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GROWTH INHIBITION OF PLASMODIUM FALCIPARUM AND INTERLEUKIN-8 PRODUCTION BY PERIPHERAL BLOOD MONONUCLEAR AND POLYMORPHONUCLEAR CELLS IN VITRO

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Abstract: Peripheral blood mononuclear and polymorphonuclear cells have been demonstrated to kill and inhibit malaria parasite proliferation in vitro, but most of the reports required activation of the cells by cytokines or presence of immune sera or opsonins. In our study, peripheral blood leukocytes from non-immune donor efficiently inhibited *Plasmodium falciparum* growth, depending on the effecter/target ratio. Moreover, these cells produced a large amount of interlenkin-8 (IL-8) under stimulation with infected erythrocytes or supernatant of the *Plasmodium falciparum* culture. IL-8 secretion in the culture supernatant of polymorphonuclear cells was noted from 6-9 hr of stimulation with the parasites, with a substantial increase over 24 hours of culture. Attempt to elucidate whether IL-8 was involved in malaria suppression was done, and the result suggested that IL-8 was not directly involved in the anti-malarial activity of the mononuclear and polymorphonuclear cells. The other mechanism besides IL-8 may work for inhibiting parasite growth in the culture system of malaria parasites associated with leukocytes.

Key words: cytokine, interleukin-8, malaria, mononuclear cell, *Plasmodium falciparum*, polymorphonuclear cell

INTRODUCTION

Anti-malarial activity of peripheral blood mononuclear cells (MNC) and polymorphonuclear cells (PMN) have been demonstrated (Vernes, 1980; Celada *et al.*, 1983; Nnalue and Friedman 1988; Bouharoun-Tayoun *et al.*, 1990; Kumaratilake *et al.*, 1997), but in most of the reports, presence of opsonising agents (Nnalue and Friedman 1988), antibodies (Celada *et al.*, 1983; Bouharoun-Tayoun *et al.*, 1990) or complements (Kumaratilake *et al.*, 1997) were required. There are considerable evidences showing involvement of cytokines such as tumor necrosis factor α (TNFα), interferon-gamma (IFNγ), interleukin lα (IL-Iα), and others in the immunity and pathophysiology of malaria (Ockenhouse *et al.*, 1984; Kossodo and Grau, 1993; Kwiatkowski, 1995). The involvement of interleukin-8 (IL-8) and its ligand in *Plasmodium vivax* infection has been suggested (Horuk, 1994), and this chemokine has been detected in the serum of patients infected with *Plasmodium falciparum* (P. f.) (Friedland *et al.*, 1993; Looareesuwan *et al.*, 1999), but the roles of IL-8 in falciparum malaria are not clear.

We performed in vitro experiments to study the anti-malarial activity of the non-immune peripheral blood leukocytes. Moreover, we studied the IL-8 production of peripheral blood MNC and PMN induced by P.f., and tried to elucidate the role of this cytokine in immunity to malaria.

MATERIALS AND METHODS

Malaria parasites

Plasmodiun falciparum FCR-3 strain has been maintained in our laboratory in a continuous culture according to previous described methods (Trager and Jensen, 1976), except for the serum supplemented to the medium. The parasites were sowed in red blood cells (RBC) at an initial hematocrit of 5% in RPMI 1640 medium (Sigma Chemical Co, St. Louis, MO, USA) supplemented with l0% heat-inactivated horse serum (Cosmo Bio Co., Tokyo, Japan). Substitution of the human sera with horse sera greatly facilitate the malaria culture, because this permitted the use of all types of human erythrocytes, without checking for compatibility.

Separation of MNC and purification of monocytes

50 ml of peripheral venous blood was collected from healthy non-immune donors in a heparinized syringe. MNC were separated by Ficoll/Urografin density gradient centrifugation, and suspended in a plastic dish with RPMI 1640

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supplemented with 5% fetal calf serum. Two hours after incubation at 37 ℃, non-adherent cells were removed from the plate by washing with phosphate buffered saline and adherent cells (mainly monocytes) were detached from the plate with a rubber policeman and resuspended in the same medium of MNC.

Separation of PMN

Once the MNC removed, PMN (about 95% neutrophils) were separated from RBC by sedimentation in 1.25% gelatin. Mingled erythrocytes were hemolysed in lysing reagent (Ortho Diagnostic Systems, New Jersey, NJ, USA) and PMN were resuspended in the same medium of MNC.

P.f. growth inhibition assay

Growth inhibition assay was done by a radiometric method, described by Kumaratilake *et al.* (1997). Briefly, the experiments were conducted in 96 well flat-bottomed microplates (Becton Dickinson Co., Franklin Lakes, NJ, USA). The cells (MNC or PMN) were subsequently diluted and placed to the wells in 100 μl aliquots, where the parasites ($1x10^5$ parasites in $5x10^7$ RBC in 100 μl of medium) were added 2 hours later. Following 48 hours incubation at 37 ℃ in a CO_2 incubator containing a gas mixture of 5% CO_2 in air, each well was pulsed with l μCi of [^{3}H] hypoxanthine and harvested 24 hours later on a glass fiber sheet. The disks on the sheet were air dried, placed in a 96 well polyethylene plate with 100 μl of toluene scintillant and counted by a microplate scintillation counter (TopCount, Packard Instrument Co., Meriden, CT, USA). Each dilution was made in triplicate and the average was calculated. Growth inhibition rate was calculated using the formula as follows:

$$\text{Growth inhibition}(\%)=100-\frac{\text{scintillation count of experimental well}}{\text{scintillation count of P.f. alone well}}x100$$

Microscopic studies

Interaction of the phagocytic cells with parasites was observed by preparing cytocentrifuged smears of the culture. Parasites were cultured together with PMN or MNC and smears were made 48 hours later. MNC were detached from the culture plate with a rubber policeman before cytocentrifugation. The smears were air dried and stained with Giemsa for microscopic study at 1000x magnification.

Stimulation of the PMN and MNC with parasites and parasite-derived products

P. faciparum induced IL-8 secretion by the PMN and MNC was investigated by measuring IL-8 in the supernatant of the culture. PMN or MNC ($1x10^5$ cells in 100 μl of medium) were cultured with viable parasitized erythrocytes ($1x10^5$ parasites in $5x10^7$ RBC in 100 μl of medium), and the supernatant was collected by 24 hr. For positive control, PMN ($1x10^5$ cells in 100 μl of medium) were stimulated with human recombinant TNFα (20 ng in 100 μl of medium), which was kindly provided by Dainihon Pharmaceutical Co. (Osaka, Japan).

To investigate the fraction responsible for IL-8 induction, PMN ($1x10^5$ cells in 100 μl of medium) were stimulated respectively with intact infected erythrocytes ($1x10^5$ parasites in $5x10^7$ RBC in 100 μl of medium), 100 μl of supernatant of the P.f. culture, 100 μl of hemolysate of the infected erythrocytes (originally $1x10^5$ parasites in $5x10^7$ RBC), and partially purified $1x10^5$ parasites obtained by hemolysing and washing out the RBC ghost. As negative control, uninfected $5x10^7$ RBC in 100 μl of medium were added to the cell culture.

IL-8 assay

IL-8 in the supernatant was measured by an enzyme-linked immunosorbent assay (ELISA) method as described elsewhere (Ko *et al.,* 1992).

Neutralization of IL-8 activity

To study the role of IL-8 on parasite suppression by PMN, the cells were pretreated, 2 hours before addition of the parasites, with a mouse anti-human IL-8 monoclonal antibody WS-4 (Ko *et al.,* 1992) at a concentration of 50 μg /ml. For the control groups, we used monoclonal antibodies for IL-1α (Kasahara *et al.,* 1987) and an unspecific IgG. After 3 days culture, proliferation of the parasite was assessed by incorporation of [^{3}H] hypoxanthine.

RESULTS

P. f. growth inhibition

Inhibitory effect of the MNC or PMN on P.f. proliferation depended upon the effecter to target ratio (E/T) (Fig. 1 a). $1x10^5$ MNC almost inhibited the parasite proliferation (parasite number at the start was $1x10^5$/well), and to get 50% growth inhibition of the parasites, we needed $0.2x10^5$ cells, meaning the E/T for IC_{50} was about 0.2. PMN also efficiently inhibited parasite growth with the E/T for IC_{50} of 0.7 (Fig. 1b). Monocytes (adherent cells) were then separated from non-adherent cells (mainly lymphocytes) and respectively cultured with the parasites. The E/T of the monocytes for IC_{50} was 0.9 (Fig. 2). Non-adherent cells alone showed no or minimal inhibitory effect on parasite growth. This suggests that both monocytes and lymphocytes are required to inhibit P.f. growth.

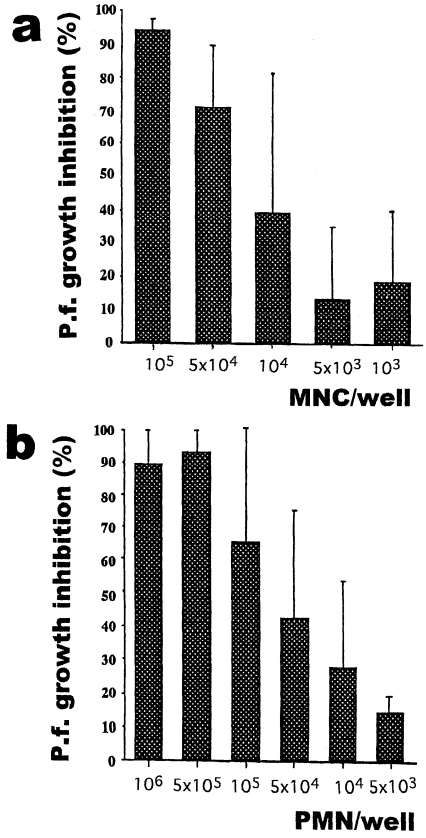

Figure 1 In vitro inhibitory effect of normal donors (a) MNC and (b) PMN on P.f. growth. The starting parasite number was $1x10^5$ /well. Results are calculated as percent inhibition in relation to the [^{3}H] hypoxanthine uptake of the P.f. alone, and presented as the mean ± standard error from 5 isolated experiments.

Morphological studies

Morphologically, 2 days after P.f. stimulation, the monocytes greatly increased in size, and most of them contained a large number of engulfed parasites. PMN also actively phagocytosed parasites, but the number of engulfed parasites per cell was not noticeable as did the monocytes.

IL-8 production of the PMN and MNC induced by P.f.

A remarkable amount of IL-8 was detected in the supernatant of P.f. stimulated MNC. After 24 hours culture, IL-8 level in the supernatant of P.f. stimulated culture was 9 times higher than that of unstimulated one (Fig. 3). Although less prominent than the MNC, P.f. stimulated PMN also secreted a considerable amount of IL-8 (Fig. 4). The IL-8 was detectable in the supernatant after 6-9 hours of stimulation, increasing progressively with time. 24 hours later, IL-8 concentration in the supernatant of P.f. stimulated PMN was 80 times higher than that of negative control. TNFα at a concentration of 1 μg/ml also induced IL-8

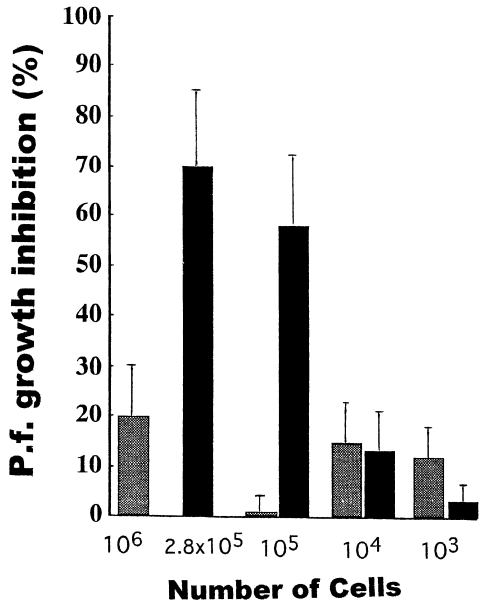

Figure 2 Effect of the monocytes (black bars) and non-adherent mononuclear cells (dotted bars) for P.f. growth inhibition.

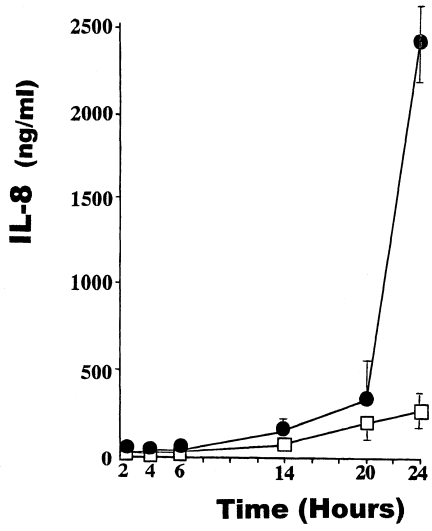

Figure 3 IL-8 detection in the supernatant of the P.f. stimulated MNC (circles). Squares represent the value of IL-8 in the supernatant of MNC cultured with uninfected RBC.

production in PMN but no stronger than P.f. parasites did.

Experiments were also designed to determine the fraction responsible for IL-8 induction. Among the fractions tested, P.f. infected erythrocytes and supernatant of the P.f. culture were the most potent stimulants for IL-8 production in the PMN cells. Hemolysate of the infected erythrocytes, partially purified parasites, and uninfected RBC had little or no stimulative effect on IL-8 production (Table 1).

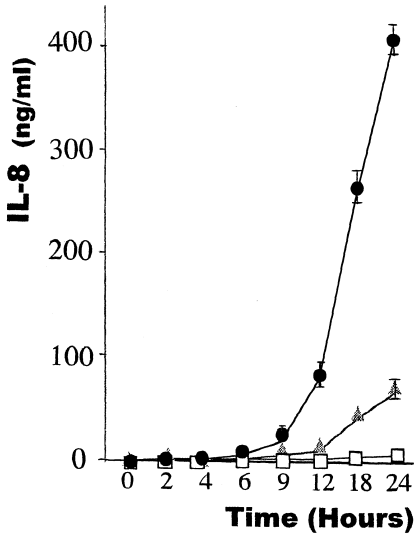

Figure 4 Time course of IL-8 secretion in1x10^5 PMN stimulated with 1x105 parasites (circles). Squares represent IL-8 detected in the PMN cultured with uninfected RBC, and triangles represent IL-8 secretion after TNF-α (100 ng/ml) stimulation.

Table 1 Concentration of IL-8 (ng/ml) in the culture supernatant of PMN stimulated with infected RBC or P.f. products

	Period of calture			
	18 hr	24 hr	32 hr	42 hr
PMN + medium	5.1	6.6	8.3	12.1
PMN + uninfected RBC	5.4	6.0	5.1	6.7
PMN + infected RBC	140	610	1100	4500
PMN + supernatant of P.f. culture	140	370	1700	2200
PMN + hemolysate of infected RBC	16	68	130	250
PMN + partially purified parasites	34	100	160	490

P.f. proliferation after neutralization of the IL-8

Treatment of the PMN or MNC with anti-IL-8 or anti-IL-1α monoclonal antibodies did not influence the inhibitory effect of these cells on parasite growth (data not shown), suggesting that IL-8 and IL-1α are not directly involved in suppression of the parasite by these cells.

DISCUSSION

Some authors have demonstrated that peripheral blood monocytes can kill malaria parasites in vivo and in vitro (Vernes, 1980; Bouharoun-Tayoun *et al.,* 1990), but monocytes required presence of immune sera (Druilhe and Khusmith, 1987; Lunel and Druilhe, 1989) or addition of cytokines (Ockenhouse *et al.,* 1984) for activation of the cells. Peripheral blood neutrophils are also reported to be effective against P.f. in the presence of immune sera (Celada *et al.,* 1983) or opsonins, and this activity was enhanced by TNF-α, lymphotoxin and IFNγ (Kumaratilake *et al.,* 1997).

We demonstrated that mononuclear cells and neutrophils from non-immune individuals efficiently inhibited P.f. growth in vitro in absence of anti-malarial antibodies or complements. Growth inhibition was dependent on the effecter/target ratio. The mechanism how the leukocytes restrain the parasite growth is not yet clear. For monocytes and macrophages, phagocytosis may be the major mechanism, as observed by morphology. The non-adherent mononuclear cells, mostly lymphocytes, did not have antiparasitic activity, but when they were incubated with monocytes, they strongly enhanced the ability of the monocytes to inhibit parasite growth. This suggests the interaction between monocytes and lymphocytes by direct contact or via cytokines. For neutrophils, the number of engulfed parasites per cell was not conspicuous. This suggests that, besides the phagocytic activity, neutrophils may produce soluble substances that may contribute to suppress parasite growth.

Cytokines have been demonstrated to be important in immunity and pathogenesis of malaria. IL-8 is a CXC type chemokine responsible for activation of a variety of cells including neutrophils (Yoshimura *et al.,* 1987), and the receptor of IL-8 has been shown to be related to *Plasmodium vivax* infection (Horuk, 1994). Duffy antigen on erythrocytes has long been reported as a target molecule for vivax malaria infection (Miller *et al.,* 1975). Interestingly, IL-8 receptor was shown to be homologous to the Duffy antigen, and the involvement of IL-8 and its receptor in infection with vivax malaria was suspected (Horuk, 1994). Some authors have detected IL-8 in the sera of P.f. infected patients (Friedland *et al.,* 1993; Looareesuwan *et al.,* 1999), and Wahlgren *et al.,* (1995) have detected various cytokines, including IL-8, in the culture supernatant of P.f. stimulated peripheral blood MNC and PMN.

Our experiments demonstrated IL-8 in the supernatant of PMN after P.f. stimulation. Since collected fraction of PMN was neutrophils by 95% and contamination with some mononuclear cells might occur, we histochemically confirmed IL-8 expression in PMN. Intracellular IL-8 was detected from 4 hours after P.f. stimulation (data not shown). Whether this IL-8 production resulted from a direct stimulation by parasites or an intermediated stimulation by a secondary cytokine has to be investigated.

In our study, we did not investigate the time required for IL-8gene expression in the P.f. stimulated PMN, but the elapsing time for IL-8 secretion may suggest presence of intermediating cytokines rather than a direct stimulation by

the parasite. Cytokines such as TNF-α , IL-1 and IFNγ are demonstrated to be produced in P.f. infections (Brown *et al.*, 1990; Wahlgren *et al.*, 1995), and we have to consider the possibility of an autocrine stimulation by these cytokines. TNF-α also induced IL-8 production in the PMN (Fig.4), but PMN responded earlier and stronger to P.f. than did to TNF-α. Thus, TNF-α should not be responsible for the induction of IL-8.

To elucidate the involvement of IL-8 in the immunity to falciparum malaria, we neutralized IL-8 with anti-IL-8 monoclonal antibody in the culture mixture of P.f. and PMN, resulting no abrogation of inhibitory ability to PMN. Therefore, we conclude that IL-8 has no direct influence on P.f. inhibitory activity of the PMN. The other mechanism besides IL-8 may work for inhibiting parasite growth in the culture system of malaria parasites associated with leukocytes.

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AN IMPORTED JAPANESE CASE OF CYCLOSPORIASIS

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Abstract: A stool examination revealed oocysts of *Cyclospora cayetanensis* in a 54-year-old Japanese man who had recently returned from the Phillipines. He suffered from watery diarrhea for about two weeks prior to visiting our hospital. An oral dose of 1,600 mg sulfamethoxazole and 320 mg trimethoprim was administered daily for nine days, and his diarrhea had disappeared by the fifth day of medication. Most Japanese physicians and laboratory technicians are unfamiliar with this protozoan disease, and need to be reminded of its existence when they encounter patients with diarrhea who have recently returned from a cyclosporiasis endemic area.
Key words: *Cyclospora cayetanensis*, diarrhea, Japanese

INTRODUCTION

The protozoan *Cyclospora cayetanensis* is recognized as causing diarrhea (Herwaldt, 2000), and has mainly been identified in residents and travelers of North, Central, and South America, the Caribbean Islands, Eastern Europe, Pakistan, India, Nepal, Southeast Asia, the Solomon Islands, Papua New Guinea, Morocco, and South Africa (Wurtz, 1994; Soave, 1996). To our knowledge, the first Japanese case of cyclosporiasis upon returning from Thailand and Cambodia was reported in 1996 (Iseki, 1996), since then, few Japanese patients with cyclosporiasis have been reported (Iseki *et al.,* 1998; Oki *et al.,* 2001; Masuda *et al.,* 2002; Ohnishi *et al.,* 2002; Shiota *et al.,* 2002; Yamaura *et al.,* 2002). This, perhaps, is due to the fact that cyclosporiasis is unfamiliar to Japanese physicians. Here we report the case of a Japanese patient with cyclosporiasis who had recently returned from the Philippines. The purpose of this report is to emphasize the importance of taking into account cyclosporiasis in the differential diagnosis of diarrhea, especially when related to countries in tropical and subtropical areas.

CASE REPORT

A 54-year-old Japanese man stayed in the Philippines from April 11 to June 30, 2002. He was admitted to a hospital in Mandaluyong City, Philippines on June 21, 2002, because of watery diarrhea and fever lasting for 2 days. He was treated with metronidazole and ciprofloxacin, but the watery diarrhea did not improve. He was discharged on June 25, 2002, and visited our hospital on July 2 because of the diarrhea. His medical history was unremarkable except for a fatty liver (bright liver had been identified by ultrasonography in another hospital in Japan), and he had been well before visiting the Philippines. When we saw him on July 2, he was afebrile, alert, and without palpable neck lymph nodes. Auscultation of the heart and lungs was also unremarkable, and bowel sounds were mildly hyperactive. Laboratory data on July 2, 2002, showed normal hemogram and blood chemistry, except for slightly increased white blood cell counts of 9,200/mm^3, AST 47 IU/l, ALT 64 IU/l, ALP 432 IU/l, and C-reactive protein 0.5 mg/dl. Serum HIV antibody was not checked. No pathogenic bacteria were cultured from the stool. Microscopic stool examination, using a formalin-ether concentration technique for helminth ova and protozoan, revealed a lot of spherical organisms that were 8-10 μm in diameter and contained numerous globules enclosed within a wall (Fig.1). The organisms appeared as bright-blue circles under ultraviolet epifluorescence microscopy (Fig.2). When unpreserved organisms isolated from the stool specimen were incubated in 2.5% potassium dichromate for 7 days at 27℃, a cluster of globules inside the organism was transformed into two ellipsoidal structures (Fig.3). On the basis of these characteristics, the organisms were identified as oocysts of *C. cayetanensis*. Our patient was treated with a daily oral dose of 1,600 mg sulfamethoxazole and 320 mg trimethoprim for

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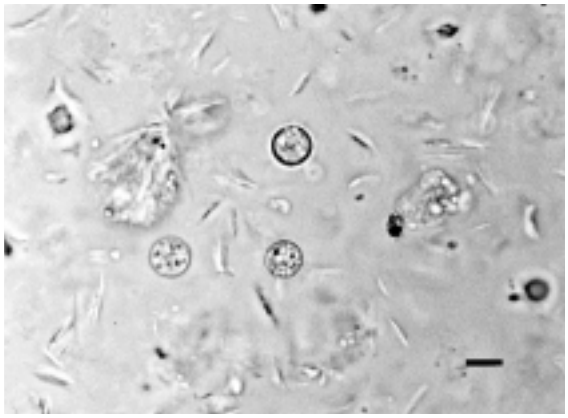

Fig. 1: Immature oocyst of *Cyclospora cayetanensis* isolated from the patient's stool specimen; bar =8 μm

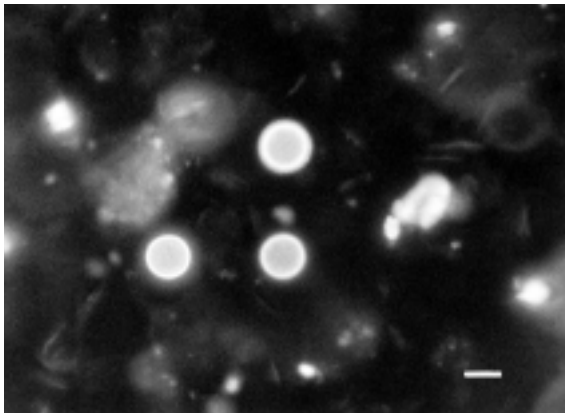

Fig. 2: Autofluorescence of the oocyst wall; bar=8 μm. (Ultraviolet epifluorescence microscopy using a 365-nm excitation filter)

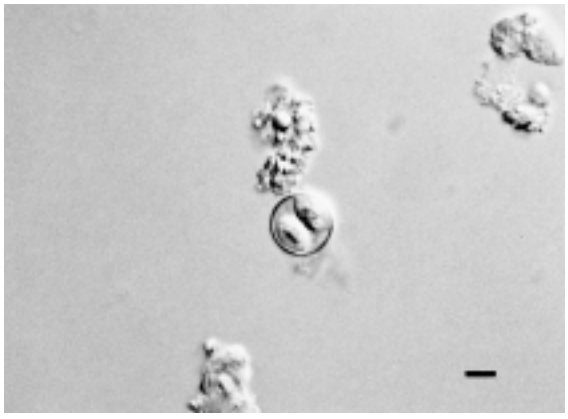

Fig. 3: Mature oocyst with two sporocysts; bar=5 μm. (Nomarski interference-contrast microscopy)

nine days, and his diarrhea disappeared by the fifth day after starting the medication.

Table 1: Reported Japanese cases of imported cyclosporiasis

Case	Age(years)	Sex	Visited countries	References
1	22	M	Thailand and Cambodia	4, 7
2	50	M	Myanmer, Singapore, Thailand, and Cambodia	7
3	26	M	Thailand	5
4	70	M	Southeast Asia	9
5	46	M	Indonesia	7
6	36	M	Thailand and Cambodia	7
7	35	F	Hong Kong and Indonesia	10
8	36	M	Philippines	8
9	53	M	Mexico	13
Present case	54	M	Philippines	

DISCUSSION

Infections with *C. cayetanensis* can be detected in 0.07 -2.8% of European diarrheal patients returning from developing countries (Gascon, *et al.,* 1995; Clarke and McIntyre, 1996; Jelinek, *et al.,* 1997). Many Japanese people have traveled to areas where cyclosporiasis is endemic, but to our knowledge, only nine other cases of Japanese travelers infected with *C. cayetanensis* upon returning from these countries have been reported (Table 1)(Iseki, 1996; Iseki *et al.,* 1998; Oki *et al.,* 2001; Masuda *et al.,* 2002; Ohnishi *et al.,* 2002; Shiota *et al.,* 2002; Yamaura *et al.,* 2002). These facts indicate that some Japanese patients with diarrhea upon returning from developing countries may, infact, be infected with *C. cayetanensis*, and some of them with cyclosporiasis may be overlooked. This oversight may be due to the fact that most Japanese physicians and laboratory technicians are unfamiliar with this protozoan disease. Our case indicates that *C. cayetanensis* is one of the causative organisms associated with diarrhea, especially when no improvement is observed, in spite of the administration of antibacterial agents and metoronidazole, as is similar to our case. Most cases with cyclosporiasis resolve themselves spontaneously in immunocompetent hosts, but cyclosporiasis is one of the diarrheal diseases which physicians should be aware of, since cyclosporiasis is fatal in immunocompromised patients. *C. cayetanensis* is probably indigenous to Japan, since a Japanese patient with cyclosporiasis who had no history of traveling to other countries was found in 2001 (Shiota *et al.,* 2002). Therefore, we have to be aware of the fact that people have a chance of contracting cyclosporiasis even in Japan.

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Seasonal differences in antimalarial activity of hot-water extract of *Dichroa febrifuga* leaves against *Plasmodium yoelii* 17XL in ICR mice, with reference to febrifugine and isofebrifugine content

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Abstract: The antimalarial activity of the hot-water extract of leaves and roots of *Dichroa febrifuga* was evaluated against *Plasmodium yoelii* 17XL in ICR mice. Untreated control mice died with a gradual body weight loss and increase of parasitemia by day 9 after infection. The hot-water extract of leaves collected in June showed an antimalarial activity, and furthermore the possible adverse side effect was also observed. All mice given orally the extract of leaves (0.1 g/ml) collected in June died by day 9 without parasite multiplication. The mice given the lower concentration (0.025 g/ml) of the same leaf extract showed low parasitemia levels during administration. Following a transient increase of malaria parasites in the bloodstream, no parasites could be detected by a microscopic examination, and all mice survived during the experiment. On the other hand, the extract of leaves (0.1 g/ml) collected in December showed no activity. The extract of roots (0.1 g/ml) of *D. febrifuga* collected in December, however, had an antimalarial activity, and three mice out of four survived during the experiment. The leaves collected in June contained about 30 times as much febrifugine and isofebrifugine mixture as those in December.

Key words: *Plasmodium yoelii* 17XL,*Dichroa febrifuga*, febrifugine, antimalarial activity

INTRODUCTION

Malaria is one of the most important tropical diseases in the world. World-wide emergence of chloroquine-resistant *Plasmodium falciparum* has stimulated the development of new effective treatments against malaria (Payne, 1987; Winstanley, 2000). Of several strategies on malaria control it is a new interest to investigate the folk medicine exhibiting a potent antimalarial activity. Isolation of artemisinin from leafy portions of *Artemisia annua* L. in 1972 has led to further trial for identifying other medicinal compounds of potential as antimalarial agents from natural products (Iwu *et al.*, 1986; Ajaiyeoba *et al.*, 1999; Isaka *et al.*, 1999; Rahman *et al.*, 1999; Boonlaksiri *et al.*, 2000).

In our continuation of investigation of plants having antimalarial activity, the hot-water extract of leaves of *Hydrangea macrophylla* Seringe var. *Otaksa* Makino showed a high *in vivo* antimalarial activity against the rodent malaria parasite *P. yoelii* 17XL though the extract of roots had no activity (Sakai *et al.*, 2000; Ishih *et al.*, 2001). Furthermore the fraction including the febrifugine and isofebrifugine mixture having an antimalarial activity was isolated from the leaves of this plant. These compounds had already been isolated from the roots of *Dichroa febrifuga* Lour. (Koepfli *et al.*, 1947) and from the leaves of *Hydrangea umbellata* Rehd. Et Wils. (Ablondi *et al.*, 1952). It is therefore of interest to clarify the distribution and/or content of these principles in the different parts of the plant. This paper deals with a preliminary *in vivo* antimalarial evaluation of the hot-water extract of leaves of *D. febrifuga* collecting in June or in December using ICR mice infected with *P. yoelii* 17XL.

MATERIALS AND METHODS

Animals and parasites

All animal experiments were performed according to the Guidelines for Animal Experimentation, Hamamatsu University School of Medicine. Outbred male ICR mice,8 weeks old, purchased from SLC Inc. (Hamamatsu, Japan), were used. For experiments, the blood stage parasites of *Plasmodium yoelii* 17XL from frozen stock were injected into two mice. The mouse showing 10-15% of parasitemia was bled under ether anaesthesia to collect *P. yoelii* 17XL-parasitized blood. Experimental mice were infected intraperitoneally with$1x10^5$ parasitized erythrocytes. Infected mice

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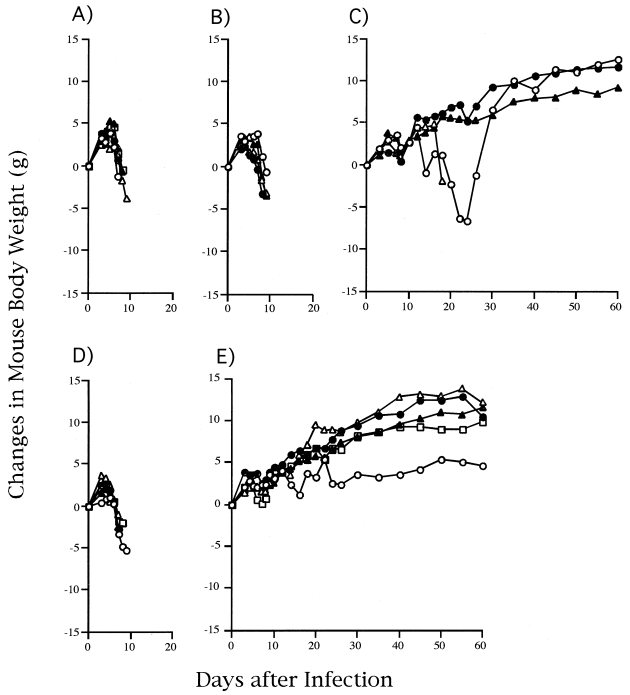

Fig.1. Body weight profile of each mouse in response to treatment with the hot-water extract of roots or leaves of *D. febrifuga*. *P. yoelii* 17XL-infected mice were orally given each extract at 75 µl/10 g body weight, twice a day, for 5 consecutive days from day 3 to day 7 after inoculation intraperitoneally with 10^5 parasitized erythrocytes. Control group (A, n=5) of mice received an equivalent volume of distilled water. Each symbol represents an individual mouse. Extract of leaves collected in December (B, n=5;0.1 g/ml), extract of roots collected in December (C, n=4;0.1 g/ml) and extract of leaves collected in June (D, n=5;0.1 g/ml and E, n=5; 0.025 g/ml).

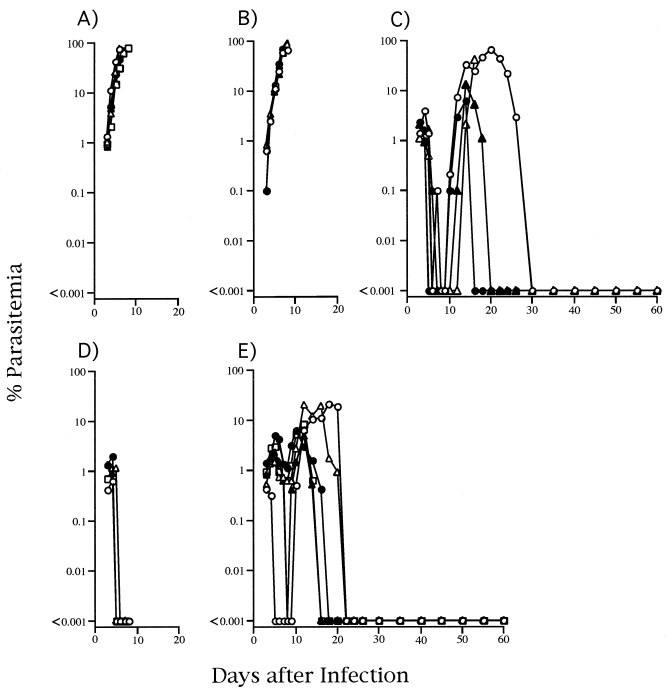

Fig.2. Parasitemia profile of each mouse in response to treatment with the hot-water extracts of roots or leaves of *D. febrifuga*. *P. yoelii* 17XL-infected mice were orally given each extract at 75 µl /10 g body weight, twice a day, for 5 consecutive days from day 3 to day 7 after inoculation intraperitoneally with 10^5 parasitized erythrocytes. Control group (A, n=5) of mice received an equivalent volume of distilled water. Each symbol represents an individual mouse. Extract of leaves collected in December (B, n=5;0.1 g/ml), extract of roots collected in December (C, n=4;0.1 g/ml) and extract of leaves collected in June (D, n=5;0.1 g/ml and E, n=5; 0.025 g/ml).

treated with extracts were monitored for % parasitemia and days of survival relative to control mice up to 60 days post-infection.

Preparation of the hot-water extract of leaves and roots of D. febrifuga

Leaves (before fallen, dark green and hard) and roots of *D. febrifuga* were collected from the Botanical Garden, University of Shizuoka in December, 1999, and leaves (in full bloom, green and soft) of the same plant were also collected in June, 2000. Five g of air-dried leaves or roots of the plant put in a herbal bag (Tokiwa Industry, Ehime, Japan) was added with 500 ml of distilled water and boiled. When the fluid volume came to about 250 ml, leaves or roots were taken out and the extract was finally concentrated to 50 ml. Each hot-water extract (equivalent to the 0.1 g of dried material /ml extract) was stored at 4℃ until use.

Quantitative analysis of febrifugine and isofebrifugine mixture

Dried leaves or roots (1 g) of *D. febrifuga* collected in December, 1999 and leaves in June, 2000 were extracted with 50 ml of water under boiling for1hr and the extract was filtered and the residue was washed with 20 ml of boiling water. The combined filtrate was acidified with hydrochloric acid (1 ml) and extracted with chloroform (10 ml) three times. To the water layer, 40% of potassium hydroxide aq. was added until the solution became to alkali. The alkaline solution was extracted with chloroform (10 ml) three times. The chloroform layer was concentrated to dryness after washing with water. The residual chloroform extract was analysed by liquid chromatography (HPLC) comparing the peak area. HPLC condition: Column, Develosil ODS - 74.6 mm x 25 cm (Nomura Chemical, Aichi, Japan); Solvent, 20 mM NaH_2PO_4- 20 mM H_3PO_4 (pH 2.1) / CH_3CN (9:1); Flow rate 1.0 ml/min; UV, 276 nm; Retention times; 7.4 min, 9.2min.

In vivo antimalarial activity of the hot-water extract of

leaves collected in June and of leaves or roots collected in December

To evaluate antimalarial activity of hot-water extract, infected ICR mice were divided into four to five per group for activity assay of each extract and untreated control. From3days after injection of parasitized erythrocytes, mice were orally given each hot-water extract at 75 μl/10 g body weight in the treated group and distilled water in the control one, respectively, twice a day for5consecutive days.

RESULTS

Antimalarial activity of the hot-water extract of leaves or roots of D. febrifuga

As shown in Fig.1, mice in the untreated control and groups treated with the extract of leaves (0.1 g/ml) collected either in June or in December died from day 7 to day 9 with a gradual body weight loss. However, in the group given the root extract (0.1 g/ml), the mouse body weight transiently decreased, but turned to increase in several days, and three mice out of four survived during the experiment. All mice given the extract of leaves (0.025 g/ml) collected in June showed a transient body weight loss but survived during the experiment. Malaria parasites appeared from day 3 of infection in the bloodstream of the control and treated groups (Fig.2). Parasitemia of each mouse in the control and the group given the extract of leaves collected in December gradually increased, and all mice died by day 9. On the other hand, mice in the group given the extract of leaves (0.1 g/ml) collected in June showed low parasitemia levels during medication but all mice died by day 9. Autopsy of dead mice showed that the livers became whitish wholly, indicating the hepatotoxicity by higher concentration of the leaf extract. Mice treated with the lower concentration (0.025 g/ml) of the extract of leaves collected in June showed low parasitemia levels during medication. After a transient increase of parasitemia, malaria parasites in the bloodstream of mice could not be detected by a microscopic examination from day 16 to day 22, and all mice survived during the experiment. Mice treated with the root extract also showed low parasitemia levels during medication. On day 10, malaria parasites increased in the bloodstream of the treated mice and one mouse died on day 17. Then the parasitemia level of three surviving mice decreased and the parasites in the bloodstream could not be detected by a microscopic examination from day 16 to day 30.

Preparation of alkaloid fraction from leaves and roots

One g of dried leaves of *D. febrifuga* collected in December, 1999 and in June, 2000 afforded 0.024 and 0.718 mg of febrifugine and isofebrifugine mixture, respectively. One g of dried roots collected in December, 1999 afforded 0.14 mg of alkaloids.

DISCUSSION

The roots of *D. febrifuga* have been used as a treatment for malaria in Chinese traditional remedies, and the mixture of febrifugine and isofebrifugine having an antimalarial activity was first isolated from the roots of *D. febrifuga* (Koepfli *et al.*, 1947). In the present study, the hot-water extract of the roots of *D. febrifuga* collected in December had a direct antimalarial effect on *P. yoelii* 17XL infection in ICR mice; namely, suppression of multiplication of parasites during medication. Besides the extract of the roots, this effect was shown in the hot-water extract of the leaves of *D. febrifuga* collected in June, but not in December. The leaves collected in June contained the febrifugine and isofebrifugine mixture about 5 times as much as the roots collected in December did. Furthermore, the former contained the alkaloid mixture about 30 times as much as the leaves in December did. Kuehl *et al*. (1948) reported that the total alkaloidal contents having the antimalarial activity were 0.1 to 0.15% of the dried roots of *D. febrifuga* but the yield of alkaloids from the stem and leaf material was invariably much lower. There is no description available about a season of harvesting leaves, and if the leaves were collected after flowering, the present results coincide with their report. Previously, a solid extract of the leaves of *D. febrifuga* (Shuu Chi) were found equally effective against *P. gallinaceum* infection in chicks in doses of only about one-fifth of the root extract (Ch'ang Shan) (Jang *et al.*, 1946). Our results also agree with their results. It was also reported that the root samples from India contained only about one-tenth of the alkaloidal fraction present in the Chinese samples. The present results hence suggest that the seasonal changes in activity of hot-water extract of leaves of *D. febrifuga* might be due to the movement of active compounds from leaves to other parts or the biological and/or chemical inactivity of compounds.

Acute toxicity tests of Ch'ang Shan, the roots of *D. febrifuga*, made on dogs showed a 50% lethal dose value of 20 g/kg (Jang *et al.*, 1946). Fatal dose produced in dogs intense congestion with numerous hemorrhagic patches throughout the whole gastrointestinal tract. Aside from some congestion, no specific lesions were found histologically in the liver, spleen and kidneys. Toxic deaths in mice were observed at 10 mg/kg for febrifugine (Chien and Cheng, 1970). In the present experiment, the hepatotoxicity of alkaloids is suggested by autopsy of mice given the higher dose of the hot-water extract (0.1 g of dried material /ml extract) of leaves collected in June. Further experiments are at present being undertaken to examine histologically

and pharmacologically the toxicity of febrifugine and isofebrifugine mixture in detail.

Recently, the evaluation of antimalarial activity of medicinal plants against malaria was extensively studied, and various active natural plant products were discovered (Iwu *et al.,* 1986; Ajaiyeoba *et al.,* 1999; Rahman *et al.,* 1999; Boonlaksiri *et al.,* 2000). The present study showed that the marked difference in the antimalarial activity of the hot-water extract of *D. febrifuga* leaves collected in between June and December, and thus it should be very important to consider the material conditions such as parts of plants, the geographic conditions, and the season of harvesting, in an ongoing effort to detect the antimalarial activity of medicinal plants and to identify novel antimalarial agents from them.

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Short communication

COMPARATIVE STUDIES ON THE DEVELOPMENT OF LARVAL *ANGIOSTRONGYLUS CANTONENSIS* IN PUERTO RICAN AND BRAZILIAN *BIOMPHALARIA* SNAILS.

YUZURU IWANAGA

Accepted November, 28, 2002

Since the first report of angiostrongylosis cantonensis by Nomura and Lin (1945), *Angiostrongylus cantonensis* has been recognized as a causative agent of eosinophilic meningoencephalitis in humans in South Asian and Pacific areas (Roux *et al.,* 1987; Purohit *et al.,* 1991; Alibhoy *et al.,* 1999; Cooke-Yarborough *et al.,* 1999; Re and Gluckman, 2001). The diagnosis of the disease is usually based on clinical manifestations and immuno-serological test. Immuno-serological tests require a large amount of antigens. Therefore, it is a paramount importance to establish the life cycle of *A. cantonensis* in the laboratory. For this purpose, it is necessary to maintain the intermediate hosts in the laboratory. It is well known that various molluscan species can serve as intermediate hosts for *A. cantonensis* (Lim and Hyneman, 1965; Liat *et al.,* 1965; Hori *et al.,* 1976; Iwanaga *et al.,* 1983). In general, snail species used to establish the life cycle of the parasite in the laboratory is *Biomphalaria* sp. that is easy to breed them. Although the larval development of *A. cantonensis* in *Biomphalaria glabrata* (Yousif and Lammler, 1977), *Achatina fulica* (Hori and Yamaguchi, 1982) and *Ampullarium* sp (Uchikawa *et al.,* 1986) has been described, few detailed observations on the location of larvae and susceptibility of the pigmented and albino types of *Biomphalaria* snails are available.

The present paper examines this relationship using laboratory colonies of the pigmented and albino types of Puerto Rican and Brazilian *B. glabrata* and pigmented *B. straminea* collected in Brazil. Only adult snails were used. The pigmented and albino types of Puerto Rican strains of *B. glabrata* were obtained from Puerto Rico via NIH in U.S. A. The Brazilian strains of snails were from the following areas in Brazil: Pigmented *B. glabrata* from Jaboatao, Pernambuco, albino *B. glabrata* from Belo Horizonte and pigmented *B. straminea* from Sao Lourenco da Mata. The snails have been reared in our laboratory by the modified method of Iwanaga and Tsuji (1972). Albino rats, *Rattus norvegicus* experimentally infected with *A. cantonensis* served as a source of the first stage larvae (L1) for snail infection. *A. cantonensis* L1 were isolated from feces of infected rats by means of a Baeruman apparatus. About 200 snails each were individually exposed to 50 L1 in 10 ml beaker for 20 hrs at 24 - 26℃. Snails were then washed with dechlorinated tapwater and kept in 24 - 26℃ in a soil filtrated aquarium. To delineate the larval infection sites in the snail, the snail was divided into four regions: the foot (region 1), seminal receptacle sac and vas deferens (region 2), prostate gland and oviduct (region 3), and ovotestis and digestive gland (region 4). 10 snails each were examined 10, 15, 20, 25 and 30 days after larval exposure. Some snails were examined with a dissecting microscope, while others were fixed in Bouin's solution, serially sectioned at 8 - 10 μm, and stained with hematoxylin-eosin for histological observations. Furthermore, some of the third stage larvae (L3) obtained from each snail were tested for infectivity by feeding rats on them using a stomach tube. The second stage larvae (L2) and L3 were distinguished by morphological examinations after fixation in 10% formalin; The intestine of L2 is composed of large cells filled with refractile food granules, whereas L3 have two well-developed chitinous rods with expanded knob-like tips near the anterior end and their intestinal cells are clear without food granules.

The number of L1 penetrated into pigmented *B. glabrata*, Puerto Rico, ranged from 20 to 29 (avg. 26). Similarly, those numbers were 18 to 26 (avg. 24) for pigmented *B. glabrata*, Brazil, 20 to 25 (avg. 22) for albino *B. glabrata*, Puerto Rico, 19 to 25 (avg. 23) for albino *B. glabrata*, Brazil and 7 to 11 (avg. 9) for pigmented *B. straminea*, Brazil. Although there was no significant difference between pigmented and albino types of *B. glabrata*, pigmented *B. straminea* showed a markedly lower larval count than those of other snails ($p<0.0001$, Student's t-test). It

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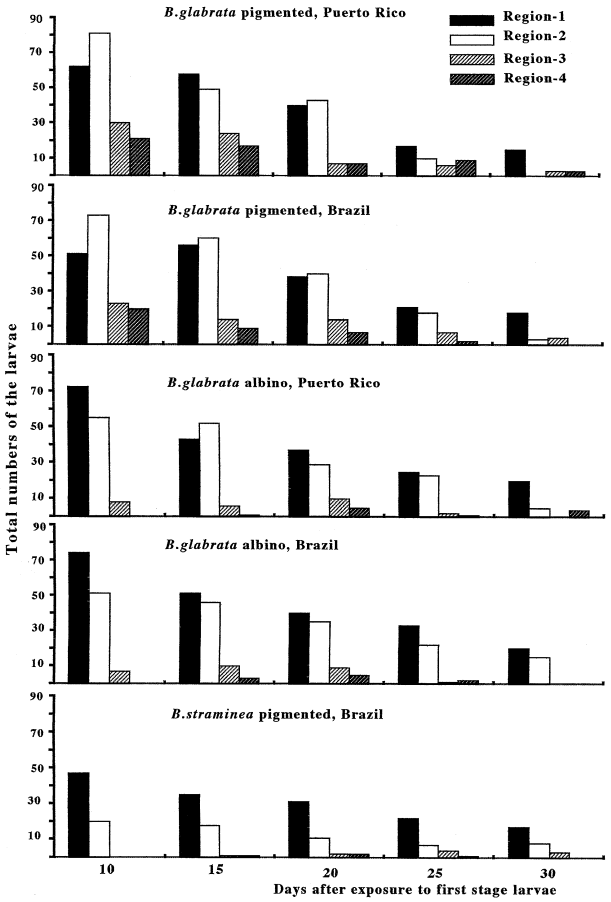

Figure 1. Distribution of the second stage larvae of *Angiostrongylus cantonensis* in *Biomphalaria* snails

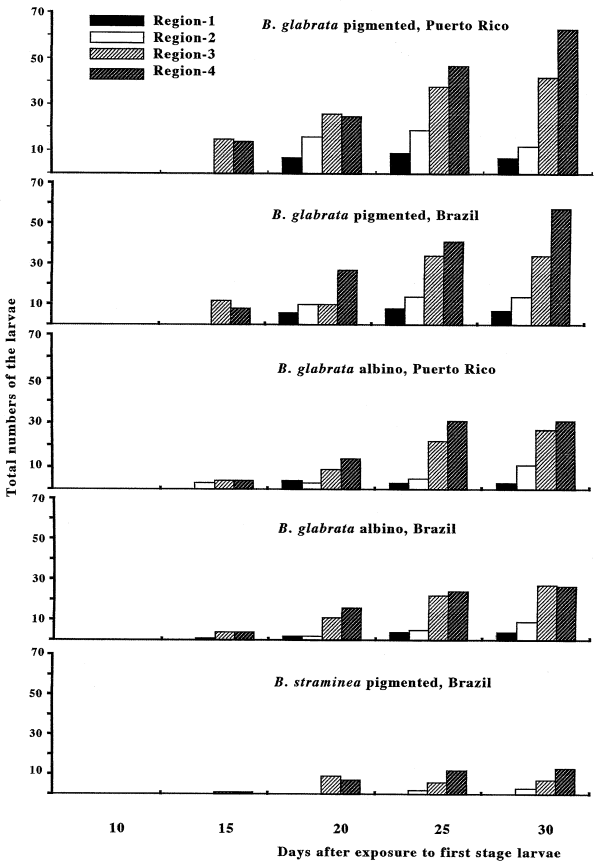

Figure 2. Distribution of the third stage larvae of *Angiostrongylus cantonensis* in *Biomphalaria* snails

may be due to differences in biochemical factors of the snails associated with invasion of the larvae. Figs.1 and 2 show the distribution in the snail bodies of L2 and L3, respectively. L2 were found in all regions, but especially in regions1and 2 at 15 days postexposure (*dpe*). On 15 *dpe*, L 3 firstly were observed in regions 3 and 4 in pigmented *B. glabrata* and *B. straminea*, and in regions 2, 3 and 4 in albino *B. glabrata*. The L3 counts increased in regions 3 and 4 in the course of time. There was a distinct difference in distribution between L2 and L3. The majority of L2 were detected in regions1and 2 at 15 *dpe*, where they generally remained without reaching to L3. Furthermore, some larvae died and mostly remained ensheathed. Especially, many dead larvae were observed in pigmented *B. straminea* and these dead larvae were readily identified as L2. Chao *et al* (1987) reported that L2 in *Ampullarium canaliculatus* were first observed on 5 *dpe* and reached the peak on day 22, mainly harboring in the head foot. In the present study, the maximum L2 count was observed during 10-15 *dpe* and most L2 were found in viscera and genital grand existing in region 2. This finding differs slightly form their report. On 25 *dpe*, most L2 developed into the L3, and the majority of the L3 were detected in regions 3 and 4. These L3 became active when transferred to 0.9% NaCl solution. In contrast, some larvae were morphologically similar to L3, but they were killed in an artificial digestive solution. It seems likely therefore that they were preinfective L3. The majority of L2 grew to L3 within 30 *dpe*. At 30 *dpe*, total numbers of L3 obtained from 10 snails each of Puerto Rican and Brazilian pigmented *B. glabrata* were 129 and 120. Similarly, 72 and 63 L3 were recovered from albino Puerto Rican and Brazilian *B. glabrata*, respectively. Interestingly, however, pigmented *B. straminea* produced only 23 L3. It seems likely that pigmented *B. glabrata* snails tend to harbor more L3 than albino *B. glabrata* and pigmented *B. straminea*. The distribution pattern of L3 was quite different from that in other reports. For instance, Richards and Merritt (1967) reported that the dispersion of L3 in *B. glabrata* occurred through digestive tract, whereas Yousif and Lammler (1977) showed that most of L3 were found in headfoot, columellar muscle and mantle collar, although it is not clear whether *B. glabrata* used in their studies are pigmented or albino. In the present study with *Biomphalaria* snails, most of L3 were found in the digestive gland and genital duct, especially, in the digestive gland in pigmented *B. glabrata*. With

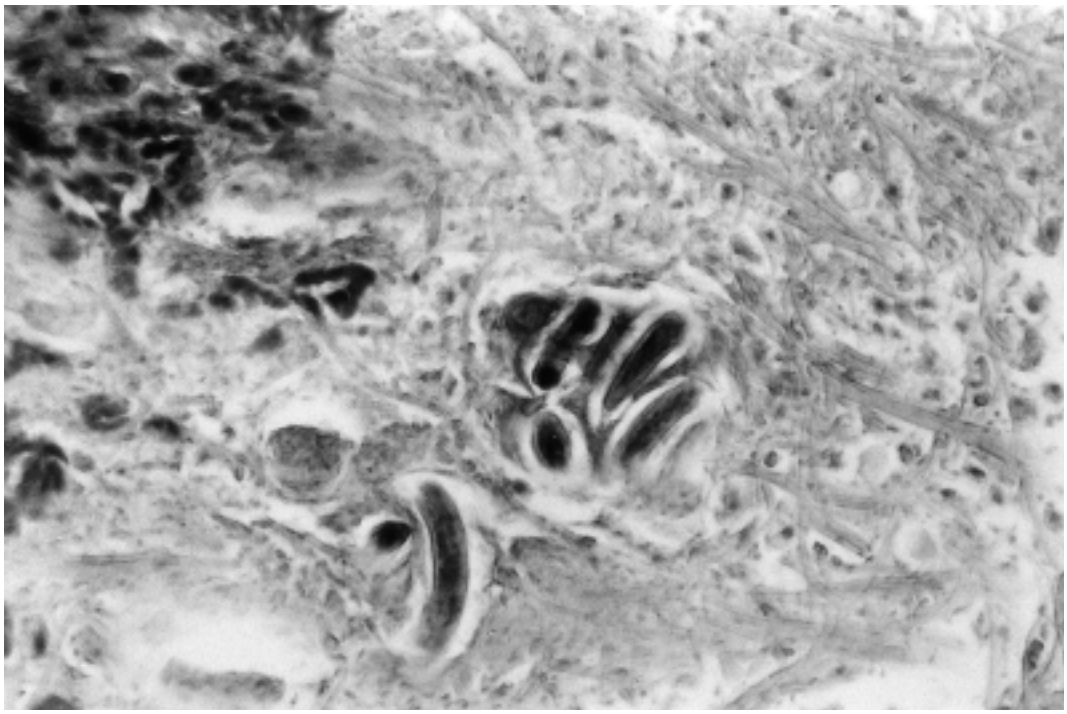

Figure 3. Section of the second stage larvae in seminal receptacle sac and vas deferens region of pigmented *Biomphalaria glabrata*, Puerto Rico on 10 day after exposure to the first stage larvae. Hematoxylin and eosin stain, x200

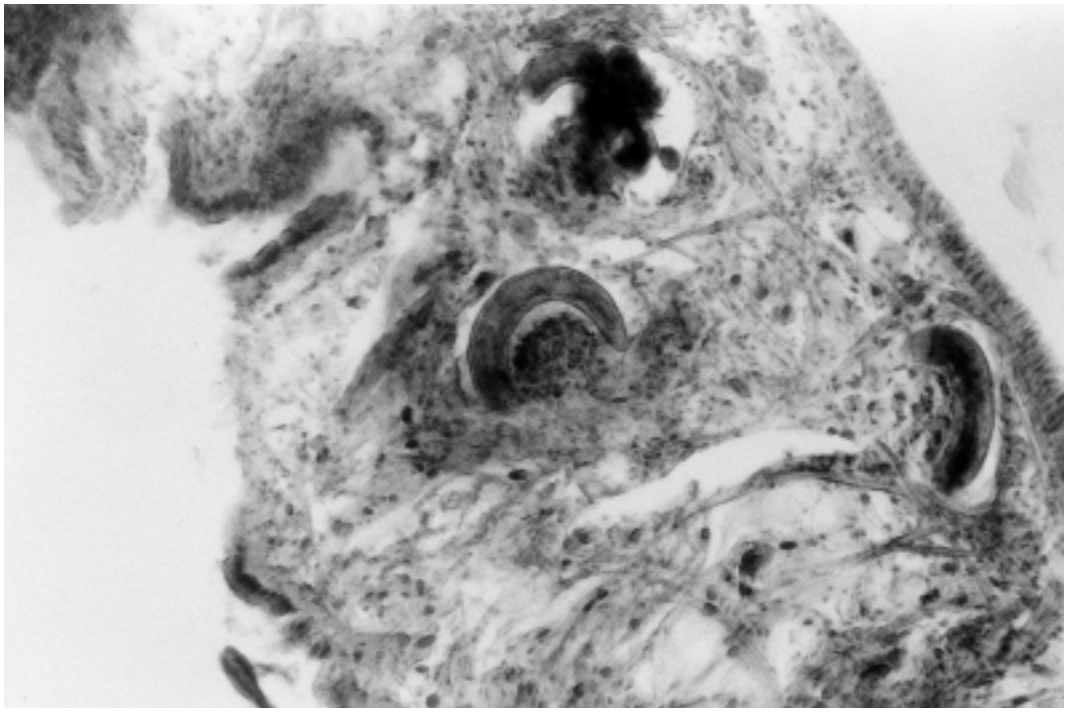

Figure 4. Section of the third stage larvae in prostate gland and oviduct region of pigmented *Biomphalaria glabrata*, Puerto Rico on 20 days after exposure to the first stage larvae. Hematoxylin and eosin stain, x200

regard to the distribution of L3, most of them were detected in the mantle, foot and digestive gland of *A. fulica* (Hori and Yamaguchi, 1982), similarly, in the head of *Ampullarium* sp. (Uchikawa *et al.,* 1986), in the body muscle of *Laevicaulis alte* (Noda *et al.,* 1985), and in the kidney and intestinal regions of *Oncomelania hupensis* snails (Iwanaga, 1995). It seems therefore that the development and distribution of L3 differ depending on snails species. Clearly, further work needs to be done on this point. Infection rates of Brazilian and Puerto Rican pigmented *B. glabrata* were 62.0 and 77.1%, respectively. Likewise, corresponding albino *B. glabrata* snails were 51.5 and 59.3%. In contrast, the infection rate of pigmented *B. straminea* was only 39.8% (data not shown). Hence, in *B. glabrata*, pigmented types show a slightly higher susceptibility than the albino types. When compared the current data with those of other experimental intermediate hosts (Iwanaga *et al.,* 1983; Iwanaga, 1995), there was a distinct difference in susceptibility among snails. There were no distributional difference of both L2 and L3 between pigmented and albino *B. glabrata*. However, numbers of L3 recovered from pigmented *B. glabrata* at 30 *dpe* were slightly higher than that from albino *B. glabrata*. Especially, it is of interest to note that pigmented *B. straminea* showed a significantly lower susceptibity than *B. glabrata* sanils ($p<0.001$). These differences are probably due to a combination of physiological and biochemical differences among the snails (Newton and Brand, 1955; Iwanaga, 1992). Host's responses against larvae were histologically examined at 10 and 20 *dpe*. No host tissue response was noted around L2 at 10 *dpe* (Fig.3). At 20 *dpe*, a few L3 were observed in region 3, and they were attacked by a definite host reaction consisting of amoebocytes surrounded by fibroblasts (Fig.4). These results agree with the fingings by Iwanaga (1995) for *O. hupensis* snails. Mean recovery rate of adult worms from rats infected with L3 grown in *Biomphalaria* snail populations tested in this study were 70 to 80%. The morphological features of these adult worms coincided with those report by Mackerras and Sandars (1955). Finally, these data suggest that *B. glabrata*, especially pigmented type, is a suitable intermediate host to establish the life cycle of *A. cantonensis* in the laboratory.

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