological observation of the advanced third stage larvae of Mexican Gnathostoma. Jpn J Parasitol 1994;.43:.18-22.

Almeyda-Artigas RJ. Finding of *Gnathostoma binucleatum* n sp (Nematoda: Spirurida) in wild felids and the role of fresh-water and oligohaline fishes as human gnathostomiasis vectors in the lower basin of Papaloapan river, Oaxaca-Veracruz, Mexico. An Inst Cienc Mar Limnol Univ Nal Auton Mexico 1991; 18: 137-55.

Anantaphruti MT. Demonstration of species-specific antigens of *Gnathostoma spinigerum*, a preliminary attempt. Southeast Asian J Trop Med Public Health 1989; 20: 305-12.

Anantaphruti MT. Immunodiagnosis of Gnathostomiasis. J Trop Med Parasitol 2002; 25: 83-90.

Anantaphruti MT, Nuamtanong S, Waikagul J. Effect of ivermectin on experimental gnathostomiasis in rabbits. Trop Med Parasitol 1992; 43: 56-7.

Anantaphruti MT, Waikagul J, Nithi-Uthai S, Pubampen S, Rojekittikhun W. Detection of humoral immune response to *Gnathostoma spinigerum* in mice. Southeast Asian J Trop Med Public Health 1986; 17: 172-6.

Anderson SE Jr, Krahenbuhl JL and Remington JS. Longitudinal studies of lymphocyte response to Toxoplasma antigen in humans infected with *T. gondii*. J. Clin. Lab. Immunol 1979 Nov; 2(4): 293-7.

Ando T. A study of *Gnathostoma spinigerum*. (Japan with English abstract) Acta Medical 1957;27: 2342-59.

Attallah AM, Smith AH, Murrell KD, Fleischer T, Woody J, Vannier WE, Scher I, Ahmed A, Sell KW. Characterization of the immunosuppressive state during *Schistosoma mansoni* infection. J Immunol. 1979 Apr; 122(4): 1413-20.

Awadzi K, Dadzie K.Y, Schulz-Key H, Gilles H.M, Fulford A.J, Aziz M.A. The chemotherapy of onchocerciasis XI A double-blind comparative study of ivermectin,

diethylcarbamazine and placebo in human onchocerciasis in Northern Ghana. Ann Trop Med Parasitol 1986; 80: 433-42.

Barriga OO. Depression of cell-mediated immunity following inoculation of Trichinella spiralis extract in the mouse. Immunology. 1978 Jan;34(1):167-73.

Beltz, L. A., M. B. Sztein, and F. Kierszenbaum. Novel mechanism for *Trypanosoma cruzi*-induced suppression of human lymphocytes. Inhibition of IL-2 receptor expression. J. Immunol 1988; 141: 289-94.

Blair, L. S., and W. C. Campbell. The rat (Rattus norvegicus) as laboratory host for the metacestode of *Taenia crassiceps*. J. Parasitol 1976; 62: 163-4.

Bodhidat P, Punyagupta S. Intracranial gnathostomiasis: A case report. Roy Thai Army M J 1956; 9: 278-87.

Bunnag T, Cosmer DS, Punyagupta S. Eosinophilic myeloencephalitis caused by *Gnathostoma spinigerum*: neuropathology of nine cases. J Neurol Sci 1970; 10: 419-34.

Boongird P, Phuapradit P, Siridej N, Chirachariyavej T, Chuahirun S, Vejjajiva A. Neulogical manifestations of gnathostomiasis. J Neurol Sci 1977;31:279-91.

Camacho SP, Ramos MZ, Torrecillas EP, Ramirez IO, Valazquez RC, Gaxiola AF. Clinical manifestations and immunodiagnosis of gnathostomiasis in Culiacan, Mexico. Am J Trop Med Hyg 1998; 5: 908-15.

Campbell WC. Ivermectin: a potent new antiparasite agent. Science 1983; 25: 823-8.

Campbell WC. Ivermectin: an update. Parasitol Today 1985; 1: 10-6.

Capron A, Capron M, Grangette C and Dessaint JP. IgE and inflammatory cells. Ciba Found Symp 1989; 147: 153-60.

Capron M, Spiegelberg HL, Prin L, Bennich H, Butterworth AE, Pierce RJ, Ouaissi MA and Capron A. Role of IgE receptors in effector function of human eosinophils. J Immunol. 1984 Jan;132(1):462-8.

Chanthchume N, Kulkhumthorn M, JongwongwutiwesS. Evaluation of skin test for human gnathostomiasis. Chula Med J 1998;32:243-8.

Chhuon H, Sangkim K, Voeunthal Y. Mobile edema of the face and neck followed by intraocular localization of Gnastosoma spinigerum. Bull Soc Pathol Exot Filiales. 1976 Jul-Aug;69(4):347-51.

Chitanondh H, Rosen L. Fatal eosinophilic encephalomyelitis caused by the nematode *Gnathostoma spinigerum*. Am J Trop Med Hyg 1967;16:638-45.

Chitchang S. The efficacy of albendazole in the treatment of human gnathostomiasis. Journal of Postgraduate Medicine 1987; 5: 39-43.

Coffman RL, Carty J. A T cell activity that enhances polyclonal IgE production and its inhibition by interferon-gamma. J Immunol. 1986 Feb 1;136(3): 949-54.

Continho AD, Dreyer G, Mediros Z. Ivermectin treatment of Bancroftian filariasis in Recife, Brasil. Am J Trop Med Hyg 1994; 50: 339-48.

Cooper ES, Spencer J., Whyte-Alleng CAM. Immidiate hypersensitivity in colon of chidren with chronic Trichuris trichiura dysentery. The Lancet 1991; 338: 1104-7.

Daengsvang S. A monograph on the genus Gnathostoma and gnathostomiasis in Thailand. SEAMIC Southeast Asian Medical Information Center. Tokyo, Japan 1980: 85.

Daengsvang S. Gnathostomiasis in Southeast Asia. Southeast Asian Journal of Tropical Medicine and Public Health. 1981 Sep;12(3): 319-332.

Daengsvang S. The world's first = story of *Gnathostoma spinigerum* Infection in the Young Thai Woman. Journal of Parasitology and Tropical Medicine Association of Thailand. 1983; 6: 1-10.

Daengsvang S. *Gnathostoma spinigerum* and human gnathostomiasis. A review. in: Sucharit's eds. The 25th Anniversary of the Faculty of the tropical Medicine, Mahidol University. Bangkok : Krung siam Press 1986: 124-47.

Dharmkrong-at A, Migasena S, Suntharasamai P, Bunnag D, Priwan R, Sirisinha S. Enzyme-linked immunosorbent assay for detection of antibody to Gnathostoma antigen in patients with intermittent cutaneous migratory swelling. J Clin Microbiol 1986; 23: 847-51.

Duke BO, Zea-Flores G, Castro J. Effects of multiple month doses of ivermectin on adult *Onchocerca volvulus*. Am J Trop Med Hyg 1990; 43: 657-64.

Dunne DW, Butterworth AE, Fulford AJC, Kariuki HC. Immunity after treatment of human schistosomiasis: association between IgE antibodies to adult worm antigens and resistance to reinfection. European Journal of Immunology 1992; 22: 1483-94.

Edda Sciutto, Gladis Fragoso, Mercedes Baca, Vera De la Cruz, Lorena Lemus, and Edmundo Lamoyi. Depressed T-Cell Proliferation Associated with Susceptibility to Experimental *Taenia crassiceps* Infection. Infection and Immunity 1995; June: 2277-81.

Edward KM, David TJ, Wojciech AK. *Gnathostoma spinigerum*. In Markell and Voge's eds, Medical Parasitology 8th ed. London : W.B.Saunders company, 1999; 35: 1-2.

Egashira M. Intracutaneous reaction in the human gnathostomiasis. II. A study on the diagnosis for human gnathostomiasis. Acta Med 1953; 23: 114-22 [Japanese, English abstract].

Elkhalifa MY, Ghalib HW, Dafa'Alla T, Williams JF. Suppression of human lymphocyte responses to specific and non-specific stimuli in human onchocerciasis. Clin Exp Immunol. 1991 Dec;86(3):433-9.

Else KJ, Grencis RK. Cellular immune responses to the murine nematode parasite Trichuris muris. I. Differential cytokine production during acute or chronic infection. Immunology. 1991 Apr;72(4): 508-13.

Encarnacion CF, Giordano MF, Murray HW. Onchocerciasis in New York City: the Moa-Manhattan connection. Arch Intern Med 1994; 154: 1749-51.

Faubert G. M. The reversal of the immunodepression phenomenon in trichinellosis and its effect on the life cycle of the parasite. Parasite Immunology 1982; 4: 13-20.

Freeman, R. S. Studies on the biology of *Taenia crassiceps* (Zeder, 1800) Rudolphi, 1810 (Cestoda) Can. J. Zool 1962; 40: 969-90.

Furuno O. An immunological study on gnathostomiasis. Acta Med Jap 1959; 29: 2802-22 [Japanese, English abstract].

Goa KL, Mctavish D, Clissold LP. Ivermectin: a review of its antifilarial activity, pharmacokinetic properties and clinical efficacy in onchocerciasis. Drugs 1991; 42: 640-52.

Goligen JE, Shevach AM. Animal health and husbandry. In: Current Protocols in immunology, vol 1, 1^{sd}ed. New York: John Viley and son, eds 1994: 1.1.1-1.8.3.

Good, A. H., and K. L. Miller. Depression of the immune response to sheep erythrocytes in mice infected with *Taenia crassiceps* larvae. Infect. Immun 1976; 14: 449-56.

Goose J. Possible role of excretory/secretory products in evasion of host defenses by *Fasciola hepatica*. Nature. 1978 Sep 21; 275(5677): 216-7.

Greene BM, Brown KR, Taylor HR. Use of ivermectin in humans. Campbell WC, ed. Ivermectin and Abamectin. New Yord: Springer-Verlag 1989: 311-23.

Greene BM, Taylor HR, Cupp EW, Murphy RP, White AT, Aziz MA, Schulz-Key H, D'Anna SA, Newland HS, Goldschmidt LP. Comparison of ivermectin and diethylcarbamazine in the treatment of onchocerciasis. N Engl J Med 1985; 313: 133-8.

Grogan JL, Kremsner PG, Deelder AM, Yazdanbakhsh M. Elevated proliferation and interleukin-4 release from CD4+ cells after chemotherapy in human *Schistosoma haematobium* infection. European Journal of Immunology 1996; 26: 1365-70.

Hagan P, Blumenthal UJ, Dunn D, Simpson AJG, Wilkins HA. Human IgE, IgG4 and resistance to reinfection with *Schistosoma haematobium*. Nature 1991; 349: 243-5.

Inkatanuvat S, Suntharasamai P, Vutikus S, Rigunti M. Change of liver function after albendazole treatment in human gnathostomiasis. J Med Assoc Thai 1998; 81: 735-9.

Isakson PC, Pure E, Vitetta ES, Krammer PH. T cell-derived B cell differentiation factor(s). Effect on the isotype switch of murine B cells. J Exp Med. 1982 Mar 1;155(3): 734-48.

Jarrett EEE, Haig DM and Bazin H. Time course studies on rat IgE production in *N.brasiliensis* infection. Clinical and Experimental Immunology 1976; 24: 346-51.

Kalra R., Singh S. P., Boroujerdi S. R., Langley R. J., Blackwell W. B., Henderson R. F. and Sopori M. L. Subclinical Doses of the Nerve Gas Sarin Impair T Cell Responses through the Autonomic Nervous System. Toxicology and Applied Phamacology 2002; 184: 82-7.

Kamiya H, Kamiya M, Ohbayashi M, Klongkamnuankarn K, Vajrasthira S. *Gnathostoma malaysiae* Miyazaki and Dunn, 1965 from Rattus surifer in Thailand. Southeast Asian J Trop Med Public Health. 1987 Mar; 18(1): 121-6.

Kasemsuth R, Panut-Ampon P, Sanghirun C. Study on the diagnosis of Gnathostoma infection in cats by radioimmunoassay. Southeast Asian J Trop Med Public Health 1981; 12: 410.

Kittiponghansa S, Prabriputaloong A, Pariyanonda S, Ritch R. Intracameral gnathostomiasis: a cause of anterior uveitis and secondary glaucoma. Br J Opthamol 1987; 71: 618-22.

Krahenbuhl JL, Humphres RC, Henika PC. Effects of Propionibacterium acnes treatment on the course of *Mycobacterium leprae* infection in mice. Infect Immun. 1982 Jul; 37(1): 183-8.

Kraivichian K, Nuchprayoon S, Sitichalernchai P, Chaicumpa W, Yentakam S. Treatment of cutaneous gnathostomiasis with ivermectin. Am J Trop Med Hyg 2004 Nov; 71(5):623-8.

Kraivichian P, Kulkumthorn M, Yingyourd P, Akarabovorn P, Paireepai CC. Albendazole for the treatment of human gnathostomiasis. Trans R Soc Trop Med Hyg 1992; 86: 418-21.

Kraivichian P, Yingyourd P, Kulkumthorn M. Experimental treatment of *Gnathostoma spinigerum* infection in cats with albendazole. Chulalongkorn Medical Journal 1989; 33: 43.

Kurniawan-Atmadja A, Atkinson B, Sartono E, Partono E, Yazdanbakhsh M, Maizels RM. Differential decline in filaria-specific IgG1, IgG4 and IgE antibodies in *Brugia malayi-*

infected patients after Diethylcarbamazine chemotherapy. Journal of Infectious Diseases 1995; 172: 1567-72.

Lertanekawattana S, Boonsit O, Anantaphruti M. Evaluation of enzyme-linked immunosorbent assay in the diagnosis of gnathostomiasis in northeastern Thailand. J Health Sci 1991; 3: 235-41.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193: 265-75.

Luft BJ, Kansas G, Engleman EG and Remington JS. Functional and quantitative alterations in T lymphocyte subpopulations in acute toxoplasmosis. J. Infect. Dis. 1984 Nov; 150(5): 761-7.

Macario A.J, Stahl W and Miller R. Lymphocyte subpopulations and function in chronic murine toxoplasmosis. II. Cyclic immunosuppression in genetic-low-responder mice. Cell Immunol. 1980 Nov; 56(1): 235-9.

Mahanty S, Abrams JS, King CL, Limaye AP, Nutman TB. Parallel regulation of IL-4 and IL-5 in human helminth infections. Journal of Immunology 1992; 148:3567-71.

Maleewong W, Morakote N, Thamasonthi W, Charuchinda K, Tesana S, Khamboonruang C. Serodiagnosis of human gnathostomiasis. Southeast Asian J Trop Med Public Health 1988; 19: 201-5.

Maleewong W, Wongkham C, Intapan P, Mahaisavariya P, Daenseegaew W, Pipitgool V, Morakote N. Detection of circulating parasite antigens in murine gnathostomiasis by a two-site enzyme linked immunosorbent assay. American Journal of Tropical Medicine and Hygiene 1992; 46: 80-4.

Malinee TA. ELISA for diagnosis of gnathostomiasis using antigens from *Gnathostoma doloresi* and *G.spinigerum*. Southeast Asian J Trop Med Pub Hlth. 1989; 20: 297-304.

Marti H, Haji HJ, Savioli L. Comparative trial of a single-dose of ivermectin versus three days of albendazole for treatment of *Strongyloides stercoralis* and other soil-transmitted helminth infections in children. Am J Trop Med Hyg 1996; 55: 477-81.

Miyasaki I. On the genus Gnathostoma and human gnathostomiasis, with special reference to Japan. Exp Parasitol 1960;9:338-70.

Miyazaki I. Gnathostoma and gnathostomiasis in Japan. In: Morishita K, Komiya Y, Matsubayashi H, Editors. Progress of medical parasitology in Japan. Tokyo: Meguro Parasitological Museum 1966; 3: 531-86.

Moorhouse DE, Bhaibulaya M, Jones HI. Suspected human gnathostomiasis in Queensland. Med J Aust. 1970 Aug 1;2(5): 250.

Morakote N, Maleewong W, Sukhavat K, Thamasonthi W, Charchinda K, Khamboonruang C. Serological study of experimental larval gnathostomiasis in mice. J Med Tech Assoc Thai 1987; 15: 19-23.

Morakote N, Nateewatana N, Maleewong W, Uthong T. Anatomical localization of *Gnathostoma spinigerum* larval antigens by an indirect fluorescent antibody test. Southeast Asian J Trop Med Public Health 1989; 20: 291-5.

Morakote N, Navacharoen W, Maleewong W, Sukhavat K. Comparison of larval and adult worm antigens in ELISA for serodiagnosis of human gnathostomiasis. J Trop Med Parasitol 1991; 14: 9-13.

Morishita KOR. A pig nematode *Gnathostoma hispidum* Fedtschenko, as a human parasite. Ann Trop Med Parasit 1924;18:23.

Morita H, Segawa T, Nishiyama T, Yamada S, Yagi J, Chin I. Gnathostomiasis cases caused by imported loaches. J Nara Med Ass 1984; 35: 607-19 [Japanese, English abstract].

Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 1986 Apr 1;136(7): 2348-57.

Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol. 1989; 7: 145-73.

Nakao M, Mizoguchi Y, Monna T, Yamamoto S, Morisawa S. Studies on an inhibitory factor to phytohemagglutinin-induced lymphocyte transformation found in the serum of patients with various liver diseases. Acta Hepatogastroenterol (Stuttg). 1978 Oct; 25(5): 335-43.

Nakayama H, Mihara M, Tanaka K. Cutaneous gnathostomiasis: a case report of creeping disease. Rinsho Derma 1981; 23: 673-7.

Naquira C, Jimenez G, Guerra J.G, Bernal R, Nalin D.R, Neu D, Aziz M. Ivermectin for human strongyloidiasis and other intestinal helminths. Am J Trop Med Hyg 1989; 40: 304-9.

Nawa Y. Historical review and current status of gnathostomiasis in Asia. Southeast Asian Journal of Tropical Medicine and Public Health. 1991 ;22: 217-9.

Nitidandhaprabhas P, Sirikarna A, Harnsomburana K, Thepsitthar P. Human urinary gnathostomiasis: A case report from Thailand. Am J Trop Med Hyg 1975; 24-9.

Nontasut P, Bussaratid V, Chullawichit S, Charoensook N, Visetsuk K. Comparison of ivermectin and albendazole treatment for gnathostomiasis. Southeast Asian J Trop Med Public Health 2000; 31: 374-7.

Nopparatana C, Chaicumpa W, Tapchaisri P, Setasuban P, Rungkunaporn Y. Towards a suitable antigen for diagnosis of *Gnathostoma spinigerum* infection. Int J Parasitol 1992; 22: 1151-6.

Nopparatana C, Setasuban P, Chaicumpa W, Tapchaisri P. Purification of *Gnathostoma spinigerum* specific antigen and immunology of human gnathostomiasis. 1991; 21: 677-87.

Nopparatana C, Tapchaisri P, Setasuban P, Chaicumpa W, Dekumyoy P. Antibody responses in human gnathostomiasis. Southeast Asian J Trop Med Public Health 1988; 19: 219-24.

Nuamtanong S, Waikagul J, Anantaphruti MT. Gnathostome infection in swamp eels, *Fluta alba*, in Central Thailand. Asian Journal of Tropical Medicine and Public Health. 1998 ;29: 144-7.

Nuchprayoon S, Sanprasert V, Suntravat M, Kraivichian K, Saksirisampant W, Nuchprayoon I. Study of specific IgG subclass antibodies for diagnosis of *Gnathostoma spinigerum*. Parasitol Res 2003; 91: 137-43.

Ogata K, Nawa Y, Akahane H, Diaz Camacho SP, Lamothe-Argumedo R, Cruz-Reyes A.Short report: gnathostomiasis in Mexico. Am J Trop Med Hyg. 1998 Mar; 58(3): 316-8.

Ollague W, Ollague J, Guevara de Veliz A, Penaherrera S. Human gnathostomiasis in Ecuador (nodular migratory eosinophilic panniculitis). First finding of the parasite in South America. Int J Dermatol. 1984 Dec;23(10):647-51.

Ottesen EA. Efficacy of diethylcarbamazine in eradicating infection with lymphatic-dwelling filariae in humans. Rev Infect Dis. 1985 May-Jun; 7(3): 341-56.

O'Keefe SJ, El-Zayadi AR, Carraher TE, Davis M, Williams R. Malnutrition and immuno-incompetence in patients with liver disease. Lancet. 1980 Sep 20; 2(8195 pt 1): 615-7.

Ottesen EA, Skvaril F, Tripathy SP, Poindexter RW, Hussain R. Prominence of IgG4 in the IgG antibody response to human filariasis. J Immunol 1985 Apr;134(4): 2707-12.

Pier B Gerald, Jeffrey BL and Lee MW Immunology, Infection and Immunity. Antibody-Antigen Intereactions and Measurements of Immunologic Reaction. 2004 ASM Press

Prijyamonda B, Pradatsundarasar A, Viranuvatti V. Pulmonary gnathostomiasis. A case report. Ann Trop Med Parasitol 1955; 49: 121-2.

Priwan R. Humoral immune response in mice infected with *Gnathostoma spinigerum*. M Ed thesis. Faculty of science, Srinakharinwirot University, 1985

Prommas C, Daengsvang S. Preliminary report of a study on the life cycle of *Gnathostoma spinigerum*. J Parasit 1933;19:287.

Punyagupta S, Juttijudata P, Bunnag T, Comer D.S. Two fatal cases of eosinophilic myeloencephalitis a newly recognized disease caused by *Gnathostoma spinigerum*. Transaction of Royal Society of Tropical medicine and Hygeine 1968; 62: 801.

Punyagupta S. Clinical manifestations of eosinophilic meningitis. Southeast Asian J Trop Med Public Health 1978; 9: 278.

Radomyos P, Daengsvang S. A brief report on *Gnathostoma spinigerum* specimens obtained from human cases. Southeast Asian Journal of Tropical Medicine and Public Health. 1987 ;18: 215-7.

Rattanasiriwilai W, Chanthachume K, Kulkumthorn M, Yingyourd P. Studies on production of excretory-secretory products from 3rd stage larvae of *Gnathostoma spinigerum* by an in vitro cultivation. Chula Med J 1985; 29: 1115-20.

Roma Kalra, Shashi P.Singh, Seddigheh Razani-Boroujerdi, Raymond J.Langley, Walter B.Blackwell, Rogene F.Henderson, and Mohan L.Sopori. Subclinical Doses of the Nerve Gas Sarin Impair T cell Responses through the Autonomic Nervous system. Toxicology and Applied Pharmacology 2002; 184: 82-7.

Romagnani S. Regulation and deregulation of human IgE synthesis. Immunol Today. 1990 Sep;11(9): 316-21.

Romagnani S. Human Th1 and Th2 subsets: doubt no more. Immunology Today 1991; 12: 256-7.

Ronald BC Antibodies. Immunology, Infection and Immunity. Edited by Pier GB, 2004 ASM Press

Rojekittikhun W, Nuamtanong S, Pubampen S, Setasuban P. Circumoral and larval microprecipitation reactions in experimental and human gnathostomiasis. Southeast Asian J Trop Med Public Health 1989; 20: 81-6.

Rojekittikhun W, Chaiyasith T, Nuamtanong S, Pubampen S, Maipanich W, Yaemput S. Current status of Gnathostoma infection in Nakhon Nayok and Prachin Buri. Southeast Asian J Trop Med Public Health. 2002;33(3): 474-84.

Rojekittikhun W. Current status of Gnathostoma infection in Thailand. J Trop Med Parasitol 2002; 25: 47-52.

Rojekittikhun W. On the Biology of *Gnathostoma spinigerum*. J Trop Med Parasitol 2002; 25: 91-8.

Rusnak J.M, Lucey D.R. Clinical gnathostomiasis: case report and review of the English language literature. Clin Infect Dis 1993; 16: 33-50.

Saksirisampant W, Kulkaew K, Nuchprayoon S, Yentakham S, Wiwanitkit V. Ann Trop Med Parasitol. 2002 Mar; 96(2): 191-5.

Saksirisampant W, Chawengkiattikul R, Kraivichian K, Nuchprayoon S. Specific IgE antibody responses to somatic and excretory-secretory antigens of third stage *G.spinigerum* larvae in human gnathostomiasis. J Med Assoc Thai 2001; 84:173-81.

Sciutto E., Fragoso G., Baca M., de la Cruz V., Lemus L. and Lamoyi E. Depressed T-Cell Proliferation Associated with Susceptibility to Experimental *Taenia crassiceps* Infection. Infection and Immunity 1995; 63: 2277-81.

Setasuban P, Chiamratana B, Muennoo C. The effect of praziquantel, levamisol and fansidar against gnathostomiasis in hamsters. Southeast Asian J Trop Med Hyg Pub Hlth 1981; 12: 454.

Setasuban P, Nuamtanong S, Rojanakittikoon V, Yaemput S, Dekumyoy P, Akahane H. Gnathostomiasis in Thailand : a survey on intermediate hosts of *Gnathostoma spp*. With special reference to a new type of larvae found in *Fluta alba*. Southeast Asian J Trop Med Public Health 1991; 22: 220-4.

Shakib F and Stanworth DR. Human IgG subclasses in health and disease, part II. La Ricerca Clin Lab 1980; 10: 561.

Sirisinha S, Tuti S, Tawatsin A and Rattanasiriwilai W. Lack of evidence for protective immunity in hamster infected with *Opisthorchis viverrini*. In: Immunology of Tropical Parasitic Infections in Asia and Pacific Region, (editors, J. W. Mak & H. S. Yong) 1982; pp. 91-100. Kuala Lumpur.

Sirisinha S. Tuti S, Vichasri S and Tawatsin A. Humoral immune responses in hamsters infected with *Opisthorchis viverrini*. Southeast Asian Journal of Tropical Medicine and Public Health 1983; 14: 243-51.

Sirisumpan P. A survey of intestinal helminths in stay dogs in Bangkok and Dhonburi. J Med Assoc Thai 1962;45: 567-79 [Thai, English abstract].

Smithers SR and Doenhoff MJ. Schistosomiasis. In: Immunology of Parasitic Infection, (editors S. Cohen and KS Warren). 2nd edition 1982, pp. 527-607. Blackwell Scientific Publications: London.

Snapper CM, Paul WE. Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. Science. 1987 May 22; 236(4804): 944-7.

Soboslay PT, Luder CG, Riesch S, Geiger SM, Banla M, Batchassi E, Stadler A, Schulz-Key H. Regulatory effects of Th1-type (IFN-gamma, IL-12) and Th2-type cytokines (IL-10, IL-13) on parasite-specific cellular responsiveness in Onchocerca volvulus-infected humans and exposed endemic controls. Immunology. 1999 Jun;97(2): 219-25.

Soesatyo M.H.N.E, Rattanasiriwilai W, Suntharasamai P, Sirisinha S. IgE responses in human gnathostomiasis. Transactions of the Royal Society of tropical Medicine and Hygiene 1987; 81: 799-801.

Srisongkram T, Saraya P. Vagina bleeding caused by Gnathostoma spinigerum. Report of a case. J Med Ass Thailand 1969; 52: 531.

Strickland GT, Ahmed A and Sell KW. Blastogenic response of Toxoplasma-infected mouse spleen cells to T- and B-cell mitogens. Clin Exp. Immunol. 1975 Oct; 22(1): 167-76.

Suntharasamai P, Desakorn V, Migasena S, Bunnag D. ELISA for immunodiagnosis of human Gnathostomiasis. Southeast Asian J Trop Med Public Health 1985 June; 16(2): 174-9.

Suzuki Y and Kobayashi A. Macrophage-mediated suppression of immune responses in Toxoplasma-infected mice. I. Inhibition of proliferation of lymphocytes in primary antibody responses. Cell. Immunol. 1984; 85: 417-27.

Tada I, Araki T, Matsuda H, Araki K, Akahane T. A study on immunodiagnosis of gnathostomiasis by ELISA and double diffusion with special reference to the antigenicity of *Gnathostoma doloresi*. Southeast Asian J Trop Med Public Health 1987; 18: 444-8.

Tapchisri P, Nopparatana C, Chaicumpa W, Setasuban P. Specific antigen of *Gnathostoma spinigerum* for immunodiagnosis of human gnathostomiasis. Int J Parasitol 1991; 21: 315-9.

Taylor H.R, Greene B.m. The status of ivermectin in the treatment of human onchocerciasis. Am J Trop Med Hyg 1989; 41: 460-6.

Terry R and Hudson K. Immunodepression in parasitic infections. Fortshritte der Zoologie 1982; 27: 125-39.

Trpis M, Childs L.E, Fryauff D.J, Greene B.M, Williams P.N, Munoz B.E, Pacque M.C, Taylor H.R. Effect of mass treatment of a human population with ivermectin on transmission of *Onchocerca volvulus* by *Simulium yahense* in Liberia, West Africa. Am J Trop Med Hyg 1990; 42: 148-56.

Tsushima H, Numata T, Yamamoto O, Iwasaki H, Iwanaka Y. Gnathostomiasis cutis probably infected in Hiroshima City. J Hiroshima Med Assoc 1980; 33: 1183-7 [Japanese].

Tuntipopipat S, Chawengkirttikul R, Sirisinha S. A simplified method for the fractionation of Gnathostoma-specific antigens for serodiagnosis of human gnathostomosis. J Helminthol. 1993 Dec;67(4):297-304.

Tuntipopipat S. Antigens antibidies and immune complexes in cerebrospinal fluid of patients with cerebral gnathostomiasis. Southeast Asian Journal of Tropical Medicine and Public Health. 1989 ;20: 439-46.

Waikagul J, Thairungroj M, Visrassuk A, Visiassuk K. A preliminary study on the efficacy of mebendazole, thiabendazole, praziquantel and ivermectin against gnathostomiasis in rats. Ann Trop Med Parasitol 1994; 17: 76-81.

Welch JS, Dobson C, Campbell GR. Immunodiagnosis and seroepidemiology of *Angiostrongylus cantonensis* zoonoses in man. Trans R Soc Trop Med Hyg. 1980; 74(5): 614-23.

Wongratanacheewin S, Rattanasiriwilai W, Priwan R, Sirisinha S. Immunodepression in hamsters experimentally infected with *Opisthorchis viverrini*. J Helminthol. 1987 Jun; 61(2): 151-6.

Yamaguchi T. Immunological studies on human gnathostomiasis. J Kurume Med Ass 1952; 15: 26-8.

Yamaguchi T. Immunological studies on human gnathostomiasis I. On the skin test. J Kurume Med Ass 1951; 14: 317-22.

Yazdanbakhsh M, Paxton WA, Kruize YC, Sartono E, Kurniawan A, van het Wout A, Selkirk ME, Partono F, Maizels RM. T cell responsiveness correlates differentially with antibody isotype levels in clinical and asymptomatic filariasis. J Infect Dis. 1993 Apr; 167(4): 925-31.

Yazdanbakhsh M, Sartono E, Kruize Y. Elevated levels of T cell activation antigen CD 27 and increased Interleukin-4 production in human lymphatic filariasis. European Journal of Immunology 1993; 23: 3312-7.

Yingyourd P, Daengsvang S. Experimental treatment of *Gnathostoma spinigerum* infection in mice with thiabendazole. Southeast Asian J Trop Med Public Health 1983 Dec; 14(4): 560-1.

Yingyourd P, Kulkumthorn M, Kraivichian P. A preliminary report on the experimental treatment of *Gnathostoma spinigerum* infection in mice with thiabendazole. Chula. Med J. 1985; 29: 1083-95 (Thai with English abtract).

Yingyourd P, Kulkamthorn M, Yuaykwarmdee N. Study on efficacy of praziquantel therapy in mice fed *Gnathostoma spinigerum* larvae. Chulalongkorn Medical Journal 1989; 35: 349-54.

Zimmerman GL, Kerkvliet NI, Brauner JA, Cerro JE. Modulation of host immune responses by *Fasciola hepatica*: responses by peripheral lymphocytes to mitogens during liver fluke infections of sheep. J Parasitol. 1983 Jun; 69(3): 473-7.

APPENDICES

1.2% Acid-pepsin

Pepsin A (Sigma, U.S.A.)	12 gm
Distilled water to	1 liter

Adjust pH to 2.0 with 1 N HCl

RPMI 1640 culture medium

RPMI 1640 (Gibco, U.S.A.)	10.4 gm
Penicillin-Streptomycin (Gibco, U.S.A.)	10.0 ml
1 M Hepes(Sigma, U.S.A.)	10.0 ml
Deionized distilled water to	1 liter

Adjust pH to 7.00 with 10% sterile $NaHCO_3$

Sterilization : By 0.22 μ millipore membrane filtration

1 M HEPES solution

Hepes (N-2-hydroxyethylpiperazine	23.82 gm
N-2-ethanesulfonic acid, Sigma)	
Distilled water to	100.0 ml

Sterilization : By 0.22 μ millipore membrane filtration

Scintillation fluid

PPO (2,5-diphenylosazole)	5.0 g
POPOP (1,4-bis-2-(5-Phenyloxazoly) benzene)	0.1 g
Toluene	1.0 liter

Coating buffer : 0.05 M Carbonate pH 9.6

Na_2CO_3	1.59 gm
$NaHCO_3$	2.93 gm
Distilled water to	1 liter

Incubation buffer

Bovine serum albumin(Sigma, U.S.A.)	1 gm
Phosphate-buffered saline (PBS) pH 7.1	100 ml

Tween 20	0.05 ml

Washing buffer

NaCl	45 gm
Distilled water to	5 liter
Tween 20	2.5 ml

Blocking Solution

Bovine serum albumin(Sigma, U.S.A.)	0.1 gm
PBS pH 7.1	10.0 ml

Substrate solution

Orthophenylene diamine (OPD, Sigma, U.S.A.)	0.2 gm
Distilled water to	10.0 ml
35% H_2O_2	5 µl

Reaction stopping solution : 5N H_2SO_4

Conc.H_2SO_4	1.2 ml
Distilled water to	10.0 ml