Dokkyo Medical University, Japan. SEA was prepared using the method described by Matsuda *et al* (1984). Eggs of *S. mekongi* were isolated from infected mice intestines by digestion method using actinase E (No. 122, Kaken Pharmaceutical, Tokyo, Japan) and collagenase (C6885, Sigma-Aldrich, St. Louis, MO), and the collected eggs were subsequently lyophilized. SEA was extracted from lyophilized eggs with carbonate buffer (0.05 M, pH 9.6). After protein content measurement using Bradford reagent (#500-0006, Bio-Rad Laboratories, Hercules, CA), 1 ml aliquots of SEA solution were placed in small tubes and stored at -80°C until use.

ELISA

The ELISA was performed as described by Matsuda *et al* (1984) and Hirose *et al* (2005) with some modifications. In this study, the standard ELISA technique used in our laboratory was designated as conventional-ELISA (conv-ELISA). *S. mekongi* SEA was dissolved in carbonate buffer at a concentration of 2 µg protein/ml just before sensitization of the ELISA plate (No.762070, Greiner Bio-One, Frickenhausen, Germany). Each well of the ELISA plate was sensitized overnight with 0.1 ml of *S. mekongi* SEA diluent. After washing the wells with T-PBS (0.15 M phosphate buffered-saline containing 0.05% Tween 20), the inner surfaces of the wells were blocked with 0.12 ml of BSA/T-PBS solution (T-PBS containing 1% bovine serum albumin) for 10 minutes.

We used horseradish peroxidase (HRP)-conjugated anti-human IgG goat serum (55252, MP Biochemicals, LLC-Cappel Products, Soton, OH) for the enzyme-conjugated antibody and 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) di-ammonium salt (ABTS)(A-1888, Sigma-Aldrich, St. Louis, MO) as a substrate for HRP. The optimal concentrations of antigen, test sera, and enzyme conjugate were determined using checkerboard titration. The test serum was diluted to 1:200 with BSA/T-PBS, and 0.1 ml of the dilution was put in each well. After incubation of the plate at 37°C for 45 minutes, the wells were washed three times with T-PBS. Zero point one milliliter of HRP conjugate diluted to 1:1,200 was then added, and the plate was incubated at 37°C for 60 minutes. After washing, 0.2 ml of ABTS solution (0.03% ABTS, 0.25 M citric acid, 0.25 M sodium dihydrogen phosphate, 0.003% H_2O_2) was put in each well and the plate was kept at room temperature for 1 hour to allow enzyme reactions. The optical density (OD) of each well was read by a microplate reader (MTP-120, CORONA ELECTRIC, Ibaraki, Japan) at 415 nm.

For SMP-ELISA, SMP treatment of antigen was performed as described by Alarcón de Noya *et al* (2000) except for the concentration of SMP solution. The *S. mekongi* SEA-sensitized plate was prepared using the same method as conv-ELISA and was washed with 50 mM sodium acetate buffer (pH 4.5). Each well was treated with 0.1 ml of 0.5 mM SMP solution (0.5 mM SMP in sodium acetate buffer) at room temperature for 1 hour in the dark. After briefly washing the treated plate with PBS, 0.12 ml of 50 mM sodium borohydride in PBS was added to the wells and the plate was incubated for 30 minutes at room temperature. After washing three times with T-PBS, the plate was subjected to ELISA in a similar manner as conv-ELISA, except the dilution of HRP conjugate was 1:6,000. Optimal concentrations of SMP solution and enzyme conjugate were determined using checkerboard titration to make the positive reference serum produce an OD value comparable with that of the conv-ELISA.

Evaluation of cross-reaction with other parasites

A parasitological survey was conducted in Kratie Province, Cambodia in May 2005 as part of the National Schistosomiasis Control Program. In this survey, eggs from other helminths were found at positivity rates of 14.6-25.8% by stool examination in a village, Talous, located 5 km south of the city of Kratie. According to a previous survey, Talous was known as an area with low transmission of *S. mekongi*. Therefore, we compared the results of stool examination with those of the two ELISA methods in samples obtained from Talous to evaluate cross-reaction with other parasite infections by ELISA. Blood and stool were collected from 151 residents in Talous. Blood samples were collected on blood sampling filter papers (Advantec Toyo Kaisha, Tokyo, Japan) after finger pricking. The blood samples on filter paper were dried and transported to the laboratory in Japan. Discs (3 mm in diameter) were cut out from the blood sampling filter paper and placed individually into wells of deep-well microplates. Each disc with blood was immersed in 400 µl of BSA/T-PBS containing 0.5% skimmed milk, shaken vigorously, and incubated overnight at 4°C after vigorous shaking. The resulting extract was estimated to have a 1:200 dilution of the plasma specimen and was tested by the ELISA methods as described above. For reasons described in the Discussion section, we used the criterion that ELISA values ≥ 0.2 were positive. Stool examination was conducted using a formalin-detergent technique (Waikagul *et al*, 1997). In brief, 0.5 ml of each stool sample was suspended in a formalin-detergent solution (10% liquid dish-washing detergent, 2% formalin in water) and incubated for 30 minutes at room temperature. The suspension was filtered through gauze into another tube and then shaken vigorously. The suspension was allowed to settle for 3 hours, after which the supernatant was discarded. The remaining pellet was dissolved in 10% formalin to give a volume of 1 ml. The resulting specimen was mixed well, then 0.04 ml of the specimen was examined under the microscope. Each sample was examined twice.

Statistical analysis

The software program Microsoft Excel (Microsoft Office Excel 2003, Microsoft, Washington, WA) was used to calculate the correlation coefficient (r).

The two-sided probability (P) for the Fisher's exact probability test was calculated by standard statistical software (Dr. SPSS 2 for Windows, Version 11.0.1J, SPSS, Chicago, IL). P-value < 0.05 was considered significant.

RESULTS

The results of the two ELISA methods are shown in Fig 1 and Table 1. The range of ELISA values in Group A was wider with the SMP-ELISA on both the higher and lower sides. The highest ELISA value of Group B with the conv-ELISA (0.578) was markedly lower with the SMP-ELISA (0.198). The range of ELISA values from Group C was very narrow in comparison with the other groups, and there was little difference between the two ELISA methods. With conv-ELISA, the lowest value in Group A (0.330) was lower than the highest value in Group B. Therefore, 10 individuals (23.8%) from Group A and 6 individuals (6%) from Group B had ELISA values ranging from 0.330 to 0.578. In contrast, the distribution of the ELISA values from Group A was different than those from Groups B and C with SMP-ELISA.

The correlation between conv-ELISA and SMP-ELISA, from Groups A and B is

Table 1
A comparison of the ELISA methods for Schistosomiasis mekongi.

	conv-ELISA			SMP-ELISA		
	Group A	Group B	Group C	Group A	Group B	Group C
No. examined	42	100	25	42	100	25
Minimum	0.330	0.006	0.004	0.233	0.015	0.010
Maximum	1.232	0.578	0.054	1.526	0.198	0.058
Mean	0.755	0.089	0.018	0.694	0.058	0.029
SD	0.211	0.107	0.014	0.314	0.041	0.011
Mean + 3SD		0.410	0.059		0.180	0.061

Table 2
Relationship between the ELISA results and other parasitic infections detected by stool examination (n = 151).

			Stool examination[a]					
			A. lumbricoides		Hookworms		*Echinostoma* sp	
			Positive	Negative	Positive	Negative	Positive	Negative
			22	129	35	116	39	112
conv-ELISA[b]	Positive	25	3	22	7	18	9	16
	Negative	126	19	107	28	98	30	96
	Fisher's exact probability test (P)		1.000		0.605		0.217	
SMP-ELISA[b]	Positive	8	2	6	3	5	3	5
	Negative	143	20	123	32	111	36	107
	Fisher's exact probability test (P)		0.329		0.388		0.426	

[a]Stool examination was conducted using the formalin-detergent method.
[b]ELISA values ≥ 0.2 were considered positive.

shown in Fig 2. There were significant correlations between the two ELISA methods in both groups. The correlation coefficient of Group A was higher (r = 0.951, <0.001) than the correlation coefficient of Group B (r = 0.744, <0.001).

Stool examination and ELISA were conducted to determine cross-reactivity with other parasites (Table 2). Of 151 subjects, 25 (16.6%) and 8 (5.3%) had positive ELISA results with the conv-ELISA and the SMP-ELISA, respectively. No *S. mekongi* eggs were detected. Eggs from *Ascaris lumbricoides*, hookworms, and *Echinostoma* sp were detected in samples from 22, 35, and 39 individuals, respectively. Most individuals who had eggs of other parasites had negative ELISA results on both ELISAs, but some individuals had positive ELISA results. The ELISA positivity rates

Table 3
False positive and false negative in each criteria.

	conv-ELISA			SMP-ELISA		
	Group A	Group B	Group C	Group A	Group B	Group C
≥0.2	42 (100%)	9 (9%)	0 (0%)	42 (100%)	0 (0%)	0 (0%)
<0.2	0 (0%)	91 (91%)	25 (100%)	0 (0%)	100 (100%)	25 (100%)
≥X (B)[a]	41 (98%)	2 (2%)	0 (0%)	42 (100%)	2 (2%)	0 (0%)
<X (B)[a]	1 (2%)	98 (98%)	25 (100%)	0	98 (98%)	25 (100%)
≥X (C)[b]	42 (100%)	45 (45%)	0 (0%)	42 (100%)	33 (33%)	0 (0%)
<X (C)[b]	0	55 (55%)	25 (100%)	0 (0%)	67 (67%)	25 (100%)

[a]X (B) Mean + 3SD of Group B in each the method. The values are shown in Table 1.
[b]X (C) Mean + 3SD of Group C in each the method. The values are shown in Tabele 1.

among egg-positive patients were 13.6-23.1% with conv-ELISA and 7.7-9.1% with SMP-ELISA. The Fisher's exact probability test did not show bias with the ELISA for intestinal parasites.

DISCUSSION

SMP treatment of SEA was performed in order to destroy the glycosylated epitopes responsible for false-positive results (Alarcón de Noya *et al*, 2000). Because glycosylated epitopes can be recognized by antibodies in *Schistosoma mekongi* infected patients, the OD values of the patients decreased with the SMP-ELISA when we used enzyme conjugate at the same dilution rate as that used for conv-ELISA (data not shown). In this experiment, we used a higher concentration of enzyme conjugate with the SMP-ELISA. SMP treatment can increase or decrease ELISA values based on serum type and result in expansion of the range of ELISA values for Group A with SMP-ELISA. Differences in the effect of SMP treatment on ELISA values appear to depend on the composition of target epitopes in each serum sample. Most antibodies in sera with high ELISA values in Group B, which consisted of subjects from a non-endemic area in Cambodia, recognized the glycosylated epitopes in SEA.

Diagnostic criteria are established based on the protocol and/or purpose of the study. Many researchers have used mean + 3SD for the OD values of negative control sera as a cut-off limit (Alarcón de Noya *et al*, 2000). Some authors used criteria that depended on the distribution of ELISA values for positive and negative controls (Hirose *et al*, 2005). In this study, Cambodian (Group B) and Japanese (Group C) individuals were examined as negative controls. Using the mean + 3SD for the negative control for Group B as a cut-off value (Table 3), the sensitivity and specificity of conv-ELISA were both 98%, and the sensitivity and specificity with SMP-ELISA were 100% and 98%, respectively. Using the same criteria for Group C (Table 3) results in false positives with conv-ELISA of 45% and with SMP-ELISA of 33%.

The range of ELISA values for positive sera was distinct from that for negative sera with SMP-ELISA. Therefore,

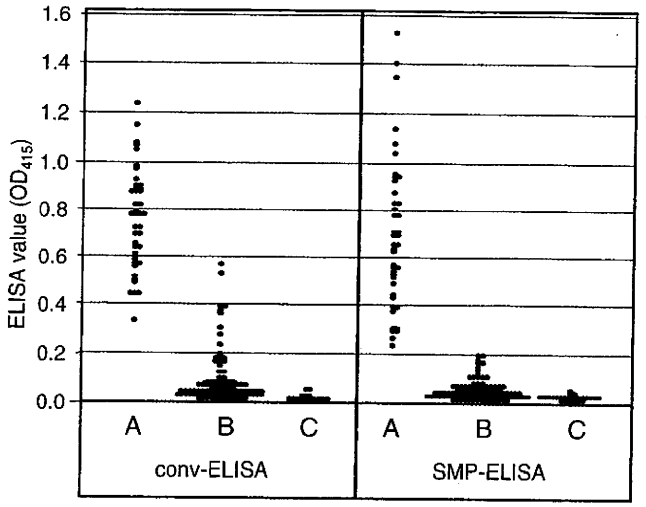

Fig 1–Distribution of ELISA values for sera from *S. mekongi* infected patients and from subjects in non-endemic areas by the two ELISA methods. A: *S. mekongi* infected patients (n = 42); B: subjects in a non-endemic area in Cambodia (n = 100); C: subjects in Japan (n = 25).

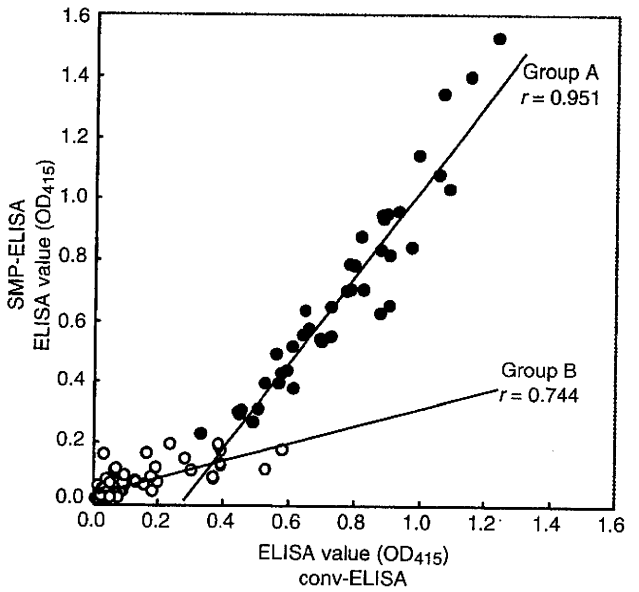

Fig 2–Plot of OD values comparing conv-ELISA to SMP-ELISA to evaluate the correlation between the two ELISA methods. The OD values of sera from *S. mekongi* infected patients (solid circle) and Cambodians living in a non-endemic area (open circle) are shown. Group A: *Schistosoma mekongi* infected patients (n = 42); Group B: persons in a non-endemic area in Cambodia (n = 100).

diagnostic criteria can be established based on the highest ELISA value for negative sera and the lowest ELISA value for positive sera. Using the criterion in which an ELISA value ≥0.2 was positive, both the sensitivity and specificity of SMP-ELISA reached 100% (Table 3).

Concerning negative control sera, the range of ELISA values for Group B (Cambodian) was higher than for Group C (Japanese). This is possibly due to a difference in antigens to which each group were exposed, both qualitatively and quantitatively. Our results suggest SMP-ELISA can reduce the influence of cross reactive antigens.

A positive ELISA reaction without schistosome eggs indicates one of three scenarios: 1) active infection with *S. mekongi* but no eggs were detected, 2) *S. mekongi* has already been treated but residual antibodies still existed, and (3) non-specific cross-reaction was detected. The lower sensitivity of the stool examination compared to the ELISA may explain why scenario 1) might occur. In low transmission areas, such as in Talous, it is difficult to detect eggs in stool samples because of lower disease intensity (Urbani *et al*, 2002; Fenwick *et al*, 2006). Scenario 2) is a characteristic feature of diagnostic methods detecting specific antibodies. This reaction indicates the subject had a risk for infection. In

general, residual antibodies tend to diminish progressively, and withdrawal periods vary between individuals (Hayashi *et al*, 2000). In scenario 3), a non-specific cross-reaction, might be one of the causalities, at least with the conv-ELISA (Fig 1). Most patients with intestinal parasite eggs had negative ELISA values, although some had positive values. The present data suggest infection with one of these three intestinal parasites should not result in a significant cross-reaction. Given the results of Table 3, it can be seen that the positive reactions on SMP-ELISA are most likely caused by scenarios 1) or 2). Assuming that all positive ELISA tests were due to cross-reaction with other parasites, the maximum rate of false positives with SMP-ELISA would be 9.1% (2/22). In another survey in a *Schistosoma mekongi* endemic area in Champasack Province, Lao PDR in 2006, we detected *Opisthorchis viverrini* eggs without *S. mekongi* eggs in 13 of 41 individuals who took the survey (Nakamura *et al*, 2006; unpublished data). Three of 13 opisthorchiasis patients had positive ELISA values, and 14 out of 41 had positive ELISA values on SMP-ELISA. The ELISA positivity rate for *O. viverrini* egg-positive persons (23%) was lower than the positivity rate of the targeted 41 persons (34%). These data suggest cross-reaction should not be significant with these helminths, although further studies are required for validation.

We conclude the use of SMP-ELISA improves diagnostic specificity and sensitivity for schistosomiasis mekongi. This method should become a powerful tool for diagnosing infection.

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REFERENCES

Alarcón de Noya B, Colmenares C, Lanz H, Caracciolo MA, Losada S, Noya O. *Schistosoma mansoni*: immunodiagnosis is improved by sodium metaperiodate which reduces cross-reactivity due to glycosylated epitopes of soluble egg antigen. *Exp Parasitol* 2000; 95: 106-12.

Bergquist R, Johansen MV, Utzinger J. Diagnostic dilemmas in helminthology: what tools to use and when? *Trends Parasitol* 2009; 25: 151-6.

Chigusa Y, Otake H, Ohmae H, *et al*. Determination of the period for establishment of a liver network echogenic pattern in *Schistosoma japonicum* infection. *Parasitol Int* 2006; 55: 33-7.

Fenwick A, Rollinson D, Southgate V. Implementation of human schistosomiasis control: challenges and prospects. *Adv Parasitol* 2006; 61: 567-622.

Hayashi M, Matsuda H, Chigusa Y, Otake H, Kubota T, Matsunaga K. Post-treatment follow-up studies on the clinical features of schistosomiasis japonica over a 33-year period in Kofu District, Japan. *J Yamanashi Med Asoc* 2000; 28: 12-9 (In Japanese with English abstract).

Hirose Y, Kirinoki M, Lipayon IL, Blas BL, Chigusa Y, Matsuda H. Development of the rapid and simplified ELISA (WHOLE BLOOD-ELISA) using samples of *Schistosoma japonicum*-infected human whole blood. *Southeast Asian J Trop Med Public Health* 2005; 36: 72-8.

Ittiprasert W, Butraporn P, Kitikoon V, *et al*. Differential diagnosis of schistosomiasis mekongi and trichinellosis in human.

Parasitol Int 2000; 49: 209-18.

Matsuda H, Tanaka H, Blas BL, Nosenas S, Tokawa T, Ohsawa S. Evaluation of ELISA with ABTS, 2-2'-azino-di-(3-ethylbenzthiazoline sulfonic acid), as the substrate of peroxidase and its application to the diagnosis of schistosomiasis. *Jpn J Exp Med* 1984; 54: 131-8.

Matsumoto J, Kirinoki M, Kawai S, *et al.* Prevalence of schistosomiasis japonica among schoolchildren and animal reservoirs in oriental Mindoro, Philippines. *Jpn J Trop Med Hyg* 1999; 27: 175-80.

Nakamura S, Matsuda H, Kirinoki M, *et al.* Evaluation of schistosomiasis mekongi prevalence after drug treatment in southern part of Khong District, Champasack Province, Lao PDR. Proceedings of International Conference on Mekong Research for the People of the Mekong, Chiang Rai, Thailand, 2006.

Ohmae H, Sinuon M, Kirinoki M, *et al.* Schistosomiasis mekongi: from discovery to control. *Parasitol Int* 2004; 53: 135-42.

Sinuon M, Tsuyuoka R, Socheat D, *et al.* Control of *Schistosoma mekongi* in Cambodia: results of eight years of control activities in the two endemic provinces. *Trans R Soc Trop Med Hyg* 2007; 101: 34-9.

Stich AHR, Biays S, Odermatt P, *et al.* Foci of schistosomiasis mekongi, northern Cambodia: II. Distribution of infection and morbidity. *Trop Med Int Health* 1999; 4: 674-85.

Tanaka H, Santos AT Jr, Matsuda H, *et al.* Epidemiological importance of COP reactions in areas lightly infested with *Schistosoma japonicum* in the Philippines. *Jpn J Trop Med Hyg* 1987; 15: 87-96.

Urbani C, Sinuon M, Socheat D, *et al.* Epidemiology and control of mekongi schistosomiasis. *Acta Trop* 2002; 82: 157-68.

Waikagul J, Anantaphruti MT, Nuamtanong S, Sanguankait S. Evaluation of the modified formalin detergent technique for detection of intestinal parasites. In: Hayashi *et al*, eds. Collected Papers on the control of Soil-transmitted Helminthiases. Vol VI. Tokyo: The Asian Parasite Control Organization, 1997: 5-11.

Zhu YC, Socheat D, Bounlu K, *et al.* Application of dipstick dye immunoassay (DDIA) kit for the diagnosis of schistosomiasis mekongi. *Acta Trop* 2005; 96: 137-41.

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Plasmodium falciparum: Differing effects of non-esterified fatty acids and phospholipids on intraerythrocytic growth in serum-free medium

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ABSTRACT

Different combinations of non-esterified fatty acids (NEFA) had variable effects on intraerythrocytic growth of *Plasmodium falciparum*. All stages of the parasite cultured in medium supplemented with *cis*-9-octadecenoic acid (C18:1-cis-9), hexadecanoic acid (C16:0), phospholipids (Pld) and bovine albumin free of NEFA were similar to those grown in complete growth medium. Three typical growth patterns indicating suppressed schizogony (SS), suppressed formation of merozoites (SMF), and inhibited invasion of merozoites (IMI) resulted from culture in other combinations of lipids. Unsaturated or saturated NEFA with longer or shorter carbon chains than C18:1-cis-9 or C16:0, higher degree of unsaturation, and *trans*-forms mainly resulted in SS and SMF effects. However, IMI or partial IMI was observed with tetradecanoic acid or octadecanoic acid enriched with C18:1-cis-9, and *cis*-9-hexadecenoic acid plus C16:0. Isoforms of C18:1-cis-9 also mainly resulted in partial IMI. SMF also occurred with C18:1-cis-9 plus C16:0 in the absence of Pld. Thus different NEFA exerted distinct roles in erythrocytic growth of the parasite by sustaining development at different stages.

1. Introduction

Malaria remains a devastating disease, particularly in the tropics. The annual incidence of malaria worldwide is estimated between 300 and 500 million clinical cases. Estimates of annual mortality from malaria, caused largely by the protozoan *Plasmodium falciparum*, range from 1.5 to 2.7 million worldwide (World Malaria Report 2008, WHO, http://apps.who.int/malaria/wmr2008/; Snow et al., 2005).

New antimalarial drugs with alternative targets are needed, because of the emergence of resistance to conventional antimalarial drugs and insecticides (Ridley, 2002). In order to create new medications, it is necessary to better understand antimalarial drugs and the biology of the parasites. The *P. falciparum* parasite develops through three distinct stages within erythrocytes (RBC) during its cycle of approximately 48 h: ring, trophozoite, and schizont (Bannister et al., 2000). It has been suggested that *P. falciparum* requires the presence of certain factors in human serum (HS) to allow its development within RBC (Jensen, 1979), although the role of HS in the growth of this parasite is unknown. The replacement of HS in *P. falciparum* culture medium with chemically-defined substances can be advantageous, not only for culturing the parasite, but also for providing critical clues to understanding the requirements for parasite proliferation during the erythrocytic phase.

We previously described an HS substitute derived from adult bovine plasma (Asahi and Kanazawa, 1994; Asahi et al., 1996, 2005) and chemically-defined culture media that used structurally-defined lipids and recombinant proteins to sustain continuous intraerythrocytic growth of *P. falciparum* (Asahi, 2009). Non-esterified fatty acids (NEFA) were found to be critical for parasite growth in the growth-promoting fraction derived from adult

Abbreviations: BSAF, bovine serum albumin free of NEFA; C12:0, dodecanoic acid; C14:0, tetradecanoic acid; C16:0, hexadecanoic acid; C16:1, *cis*-9-hexadecenoic acid; C18:0, octadecanoic acid; C18:1-cis-6, *cis*-6-octadecenoic acid; C18:1-cis-9, *cis*-9-octadecenoic acid; C18:1-trans-9, *trans*-9 octadecenoic acid; C18:1-cis-11, *cis*-11-octadecenoic acid; C18:1-cis-13, *cis*-13-octadecenoic acid; C18:2, *cis,cis*-9,12-octadecadienoic acid; C20:4, *cis*-5,8,11,14-eicosatetraenoic acid; C22:0, docosanoic acid; CRPMI, basal medium; FCM, flow cytometry; GFS, a growth-promoting fraction derived from adult bovine plasma; GFSRPMI, CRPMI containing 10% GFS; HS, human serum; IMI, inhibited invasion of merozoites; NEFA, non-esterified fatty acid; parasitemia-45 h, parasitemia at 45 h after inoculation; PC, phosphatidylcholine; Pld, phospholipids; PRBC, RBC infected with *P. falciparum*; RBC, erythrocytes; released merozoite-45 h, merozoites released into media at 45 h after inoculation; RFU, relative fluorescent units; ring form-45 h, ring forms at 45 h after inoculation; schizont-25 h, schizonts at 25 h after inoculation; SMF, suppressed formation of merozoites; SS, suppressed schizogony; SYBR Green I-basic, SYBR Green I in buffered saline at pH 8.8.

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bovine plasma (GFS) and in the chemically-defined media. The efficacies of NEFA in sustaining the growth of *P. falciparum* have been shown to vary markedly, depending on the type, total amount, and combinations used. The NEFA involved in the growth promotion of *P. falciparum* require to be at least in specific pairs; the most effective combination in GFS and HS comprises the two most abundant NEFA, *cis*-9-octadecenoic acid (C18:1-cis-9, oleic acid) and hexadecanoic acid (C16:0, palmitic acid), while the combination of C18:1-cis-9 and octadecanoic acid (C18:0, stearic acid) is slightly less effective (Asahi, 2009; Asahi et al., 2005; Mitamura et al., 2000). The growth rates in media containing even the best combination of NEFA alone were, however, much lower than in GFS-containing media (Asahi et al., 2005; Asahi, 2009). The addition of phospholipids (Pld) with specific structures, such as phosphatidylcholine (PC), into culture media containing optimal NEFA markedly increased parasite growth to a level similar to, or greater than, that seen with GFS-containig media. The mechanisms responsible for the different abilities of the various NEFA in the presence or absence of Pld, and of specific proteins such as bovine and human albumin in promoting parasite growth are, however, unknown.

In this study we investigated the distinct effects of various NEFA on each developmental stage of *P. falciparum*, including schizogony, merozoite formation, and reinvasion of RBC, to provide clues to the mechanisms underlying the growth-promoting properties of NEFA. Experiments were carried out using synchronized culture and flow cytometry (FCM).

2. Materials and methods

2.1. Parasites, culture and synchronization

Cultures of the FCR3/FMG (ATCC Catalogue No. 30932, Gambia) strain of *P. falciparum* were used in all experiments. The parasites were routinely maintained using *in vitro* culture techniques. The culture medium was devoid of whole serum, and consisted of basal medium (CRPMI) supplemented with 10% GFS (Daigo's GF21; Wako Pure Chemical Industries, Japan), as previously reported (Asahi and Kanazawa, 1994; Asahi et al., 2005). This complete medium is referred to as GFSRPMI. CRPMI consisted of RPMI-1640 containing 2 mM glutamine, 25 mM 4-(2-hydroxylethyl)-piperazine ethanesulfonic acid, 24 mM $NaHCO_3$ (Invitrogen Ltd., USA), 25 μg/ml gentamycin (Sigma–Aldrich Corp., USA) and 150 μM hypoxanthine (Sigma–Aldrich). Briefly, RBC were preserved in Alsever's solution (Asahi et al., 1996) for 3–30 days, then washed, dispensed into 24-well culture plates at a hematocrit of 2% (1 ml of suspension/well), and cultured in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 37 °C. Infected RBC (PRBC) and uninfected RBC were washed with CRPMI 3–4 days after inoculation, for subculture or growth experiments. Parasitemia was adjusted to 0.1% for subcultures by adding uninfected RBC, unless otherwise specified, and the hematocrit was adjusted to 2% by adding the appropriate volume of culture medium.

The cultures were synchronized at the ring stage by three successive exposures to 5% (W/V) D-sorbitol (Sigma–Aldrich) at 41- and 46-h intervals (Izumiyama et al., 2009). After the third sorbitol treatment, residual schizonts and cell debris were removed by isopycnic density centrifugation on 63% Percoll PLUS (GE Healthcare Bio-Science Corp., USA). Parasites at the ring stage (adjusted to 5.0% parasitemia) were maintained for growth experiments in synchronized culture.

2.2. Growth-promoting activity experiments

GFSRPMI was replaced by CRPMI supplemented with the test substances for growth experiments. Various substances were tested for their effects on growth at each developmental stage of *P. falciparum*: CRPMI containing bovine serum albumin free of NEFA (BSAF) at a final concentration of 3 mg/ml, except where otherwise stated, was further supplemented with various NEFA at concentrations of 100 μM of unsaturated NEFA and 60 μM of saturated NEFA, individually or in combination. Abbreviations, carbon-chain length, degree and position (delta) of unsaturation, isomerism, and common name of tested NEFA are shown in supplementary data. These include dodecanoic acid (C12:0), tetradecanoic acid (C14:0), C16:0, *cis*-9-hexadecenoic acid (C16:1), C18:0, *cis*-6-octadecenoic acid (C18:1-cis-6), C18:1-cis-9, *cis*-11-octadecenoic acid (C18:1-cis-11), *cis*-13-octadecenoic acid (C18:1-cis-13), *trans*-9-octadecenoic acid (C18:1-trans-9), *cis,cis*-9,12-octadecadienoic acid (C18:2), *cis*-5,8,11,14-eicosatetraenoic acid (C20:4), and docosanoic acid (C22:0). Unless stated otherwise, Pld supplements were added at concentrations of 15 μM 1,2-dioleoyl phosphatidic acid sodium salt, 130 μM 1,2-dioleoyl-sn-glycerol-3-phosphocholine, 25 μM 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (Fluka Biochemica, Switzerland), and 15 μM 1,2-dioleoyl-sn-glycero-3-phosphoserine, sodium salt. All the compounds were obtained from Sigma–Aldrich, except where otherwise specified.

Dried lipid precipitates were prepared, added to the culture media, and sterilized to reconstitute the lipids, as previously described (Asahi et al., 2005; Asahi, 2009).

2.3. Assessment of parasite growth

Samples were taken at 25 and 45 h (synchronized parasites), or at 96 h (asynchronized parasites) after inoculation, except where otherwise indicated. Thin smears were made and stained with Giemsa. Parasitemia was determined based on examination of more than 10,000 PRBC/RBC. The growth rate was estimated by dividing the parasitemia of the test sample 96 h after inoculation by the initial parasitemia. The numbers of PRBC were also measured by FCM (PAS, Partec Co. Ltd., Germany), as described previously (Izumiyama et al., 2009). Briefly, after fixation by the addition of 1% paraformaldehyde combined with Alsever's solution, PRBC (8×10^5 cells in a 16 μl aliquot of 0.5% PRBC/RBC suspension) were stained by mixing with 1 ml SYBR Green I (1× dilution, Invitrogen) prepared in 20 mM Tris (hydroxy-methyl) aminomethane hydrochloride at pH 8.8 (SYBR Green 1-basic). The levels of nucleic acid synthesis by PRBC were determined by examining more than 10,000 stained PRBC/RBC. Cell counts were analyzed with FCS express software (*De Novo* Software Inc., Canada). The number of parasite nuclei determined by the Giemsa method was proportional to the fluorescence intensity (RFU) of PRBC stained with SYBR Green I-basic. PRBC were located as three clusters in two-parameter dot plot presentations of PRBC/RBC from *P. falciparum* cultures: (1) cluster 1 contained predominantly ring forms with low DNA content (low RFU), (2) cluster 2 contained predominantly late trophozoites and young schizonts with moderate DNA content (moderate RFU), and (3) cluster 3 contained late schizonts and segmenters with high DNA content (high RFU).

Merozoites released from mature schizonts into the surrounding medium were also measured. The number of released merozoites was counted in more than 5000 PRBC. The number of merozoites in each schizont of the segmenter stage was examined by microscopic examination of Giemsa-stained slides.

For each experiment, PRBC were divided into identical aliquots and different treatments were performed simultaneously. To make the results comparable across experiments, untreated control cultures in GFSRPMI were prepared for each test. All experiments were repeated two to four times.

2.4. Statistical analysis

The significance of differences between means was evaluated using multifactorial analysis of variance. All calculations were performed using GraphPad PRISM 5 (GraphPad Software, Inc., USA). The *P* value for significance was 0.05, and all pairwise comparisons were made post hoc with Bonferroni's test. *y*-Axis error bars were added to indicate the standard deviation for each point in graphs, except where otherwise indicated.

3. Results

3.1. Different effects of various NEFA on growth of P. falciparum

The optimal and other representative combinations of NEFA were chosen on the basis of previous results (Asahi, 2009). Their growth-promoting activities were tested in parasites maintained in asynchronous cultures in different media containing uniform molar concentration of NEFA (100 μM of unsaturated and 60 μM saturated), Pld and BSAF for 96 h (2 cycles of complete growth). The different types and combinations of NEFA exerted markedly different effects on parasite growth in the presence/absence of Pld: the optimal combination of NEFA was C18:1-cis-9 plus C16:0 in the presence of Pld and BSAF, followed by the combinations of C18:1-cis-9 plus C18:0, and C18:1-cis-9 plus C14:0 (Fig. 1A). The combinations of C16:1 plus C16:0, C18:2 plus C16:0, and C18:1-cis-9 alone had much lower growth-promoting effects. Combinations of C18:1-cis-9 plus C12:0, C18:1-cis-9 plus C22:0, C20:4 plus C16:0, and only C16:0 were detrimental to parasite growth (Fig. 1A). C18:1-cis-9 plus C16:0 in the absence of Pld also had a lower growth-promoting effect. These results indicate that saturated or unsaturated NEFA with longer or shorter carbon-chain lengths than the optimal combination (C18:1-cis-9 plus C16:0) promoted growth to lesser extents, or were detrimental to growth of *P. falciparum*. Higher degrees of unsaturation of NEFA were also associated with detrimental effects on parasite growth. The combination of C18:1-trans-9 plus C16:0 also inhibited parasite growth, indicating that the growth-promoting effects of NEFA with 18 carbons and one double bond is specific to the *cis*-form.

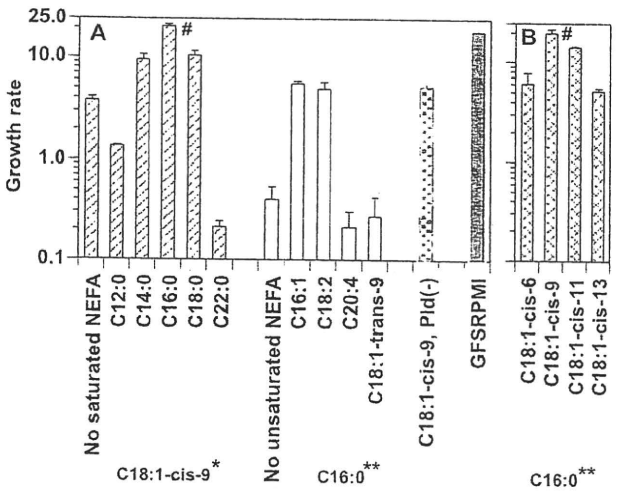

Fig. 1. Growth of *P. falciparum* in the presence of various NEFA (A) and isoforms of C18:1-cis-9 (B) added alone or in combination. Each saturated NEFA was added in the presence of 100 μM C18:1-cis-9 (*) and each unsaturated NEFA in the presence of 60 μM C16:0 (**). The culture media contained Pld and BSAF, except for GFSRPMI. NEFA (C18:1-cis-9 plus C16:0) plus BSAF in the absence of Pld, and GFSRPMI were tested for comparison. The initial parasitemia was adjusted to 0.3%. #No significant difference. Others were significantly different ($P<0.001$) compared with GFSRPMI.

Among the different NEFA isoforms, C18:1-cis-9 plus C16:0 had the highest growth-promoting effect, followed by C18:1-cis-11 plus C16:0, C18:1-cis-6 plus C16:0, and C18:1-cis-13 plus C16:0 (Fig. 1B); this indicates that the position of the double bond in NEFA with 18 carbons influences their growth-promoting effects.

To assess the effects of NEFA on each developmental stage of the parasite (schizogony, merozoite formation, and reinvasion of RBC), synchronized *P. falciparum* were cultured in the presence of Pld and BSAF, further supplemented with one or two NEFA. The distribution of the parasites among the different developmental stages was determined at 25 and 45 h during the first cycle of growth, using FCM with SYBR Green I-basic. This was based on our previous study, which demonstrated that the relative distribution of each developmental stage could be clearly defined at 25 and 45 h (Izumiyama et al., 2009). Late schizonts at 25 h (schizont-25 h), released merozoites at 45 h (released merozoite-45 h), ring forms at 45 h (ring form-45 h), and parasitemia at 45 h (parasitemia-45 h) were compared between parasites grown under test conditions and those grown in GFSRPMI (Fig. 2A and B). The types and combinations of NEFA exerted markedly different effects on parasite growth in the presence/absence of Pld and BSAF. Four typical growth patterns were defined: no inhibition (comparable growth); and three rate-determining steps in growth including suppressed schizogony (SS); suppressed formation of merozoites (SMF); and inhibited invasion of merozoites into new RBC (IMI)/formation of incomplete merozoites (Fig. 3A–D). An increase in newly formed healthy rings after invasion of RBC by released merozoites was considered to indicate normal merozoites.

As summarized in Table 1, all stages of the parasite cultured in medium supplemented with NEFA (C18:1-cis-9 plus C16:0), Pld, and BSAF were comparable to those grown in GFSRPMI. Medium containing C18:1-cis-9, C12:0, Pld and BSAF caused parasites to accumulate in cluster 1 (ring forms), by an SS effect. SS was also observed in the presence of C16:0 alone, C18:2 plus C16:0, C20:4 plus C16:0, C18:1-trans-9 plus C16:0, or Pld plus BSAF alone. Partial SS (less suppressed) was detected when C18:1-cis-13 plus C16:0, or BSAF alone were added. C18:1-cis-9 alone and C18:1-cis-9 plus C22:0 suppressed the progression of parasites to merozoites following schizont formation, by an SMF effect. SMF was also observed in parasites cultured in C18:1-cis-9 plus C16:0 in the absence of Pld, indicating that exogenous Pld was crucial for the development of complete merozoites. Adding C18:1-cis-13 plus C16:0 or C16:1 plus C16:0 to media containing Pld and BSAF caused accumulation of the

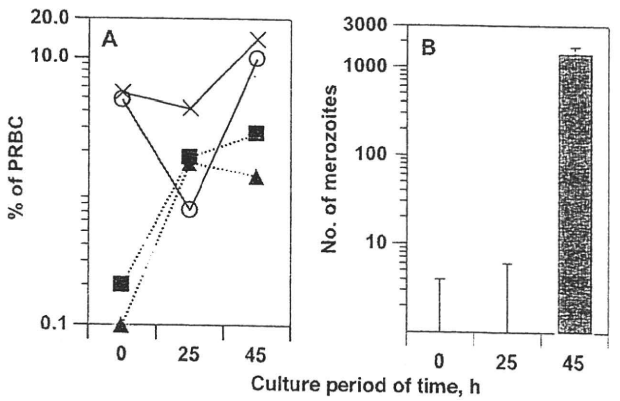

Fig. 2. Growth of *P. falciparum* in GFSRPMI. *P. falciparum* parasites were characterized by FCM at 25 and 45 h of synchronized culture: ring forms (—O—), late trophozoites and young schizonts (...■...), late schizonts (...▲...), and parasitemia (—X—) (A), and released merozoites (closed bars) (B). The numbers of merozoites are shown per 5000 PRBC. The baseline numbers of background merozoites in the cultures were subtracted.

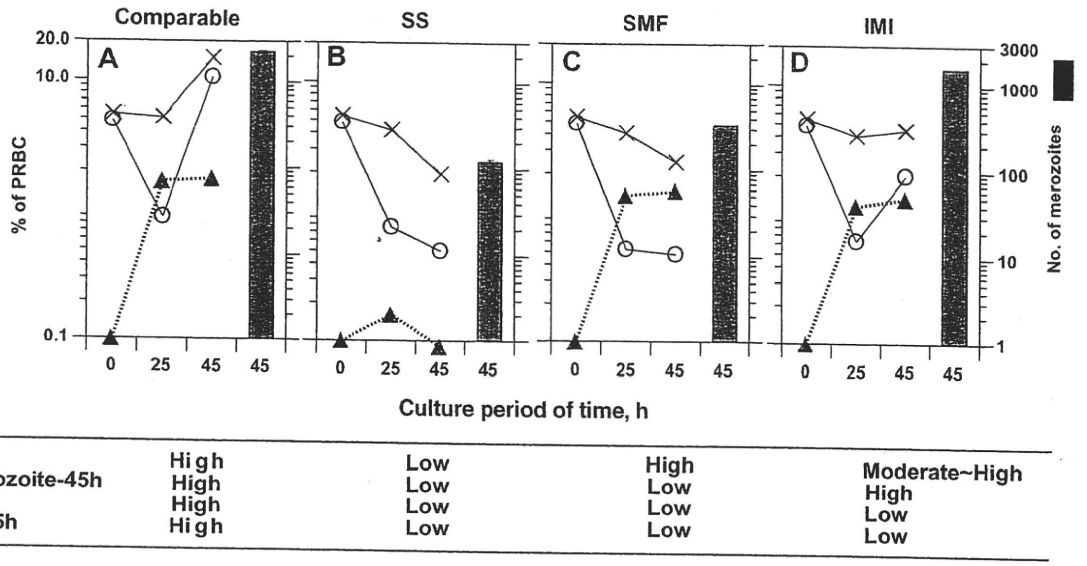

Fig. 3. Representative modification of growth of *P. falciparum* cultured in the presence of various growth promoters, indicating comparable growth (A), SS (B), SMF (C), and IMI (D). Parasites in synchronized culture were characterized by FCM and compared with complete growth in GFSRPMI: ring forms (—O—), late schizonts (...▲...), parasitemia (—X—), and released merozoites (closed bars).

Table 1
Growth-rate-determining step in development of *P. falciparum* cultured in the presence of NEFA alone or in combination.

Additives	Growth-rate-determining step	Schizont-25 h	Released merozoite-45 h	Ring form-45 h	Parasitemia-45 h
NEFA[a]					
C18:1-cis-9	SMF	83.4 ± 1.4	**23.6 ± 3.7**	4.8 ± 0.2	17.5 ± 2.4
C18:1-cis-9 + C12:0	SS	**38.3 ± 1.6**	23.2 ± 3.9	8.1 ± 0.3	24.8 ± 2.7
C18:1-cis-9 + C14:0	Partial IMI	99.4 ± 1.0	124.3 ± 10.1	**69.4 ± 1.5**	73.4 ± 3.2
C18:1-cis-6 + C16:0	Partial IMI	**75.8 ± 5.1**	106.9 ± 3.9	**72.1 ± 0.9**	62. ± 2.1
C18:1-cis-9 + C16:0	Comparable/better	102.5 ± 5.1	156.2 ± 15.7	106.3 ± 5.4	106.0 ± 8.3
C18:1-cis-11 + C16:0	Partial IMI	92.3 ± 1.9	143.2 ± 1.6	**66.7 ± 1.1**	75.8 ± 7.2
C18:1-cis-13 + C16:0	Partial SS, IMI	**66.7 ± 5.5**	89.9 ± 13.2	**38.1 ± 1.7**	52.7 ± 1.1
C18:1-cis-9 + C18:0	Partial IMI	**79.4 ± 0.5**	140.1 ± 14.6	**61.6 ± 1.8**	66.5 ± 3.4
C18:1-cis-9 + C22:0	SMF	90.2 ± 1.6	**20.1 ± 1.7**	3.7 ± 0.1	13.7 ± 2.2
C16:0	SS	**0.6 ± 0.1**	11.3 ± 1.6	10.0 ± 0.3	13.7 ± 2.2
C16:1 + C16:0	IMI	**71.8 ± 1.4**	114.2 ± 5.7	**20.1 ± 0.7**	31.8 ± 6.2
C18:2 + C16:0	SS	**28.4 ± 3.7**	64.7 ± 4.9	23.5 ± 0.5	33.9 ± 4.2
C20:4 + C16:0	SS	**15.9 ± 1.9**	46.3 ± 5.4	9.2 ± 0.5	11. ± 2.7
C18:1-trans-9 + C16:0	SS	**9.6 ± 1.4**	8.6 ± 0.5	5.1 ± 0.2	13.9 ± 0.1
C18:1-cis-9 + C16:0 + BSAF, Pld(−)[b]	SMF	109.6 ± 1.8	**18.4 ± 1.3**	13.6 ± 0.7	42.7 ± 8.4
Pld + BSAF[b]	SS	**24.1 ± 1.6**	37.2 ± 8.6	9.5 ± 0.1	19.8 ± 3.2
BSAF alone[b]	Partial SS, SMF	**54.3 ± 0.9**	**0.5 ± 0.6**	12.1 ± 0.2	25.9 ± 4.5
GFSRPMI	Complete growth	100.0 ± 2.4	100.0 ± 19.2	100.0 ± 4.2	100.0 ± 4.3

Data are expressed as % of control (GFSRPMI) ± standard error of mean.
[a] Each saturated NEFA at 60 μM and each unsaturated NEFA at 100 μM were added to CRPMI containing Pld and BSAF.
[b] The growth in C18:1-cis-9 plus C16:0 in the absence of Pld, Pld plus BSAF, and BSAF alone were tested for comparison. GFSRPMI served as a control for complete growth.
Bold letters indicate the first appearance of a marked reduction of each developmental stage.
* No significant difference; others were significantly different ($P < 0.001$) versus each developmental stage in GFSRPMI.

merozoites released from mature schizonts, but the merozoites did not invade new RBC, by the IMI effect. Partial IMI (less inhibited) was detected when C18:1-cis-9 plus C14:0, C18:1-cis-6 plus C16:0, C18:1-cis-11 plus C16:0, or C18:1-cis-9 plus C18:0 were added. Any effects on steps that governed parasite growth rate disrupted the cyclic behavior of the parasite, and reduced parasitemia-45 h. These results indicate that different NEFA exert distinct roles in parasite development by arresting development at different stages.

3.2. Microscopic examination of *P. falciparum* cultured in NEFA with IMI and SMF effects

Parasites cultured in medium containing NEFA (C18:1-cis-9 plus C16:0), Pld and BSAF were very similar, on microscopic examination, to parasites grown in GFSRPMI. In contrast, the majority of ring forms cultured in media containing C16:1 plus C16:0 or C18:1-cis-13 plus C16:0 (IMI effect) for 45 h were devoid of normal structures. The majority of schizonts cultured in media containing C18:1-cis-9 alone, C18:1-cis-9 plus C22:0, C18:1-cis-9 plus C16:0 in the absence of Pld, and BSAF alone (SMF effect) for 40–45 h were found to be degenerate.

The numbers of merozoites in each mature schizont/segmenter cultured synchronously in different media for 40–45 h were counted after staining with Giemsa. The numbers of merozoites in each schizont were only comparable to the results with GFSRPMI for cultures containing C18:1-cis-9 plus C16:0, in the presence of Pld and BSAF. The numbers of merozoites in each schizont grown in cultures containing NEFA that exerted the SMF effect (C18:1-cis-9 alone, C18:1-cis-9 plus C22:0, and C18:1-cis-9

plus C16:0 in the absence of Pld, and BSAF alone) were significantly reduced (Fig. 4). These results reflect the formation of abnormal schizonts in these culture media. The numbers of merozoites in each schizont obtained from cultures containing NEFA that exerted either IMI or partial IMI effects (C18:1-cis-9 plus C14:0, C18:1-cis-6 plus C16:0, C18:1-cis-11 plus C16:0, C18:1-cis-9 plus C18:0, or C16:1 plus C16:0) tended to be lower, but the differences were not significant, except in the case of C18:1-cis-13 plus C16:0 (Fig. 4). Ring form-45 h and parasitemia-45 h were significantly lower in these culture media, compared with those in GFSRPMI, despite the fact that the numbers of released merozoites-45 h were comparable (Table 1). These results indicate the possible formation of functionally abnormal merozoites, although the crisis form of merozoites is difficult to identify by light microscopy, because of their size and staining properties.

4. Discussion

The ability of several NEFA to sustain the growth of *P. falciparum* has been studied, with only limited success (Asahi et al., 2005; Mi-Ichi et al., 2006, 2007; Mitamura et al., 2000; Willet and Canfield, 1984). Paired NEFA, such as C18:1-cis-9 plus C16:0, and C18:1-cis-9 plus C18:0 effectively supported parasite growth (Asahi et al., 2005; Mi-Ichi et al., 2006, 2007; Mitamura et al., 2000), though mixtures of three or four NEFA were better than mixtures of two (Asahi et al., 2005). However, the maximal effectiveness of NEFA mixtures alone in sustaining parasite growth was much lower than that of GFSRPMI. The addition of Pld, essentially PC containing a specific fatty acid moiety, amplified the growth promoting ability of NEFA to an extent similar to GFSRPMI (Asahi, 2009). Nevertheless, NEFA could represent the dominant factors involved in growth promotion of *P. falciparum* in serum-free media, because Pld plus BSAF were unable to promote growth in the absence of NEFA. Also types, combinations and concentrations of NEFA effective for sustaining growth of the parasite were shown

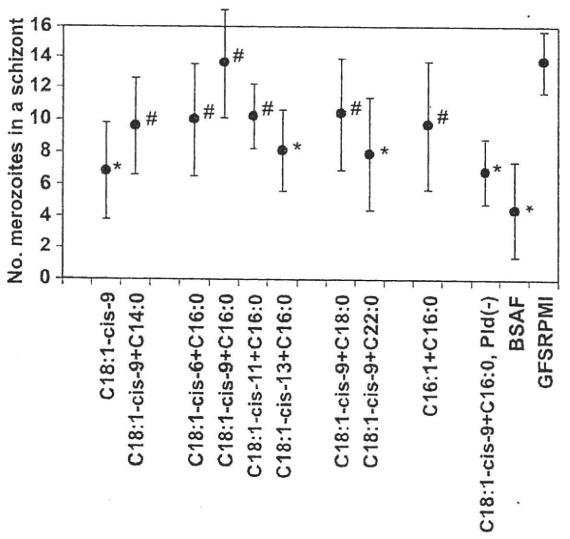

Fig. 4. Difference in numbers of merozoites in each mature schizont/segmenter. Parasites were cultured synchronously for 40–45 h in different media. All culture media, except for GFSRPMI, contained Pld and BSAF. Merozoites in schizonts cultured in NEFA (C18:1-cis-9 plus C16:0) + BSAF in the absence of Pld, BSAF alone, and GFSRPMI were also counted for comparison. Culture media containing C18:1-cis-9 plus C12:0, C16:0 alone, C18:2 plus C16:0, C20:4 plus C16:0, C18:1-trans-9 plus C16:0, or Pld plus BSAF were not included because they did not produce merozoites. Each bar represents the range of the numbers. #No significant difference; *significant difference ($P < 0.001$) vs. numbers of merozoites in a schizont cultured in GFSRPMI.

to be no difference between culture media enriched with and without Pld, though growth rates in the presence of Pld were much higher (Asahi, 2009; Asahi et al., 2005).

In the current study, we further investigated the effects of NEFA and Pld on the growth of *P. falciparum* at each developmental stage of the parasite. Distribution of the parasite among the different developmental stages was achieved in tightly synchronized cultures, and growth was compared with complete growth in GFSRPMI. The addition of different NEFA, individually or in combination, affected different steps that determined the rate of intraerythrocytic development of *P. falciparum*. Four typical growth patterns (SS, SMF, IMI, and no inhibition) were noted, and an effect on any growth step disrupted the cyclic behavior of the parasite and reduced parasitemia at 45 h. Thus different NEFA played distinct roles during intraerythrocytic development by sustaining development at different stages. These results reflect the varied parasitemia obtained in asynchronous 4-day-long cultures of the parasite in the presence of various combinations of NEFA. Certain structural characteristics of NEFA, such as carbon-chain length, degree and position of unsaturation, and isomerism are important for the development of the different stages of the parasite in RBC, as well as for general growth of the parasite. For example, unsaturated or saturated NEFA with longer or shorter carbon-chain length than C18:1-cis-9 or C16:0, higher degrees of unsaturation, and *trans*-forms resulted in much lower parasite growth, mainly through SS and SMF effects, although C18:1-cis-9 plus C14:0 and C18:1-cis-9 plus C18:0 sustained moderate growth by a partial IMI effect. Culture media producing IMI or partial IMI effects produced relatively high numbers of released merozoites at 45 h after inoculation, but the merozoites appeared to be unable to invade new RBC, because ring form-45 h was low. These results suggest that IMI may result from the formation of abnormal merozoites. The position of unsaturation of NEFA with 18 carbons and one double bond also influenced the growth of the parasite by IMI or partial IMI effects, as noted with isoforms of C18:1-cis-9.

While developing in RBC, malarial parasites may satisfy their requirements for nutrition and membrane formation using Pld from intrinsic metabolism (Holz, 1977; Maguire and Sherman, 1990; Vial and Ancelin, 1998). In addition to the *de novo* synthesis of Pld, it has also been demonstrated that RBC infected with *P. falciparum* or *P. knowlesi* readily take up intact Pld from exogenous sources (Grellier et al., 1991, Haldar, 1992; Haldar et al., 1989; Moll et al., 1988; Simoes et al., 1991, 1992). We previously tested various PC and other Pld, which, in combination with NEFA mixtures, are crucial components for complete parasite growth in chemically-defined culture media (Asahi, 2009). PC containing two C18:1-cis-9 markedly increased the low ability of the NEFA mixture to promote parasite growth, while other Pld exerted different effects on parasite growth. This suggests that certain structural characteristics of Pld, as well as NEFA, are important for parasite growth. It is also likely that Pld satisfies the requirements of merozoites in PRBC for membrane construction, because SMF was noted in the absence of Pld, despite the presence of the optimal combination of NEFA (C18:1-cis-9 plus C16:0). Further studies are needed to determine the mechanism(s) that underlie the actions of Pld in association with NEFA.

There is increasing evidence to suggest that NEFA are involved in numerous biological processes, including the activation of protein kinases, and cell proliferation, differentiation, and death (Diaz-Guerra et al., 1991, Hardy et al., 2005; Leroy et al., 2008; Malhi et al., 2006; Murakami et al., 1986). Saturated NEFA have been reported to increase oxidative stress, activate protein kinases associated with stress, and increase apoptosis of myocytes. Furthermore, low concentrations of C18:1-cis-9 or *cis*-5,8,11,14,17-eicosapentaenoic acid completely prevented cytotoxic stress induced by C16:0 (Hardy et al., 2000; Miller et al., 2005; Leroy

et al., 2008). Ring forms cultured in medium containing C16:0, Pld and BSAF did not develop into trophozoites and schizonts, as a result of the SS effect, while media containing C18:1-cis-9, Pld and BSAF allowed the development of ring forms into trophozoites and schizonts. The schizonts formed, however, had an abnormal morphology, and the merozoites released from mature schizonts were markedly suppressed by the SMF effect. In contrast, a mixture of two NEFA (C18:1-cis-9 plus C16:0) in the presence of Pld promoted parasite growth comparable to that seen in GFSRPMI. Similar observations have been described in non-optimal media, with poor overall parasite growth (Mi-Ichi et al., 2007). A range of apoptic markers has been described for *Plasmodium* spp., suggesting that *P. falciparum* experiences programmed cell death. However, the putative machinery for death of malaria parasite cells differs significantly from that in their human host (Deponte and Becker, 2004). Further investigations are needed to explain the mechanisms underlying the various actions of NEFA and their distinct roles during each developmental stage in *P. falciparum*.

Malarial parasites have long been believed to be unable to synthesize fatty acids via *de novo* biosynthesis; instead they were thought to depend on fatty acids scavenged from the host plasma and RBC to synthesize membrane lipids (Holz, 1977; Vial and Ancelin, 1998). However, on the basis of evidence indicating that machinery to synthesize type II fatty acids does exist in *P. falciparum*, we suggest that the parasite can itself synthesize fatty acids (Surolia and Surolia, 2001; Waller et al., 2003; Yeh and Altman, 2006). *P. falciparum* may thus satisfy its fatty acid requirements via two independent mechanisms: (1) by scavenging NEFA from the host plasma or from fatty acids released by the enzymatic action of lipases on the lipids, and (2) by *de novo* synthesis using the type II synthetase system. The results of the current study show that *P. falciparum* predominantly scavenges NEFA from the external milieu to promote growth, although it is unclear whether the NEFA are subsequently modified in any way (Krishnegowda and Gowda, 2003).

Acknowledgment

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.exppara.2010.11.001.

References

Asahi, H., 2009. *Plasmodium falciparum*: chemically defined medium for continuous intraerythrocytic growth using lipids and recombinant albumin. Experimental Parasitology 121, 22–28.

Asahi, H., Kanazawa, T., 1994. Continuous cultivation of intraerythrocytic *Plasmodium falciparum* in a serum-free medium with the use of a growth-promoting factor. Parasitology 109, 397–401.

Asahi, H., Kanazawa, T., Hirayama, N., Kajihara, Y., 2005. Investigating serum factors promoting erythrocytic growth of *Plasmodium falciparum*. Experimental Parasitology 109, 7–15.

Asahi, H., Kanazawa, T., Kajihara, Y., Takahashi, K., Takahashi, T., 1996. Hypoxanthine: a low molecular weight factor essential for growth of erythrocytic *Plasmodium falciparum* in a serum-free medium. Parasitology 113 (Pt 1), 19–23.

Bannister, L.H., Hopkins, J.M., Fowler, R.E., Krishna, S., Mitchell, G.H., 2000. A brief illustrated guide to the ultrastructure of *Plasmodium falciparum* asexual blood stages. Parasitology Today 16, 427–433.

Deponte, M., Becker, K., 2004. *Plasmodium falciparum* – do killers commit suicide? Trends in Parasitology 20, 165–169.

Diaz-Guerra, M.J., Junco, M., Bosca, L., 1991. Oleic acid promotes changes in the subcellular distribution of protein kinase C in isolated hepatocytes. The Journal of Biological Chemistry 266, 23568–23576.

Grellier, P., Rigomier, D., Clavey, V., Fruchart, J.C., Schrevel, J., 1991. Lipid traffic between high density lipoproteins and *Plasmodium falciparum*-infected red blood cells. The Journal of Cell Biology 112, 267–277.

Haldar, K., 1992. Lipid transport in *Plasmodium*. Infectious Agents and Disease 1, 254–262.

Haldar, K., de Amorim, A.F., Cross, G.A., 1989. Transport of fluorescent phospholipid analogues from the erythrocyte membrane to the parasite in *Plasmodium falciparum*-infected cells. The Journal of Cell Biology 108, 2183–2192.

Hardy, S., Langelier, Y., Prentki, M., 2000. Oleate activates phosphatidylinositol 3-kinase and promotes proliferation and reduces apoptosis of MDA-MB-231 breast cancer cells, whereas palmitate has opposite effects. Cancer Research 60, 6353–6358.

Hardy, S., St-Onge, G.G., Joly, E., Langelier, Y., Prentki, M., 2005. Oleate promotes the proliferation of breast cancer cells via the G protein-coupled receptor GPR40. The Journal of Biological Chemistry 280, 13285–13291.

Holz Jr., G.G., 1977. Lipids and the malarial parasite. Bulletin of the World Health Organization 55, 237–248.

Izumiyama, S., Omura, M., Takasaki, T., Ohmae, H., Asahi, H., 2009. *Plasmodium falciparum*: development and validation of a measure of intraerythrocytic growth using SYBR Green I in a flow cytometer. Experimental Parasitology 121, 144–150.

Jensen, J.B., 1979. Some aspects of serum requirements for continuous cultivation of *Plasmodium falciparum*. Bulletin of the World Health Organization 57 (Suppl. 1), 27–31.

Krishnegowda, G., Gowda, D.C., 2003. Intraerythrocytic *Plasmodium falciparum* incorporates extraneous fatty acids to its lipids without any structural modification. Molecular Biochemical Parasitology 132, 55–58.

Leroy, C., Tricot, S., Lacour, B., Grynberg, A., 2008. Protective effect of eicosapentaenoic acid on palmitate-induced apoptosis in neonatal cardiomyocytes. Biochimica et Biophysica Acta 1781, 685–693.

Maguire, P.A., Sherman, I.W., 1990. Phospholipid composition, cholesterol content and cholesterol exchange in *Plasmodium falciparum*-infected red cells. Molecular Biochemical Parasitology 38, 105–112.

Malhi, H., Bronk, S.F., Werneburg, N.W., Gores, G.J., 2006. Free fatty acids induce JNK-dependent hepatocyte lipoapoptosis. The Journal of Biological Chemistry 281, 12093–12101.

Mi-Ichi, F., Kita, K., Mitamura, T., 2006. Intraerythrocytic *Plasmodium falciparum* utilize a broad range of serum-derived fatty acids with limited modification for their growth. Parasitology 133, 399–410.

Mi-Ichi, F., Kano, S., Mitamura, T., 2007. Oleic acid is indispensable for intraerythrocytic proliferation of *Plasmodium falciparum*. Parasitology 134, 1671–1677.

Miller, T.A., LeBrasseur, N.K., Cote, G.M., Trucillo, M.P., Pimentel, D.R., Ido, Y., Ruderman, N.B., Sawyer, D.B., 2005. Oleate prevents palmitate-induced cytotoxic stress in cardiac myocytes. Biochemical and Biophysical Research Communications 336, 309–315.

Mitamura, T., Hanada, K., Ko-Mitamura, E.P., Nishijima, M., Horii, T., 2000. Serum factors governing intraerythrocytic development and cell cycle progression of *Plasmodium falciparum*. Parasitology International 49, 219–229.

Moll, G.N., Vial, H.J., Ancelin, M.L., Op den Kamp, J.A., Roelofsen, B., van Deenen, L.L., 1988. Phospholipid uptake by *Plasmodium knowlesi* infected erythrocytes. FEBS Letters 232, 341–346.

Murakami, K., Chan, S.Y., Routtenberg, A., 1986. Protein kinase C activation by cis-fatty acid in the absence of Ca2+ and phospholipids. The Journal of Biological Chemistry 261, 15424–15429.

Ridley, R.G., 2002. Medical need, scientific opportunity and the drive for antimalarial drugs. Nature 415, 686–693.

Simoes, A.P., Moll, G.N., Slotboom, A.J., Roelofsen, B., Op den Kamp, J.A.F., 1991. Selective internalization of choline-phospholipids in *Plasmodium falciparum* parasitized human erythrocytes. Biochimica et Biophysica Acta 1063, 45–50.

Simoes, A.P., Roelofsen, B., Op den Kamp, J.A.F., 1992. Lipid compartmentalization in erythrocytes parasitized by *Plasmodium* spp. Parasitology Today 8, 18–20.

Snow, R.W., Guerra, C.A., Noor, A.M., Myint, H.Y., Hay, S.I., 2005. The global distribution of clinical episodes of *Plasmodium falciparum* malaria. Nature 434, 214–217.

Surolia, N., Surolia, A., 2001. Triclosan offers protection against blood stages of malaria by inhibiting enoyl-ACP reductase of *Plasmodium falciparum*. Nature Medicine 7, 167–173.

Vial, H.J., Ancelin, M.L., 1998. Malarial lipids. In: Sherman, I.W. (Ed.), Malaria: Parasite Biology, Pathogenesis, and Protection. AMS Press, Washington, DC, USA, pp. 159–175.

Waller, R.F., Ralph, S.A., Reed, M.B., Su, V., Douglas, J.D., Minnikin, D.E., Cowman, A.F., Besra, G.S., McFadden, G.I., 2003. A type II pathway for fatty acid biosynthesis presents drug targets in *Plasmodium falciparum*. Antimicrobial Agents and Chemotherapy 47, 297–301.

Willet, G.P., Canfield, C.J., 1984. *Plasmodium falciparum*: continuous cultivation of erythrocyte stages in plasma-free culture medium. Experimental Parasitology 57, 76–80.

Yeh, I., Altman, R.B., 2006. Drug Targets for *Plasmodium falciparum*: a post-genomic review/survey. Mini-Reviews in Medicinal Chemistry 6, 177–202.

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SHORT COMMUNICATION

A human case of subcutaneous dirofilariasis caused by *Dirofilaria repens* in Vietnam: histologic and molecular confirmation

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Abstract Human dirofilariasis caused by infection with *Dirofilaria* worms has been frequently reported. The symptoms associated with infection by these filarial parasites, which are transmitted to humans by zooanthropophilic mosquitoes, are characterized by mainly pulmonary and subcutaneous nodules. Here, we report the first case in Vietnam of a subcutaneous dirofilariasis with a painful nodule in the right eyelid. An immature female worm was removed by excisional biopsy and identified as *Dirofilaria repens* by histology and DNA analysis.

Nucleotide sequence data reported in the present paper are deposited in the DDBJ/GenBank/EMBL databases under the accession numbers AB547465 and AB547466 for the cytochrome c oxidase subunit 1 and 12S rRNA genes, respectively.

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Introduction

The genus *Dirofilaria* (Nematoda; Filarioidea, Onchocercidae), which includes etiologic agents such as *Dirofilaria immitis*, *Dirofilaria repens*, *Dirofilaria tenuis*, and *Dirofilaria ursi*, is responsible for the increased occurrence of zoonotic dirofilariasis in humans around the world (Pampiglione and Rivasi 2000). Of these species, *D. repens* is a habitual parasite in the subcutaneous tissue of dogs and other carnivores that are transmitted by zooanthropophilic mosquitoes (Culicidae). Human infections by this parasite also occur and more than 780 cases have been reported in over 37 countries in Europe, Southeast Asia, and Africa (Pampiglione et al. 1995; Pampiglione and Rivasi 2000). In Asia, human cases of *D. repens* infection have been reported in Iran, India, Sri Lanka, Malaysia, Thailand, China (Pampiglione and Rivasi 2000) and Japan (McLean et al. 1979), but such case has not yet been reported in Vietnam. In its definitive hosts, *D. repens* can easily be identified by its morphological features; however, in non-definitive hosts such as humans, these filarial worms may be more difficult to identify using morphological and histological characteristics, particularly when the clinical samples that have been removed from the subcutaneous nodules have become damaged and/or immature worms were found (Pampiglione et al. 1999). Serological examination is a useful tool for differentiating between *D. repens*

and *D. immitis* infections (Santamaría et al. 1995), while elevated levels of IgM, IgG and IgE have been used in patients exposed to other *Dirofilaria* species (Simón et al. 1997; Orihel and Eberhard 1998). In order to overcome the limitations associated with serological and histological examinations, polymerase chain reaction (PCR)-based methods capable of differentiating between *Dirofilaria* species have been developed (Favia et al. 1996, 1997a, b, 2000; Rivasi et al. 2006; Marušić et al. 2008). Here, we report the first human case of *D. repens* infection in Vietnam, diagnosed definitively by histology and PCR-based DNA analysis.

Case report

The patient was a 30-year-old Vietnamese man residing in the Ha Dong district of Hanoi, the capital of Vietnam. In 2008, the patient presented at Ha Dong Hospital complaining of a painful, itchy, swollen and tangible nodule, measuring 1.0×0.5 cm on the right eyelid. The patient was treated with an antibiotic for the first week, but the clinical symptoms did not change. No routine laboratory data, such as white blood and eosinophil counts in blood were available. On April 19, 2008, surgical biopsy was performed with the aim of diagnosis and treatment. As a result, a long, slender, whitish and living worm, approximately 4.5 cm in length and 0.5 mm in width, was removed (Fig.1a) and clinical signs disappeared after the biopsy. The removed worm was fixed in 10% formalin and was kept at the National Institute of Malariology, Parasitology and Entomology (NIMPE) in Hanoi, Vietnam before being sent to the Department of Parasitology, National Institute of Infectious Diseases in Tokyo, Japan for identification of the parasite to the species level in 2009.

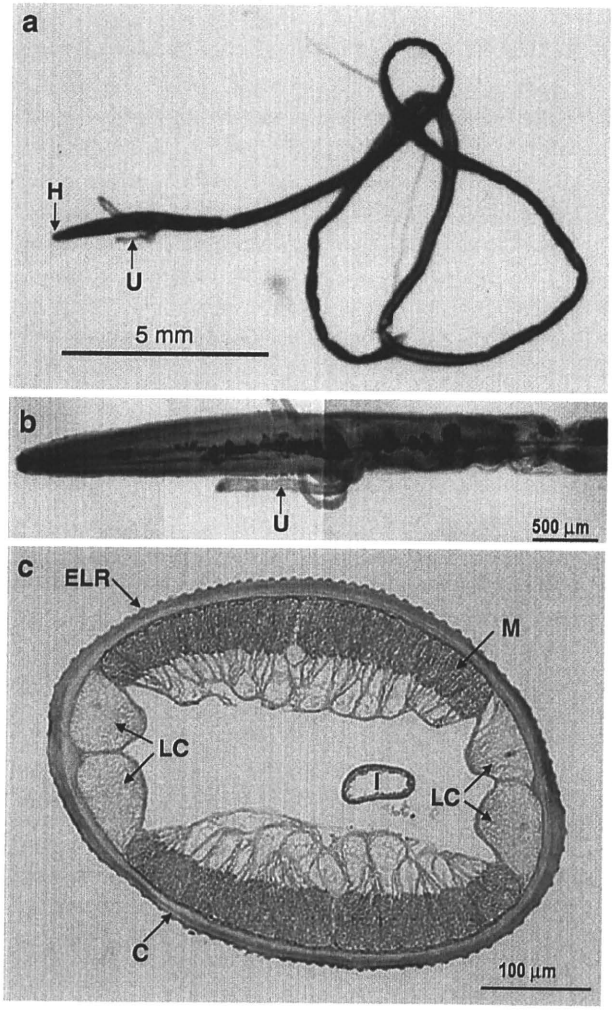

Fig. 1 Microscopic appearance and transverse section of the filarial worm surgically removed from the subcutaneous tissue of a human eyelid. **a** Removed immature female measuring approximately 4.5 cm in length and 500 µm in width. *H* head, *U* uterus. **b** Magnification of the anterior end of the worm. **c** Transverse section of the worm. Note the conspicuous external longitudinal ridges (*ELR*), polymyarian-type musculature (*M*), enlarged lateral chords (*LC*), multilayered cuticle (*C*), and intestine (*I*). Hematoxylin-eosin stain

Materials and methods

A part of the formalin-fixed worm kept at the NIMPE was processed for paraffin-embedded specimen and then transverse sections were stained with hematoxylin and eosin and were observed microscopically.

To confirm the morphological identification, molecular analysis was performed as follows: the remaining worm was homogenized in a small amount of the ATL buffer supplied with a DNeasy Blood & Tissue kit (Qiagen, Germany) before DNA was extracted overnight using the same kit. The mitochondrial cytochrome c oxidase subunit 1 (cox1) and 12S ribosomal RNA (12S rRNA) genes of the parasite were then amplified by PCR. Since the DNA samples were degraded by formalin fixation, short DNA sequences were amplified using the following primer pairs: Diro cox1/F255 (5′-GGTGCTATTAATTTTATGGTTACT-3′) and Diro cox1/R434 (5′-AAAAGAAGTATTAAAATTACGATC-3′), which were designed based on the cox1 gene sequences of *D. repens* (AM749234) and *D. immitis* (EU159111); and Diro 12S rRNA/F196 (5′-GTTTTGTTTAAACCGAA AAAATATT-3′) and Diro 12S rRNA/R374 (5′-TAAGCCA AATATATATCTGTTTTAA3′), which were designed based on the 12S rRNA gene sequences of *D. repens* (AJ544832) and *D. immitis* (EU182327). In addition, the following three primer pairs specific for 12S rRNA gene of *D. repens* were also used: Dr/F1 (5′-TCATTTTAATTTTTAACTC TATTT-3′) and Dr/R160 (5′-ATTAATAAACTTTGATTA

CCTGGG-3′), Dr/F121 (5′-TTGAACTGGATTAG TAACCCAGGT-3′) and Dr/R284 (5′-CTAAACAATCA TACATGTGCCAATA-3′), and Dr/F260 (5′-TATTGGCA CATGTATGATTGTTTAG-3′) and Dr/R443 (5′-CACAT AAGAAAAAATTCTTTCTT-3′), which were designed based on accession number AJ544832. PCR amplification of the target DNAs was performed in a 50-μL reaction mixture with Ex Taq DNA polymerase (Hot Start version, Takara Bio, Japan) and 35 cycles was performed consisting of denaturation (94°C, 30 s), annealing (58°C, 30 s) and extension (72°C, 60 s), with a final elongation step of 72°C for 5 min. The amplicons were confirmed by capillary electrophoresis (HAD-GT12, eGene Inc., CA) and were purified for use as templates for direct DNA sequencing using a NucleoSpin Extract II kit (Macherey-Nagel, Germany). Samples for sequencing were prepared using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems Inc., CA) and sequencing was performed on an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems Inc., CA). Sequence data were analyzed using the EditSeq and MegAlign programs of the DNASTAR package Inc. (DNASTAR, Madison, WI).

Results and discussion

The parasite exhibited movement at the time of surgical extraction. The body length measured 4.5 cm because the posterior part of the body was lacking (Fig. 1a). The uterus, which was protruding from the anterior end of the body at the time of removal, did not contain any larval microfilariae (Fig. 1a, b). A transverse section of the parasite (Fig. 1c) revealed that the parasite had a diameter of approximately 400 μm, a thick, laminated cuticle with external longitudinal ridges (99 ridges all around the nematode surface), and a well-developed, polymyarian-type musculature interrupted by two large lateral chords, all of which are characteristic of immature *D.* (*Nochtiella*) *repens* female worms.

In the molecular analysis, the target DNAs were successfully amplified by nested PCR using primer pairs specific for *D. repens*, but not primer pairs specific for *D. immitis* (data not shown). Figure 2 shows the alignment of the PCR-amplified cox1 (a, 123 bp) and 12S rRNA genes (b, 362 bp) nucleotide sequences. Homology search revealed sequence similarities of 97.6% and 91.1% against the cox1 genes of the *D. repens* (AM749233) and *D. immitis* reference sequences (EU159111), respectively. For the 12S rRNA gene, homology with the *D. repens* reference sequence (AM779772) reached as high as 99.2%; whereas, homology with the *D. immitis* reference sequence (EU182327) was lower at 85.9 %. In addition, the obtained sequence data also confirmed that the filarial worm in this case was *D. repens*.

Considerable variation has been observed in the dimensions of male and female *D. repens* recovered from a variety of definitive hosts around the world. Compared with other *Dirofilaria* worms, the *D. repens* is a robust parasite with a maximum female and male body length of 17 cm and approximately 7 cm, respectively and a diameter of 650 μm and 450 μm, respectively, have been reported

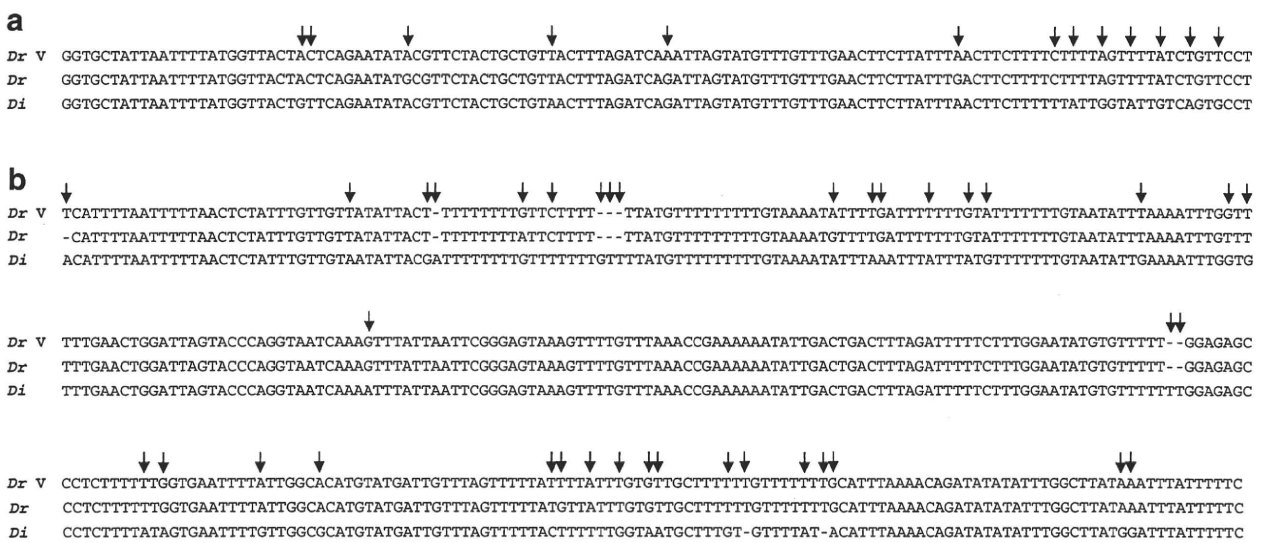

Fig. 2 Alignment of the PCR-amplified cox1 and 12S rRNA gene sequences. **a** Cox1 gene; *Dr V, D. repens* obtained from a Vietnamese patient; *Dr*, *D. repens* (AM749233); and *Di*, *D. immitis* (EU159111). **b** 12S rRNA gene; *Dr V, D. repens* from this case; *Dr*, *D. repens* (AM779772); *Di*, *D. immitis* (EU182327). *Arrows* indicate differences between nucleotides of *D. repens* and *D. immitis*

(Orihel and Eberhard 1998). In cases of human infection, *D. repens* measuring up to approximately 660 μm in diameter have been observed (Gutierrez 1984; Pampiglione et al. 1995). In this case, the maximum body width measured approximately 500 μm. Although an extensive review of human infections by *D. repens* found that female worms were more prevalent than males (Pampiglione and Rivasi 2000), a male and a female worm have been observed in the same nodule (Mrad et al. 1999), and also in separate nodules (Fernando et al. 2000). In the majority of cases, nodules are located in subcutaneous and subconjunctival tissues, deep dermis and submucosa. According to Pampiglione and Rivasi (2000), localization is more frequent in the upper half of the body (74%), mostly in the ocular region (35.3%) and the upper limbs (11%). Although rare, localization in muscle tissue, lymph nodes (Maltezos et al. 2002) and in the deep viscera (Pampiglione and Rivasi 2000) has been reported. In this case, the nodule was located in the eyelid.

The identification of *Dirofilaria* worms can only be made with certainty after a biopsy. The morphological features of individual parasites are as follows: body length and width, thickness of the laminated cuticle, the presence and/or absence of external longitudinal ridges on the surface of the cuticle, lateral chords, and number and type of circumferential muscle cells (Orihel and Eberhard 1998; Ratnatunga and Wijesundera 1999; Pampiglione et al. 1999). However, depending on the quality of the histological sections, some of these morphological features may not be absolute. In addition, identification of the causative *Dirofilaria* agent is also closely correlated with geographic areas affected with individual *Dirofilaria* species (Orihel and Eberhard 1998).

The most precise method for identifying *Dirofilaria* worms is, therefore, molecular identification using biopsied tissues and/or parasite material. Several PCR-based diagnostic methods have been developed using DNA samples prepared from fresh and ethanol-fixed tissues (Chandrasekharan et al. 1994; Favia et al. 1996, 1997a, 2000; Cancrini et al. 1998; Vakalis et al. 1999, 2002), as well as formalin-fixed and paraffin-embedded specimens (Favia et al. 1997a; Vakalis et al. 1999, 2002; Rivasi et al. 2006; Marušić et al. 2008). These PCR-based approaches target the internal transcribed spacer two region and tandemly repeated sequence of the cuticular surface antigen gene. The combination of the PCR-based assay with DNA sequencing of the mitochondrial DNA (cox1 and 12S rRNA genes) using formalin-fixed specimens reported here is considered to be useful for the precise identification of the *Dirofilaria* species. Such DNA analyses are useful not only for diagnostic investigations, but also for retrospective studies using histopathological specimens fixed in formalin (Yamasaki et al. 2007).

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References

Chandrasekharan NV, Karunanayake EH, Franzen L, Abeyewickreme W, Pettersson U (1994) *Dirofilaria repens*: cloning and characterization of repeated DNA sequence for the diagnosis of dirofilariasis in dogs, *Canis familiaris*. Exp Parasitol 78:279–286

Cancrini G, Favia G, Giannetto S, Merulla R, Russo R, Ubaldino V, Tringai R, Pietrobelli M, Del Nero L (1998) Nine more cases of human infection by *Dirofilaria repens* diagnosed in Italy by morphology and recombinant DNA technology. Parassitologia 40:461–466

Favia G, Lanfrancotti A, Della Torre A, Cancrini G, Coluzzi M (1996) Polymerase chain reaction—identification of *Dirofilaria repens* and *D. immitis*. Parasitology 113:567–571

Favia G, Lanfrancotti A, Della Torre A, Cancrini G, Coluzzi M (1997a) Advances in the identification of *Dirofilaria repens* and *Dirofilaria immitis* by a PCR-based approach. Parassitologia 39:401–402

Favia G, Tringali R, Cancrini G (1997b) Molecular diagnosis of human dirofilariasis. Ann Trop Med Parasitol 91:961–962

Favia G, Cancrini G, Ricci I, Bazzocchi C, Magi M, Pietrobelli M, Genchi C, Bandi C (2000) 5S ribosomal spacer sequences of some filarial parasites: comparative analysis and diagnostic applications. Mol Cell Probes 14:285–290

Fernando SD, Ihalamulla RL, De Silva WAS (2000) Male and female filarial worms *Dirofilaria* (*Nochtiella*) *repens* recovered from the scrotum. Ceylon Med J 45:131–132

Gutierrez Y (1984) Diagnostic features of zoonotic filariae in tissue sections. Hum Pathol 15:514–525

Maltezos ES, Sivridis EL, Giatromanolaki AN, Simopoulos CE (2002) Human subcutaneous dirofilariasis: a report of three cases manifesting as breast or axillary nodules. Scott Med J 47:86–88

Marušić Z, Stastny T, Kirac I, Stojčević D, Krušlin B, Tomas D (2008) Subcutaneous dirofilariasis caused by *Dirofilaria repens* diagnosed by histopathologic and polymerase chain reaction analysis. Acta Dermatovenerol Croat 16:222–225

McLean JD, Beaver PC, Michalek H (1979) Subcutaneous dirofilariasis in Okinawa, Japan. Am J Trop Med Hyg 28:45–48

Mrad K, Romani-Ramah S, Driss M, Bougrine F, Hechiche M, Maalej M, Romdhane KB (1999) Mammary dirofilariasis. A case report. Int J Surg Pathol 7:175–178

Orihel TC, Eberhard ML (1998) Zoonotic filariasis. Clin Micrbiol Rev 11:366–381

Pampiglione S, Rivasi F (2000) Human dirofilariasis due to *Dirofilaria* (*Nochtiella*) *repens*: an update of world literature from 1995 to 2000. Parassitologia 42:231–254

Pampiglione S, Canestri Trotti G, Rivasi F (1995) Human dirofilariasis due to *Dirofilaria* (*Nochtiella*) *repens*: a review of world literature. Parassitologia 37:149–193

Pampiglione S, Rivasi F, Canestri-Trotti G (1999) Pitfalls and difficulties in histological diagnosis of human dirofilariasis due to *Dirofilaria* (*Nochtiella*) *repens*. Diagn Microbiol Infect Dis 34:57–64

Ratnatunga NVI, de Wijesundera MS (1999) Histopathological diagnosis of subcutaneous *Dirofilaria repens* infection in humans. Southeast Asian J Trop Med Public Health 30:375–378

Rivasi F, Boldorini R, Criante P, Leutner M, Pampiglione S (2006) Detection of *Dirofilaria* (*Nochtiella*) *repens* DNA by polymerase chain reaction in embedded paraffin tissues from two human pulmonary locations. APMIS 114:567–574

Santamaría B, Di Sacco B, Muro A, Genchi C, Simón F, Cordero M (1995) Serological diagnosis of subcutaneous dirofilariasis. Clin Exp Dermatol 20:19–21

Simón F, Prieto G, Muro A, Cancrini G, Cordero M, Genchi C (1997) Human humoral response to *Dirofilaria* species. Parassitologia 39:397–400

Vakalis N, Spanakos G, Patsoula E, Vamvakopoulos NC (1999) Improved detection of *Dirofilaria repens* DNA by direct polymerase chain reaction. Parasitol Int 48:145–150

Vakalis N, Vougioukas N, Patsoula E, Spanakos G, Sioutopoulou DO, Vamvakopoulos NC (2002) Genotypic assignment of infection by *Dirofilaria repens*. Parasitol Int 51:163–169

Yamasaki H, Nakaya K, Nakao M, Sako Y, Ito A (2007) Significance of molecular diagnosis using histopathological specimens in cestode zoonoses. Trop Med Health 35:307–321

寄生虫症の臨床検査—外来でできる検査手法と依頼検査の依頼法の実際—

免疫血清検査と遺伝子検査で確認できる寄生虫と依頼方法

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はじめに●

寄生虫症の検査法には，糞便検査によって寄生虫の虫卵や原虫の嚢子などを検出する方法や駆虫や手術によって虫体が得られた場合にはそれを調べて直接的に寄生虫の存在を確認する方法がある．一方，幼虫移行症のようにヒト体内で成虫に発育できない寄生虫の場合には，虫卵が排出されないので，寄生虫に対する特異抗体や寄生虫抗原を検出することによって，間接的に寄生虫感染を証明する方法がある．間接的な方法は悪性腫瘍など外科手術が適用になる疾患との鑑別などにも用いられる．本稿では，紙面の都合上，寄生蠕虫症に絞った抗体検出による免疫血清学的方法(以下，血清検査)と寄生虫のDNA検出とその解析に基づいた遺伝子検査法について実例を紹介する．

免疫血清学的検査●

抗体検出による血清検査が有効とされる寄生虫症は**表1**に示した．ここで述べる血清検査は寄生虫抗原に対して産生される特異抗体を，ペルオキシダーゼあるいはアルカリホスファターゼなど酵素を標識した二次抗体と順次反応させ，最後に酵素に対する基質を加えて発色させ，可視化するという原理に基づいている．しかし，検査に必要な抗原調製法や検査法，あるいは判定基準は検査実施機関ごとに異なり，標準化されていないのが現状である．血清検査の実践的手技，感度や特異性などに関してはすでにいくつかの総説があるので[1]，それらを参照願いたい．

1.　酵素抗体法(ELISA)

本法は簡便で検出感度が高く，寄生虫症の検査に汎用されている．ポリスチレン製96穴マイクロプレートの穴(well)に抗原を結合させ，被検者血清(胸水，硝子体液など)，標識二次抗体と順次反応させ，最後に基質を加え，吸光度を測定して判定する方法である．一度に多検体の検査が可能であるので，スクリーニング検査に向いている．**図1**は胸水貯留が認められた患者について，肺に寄生する可能性のある4種類の寄生虫を想定して行ったスクリーニング検査例である．この患者の場合，ウェステルマン肺吸虫抗原に対する特異抗体が検出された．筆者らの研究室では，標識二次抗体として抗ヒトIgG抗体に替わり，ペルオキシダーゼ標識Protein Gを用いている．

表1　抗体検出による免疫血清学的検査が有効とされる寄生虫症

原虫症	
	赤痢アメーバ症
	トキソプラズマ
	マラリア
蠕虫症	
	イヌ回虫症・ネコ回虫症(トキソカラ症)
	アニサキス症
	広東住血線虫症
	顎口虫症
	旋尾線虫症
	イヌ糸状虫症
	旋毛虫症
	ウェステルマン肺吸虫症
	宮崎肺吸虫症
	肝蛭症
	住血吸虫症
	マンソン孤虫症
	有鉤嚢虫症
	多包虫症・単包虫症(エキノコックス症)

2.　ウェスタンブロット(イムノブロット)

寄生虫抗原成分(蛋白質)をSDS-ポリアクリルアミドゲル電気泳動によって分離した後，抗原を物理的，化学的耐久性に富むポリフッ化ビニリデン(PVDF)膜やニトロセルロース膜に電気的に転写する．さらに，膜上の抗原と被検者血清，標識二次抗体，発色基質を順次加えると，膜上に着色

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●抗体検出を目的とした血清検査でも感染初期や単数寄生例では抗体は検出されにくい.
●抗体陽性の場合，寄生虫症の既往をみている可能性も考えられる.

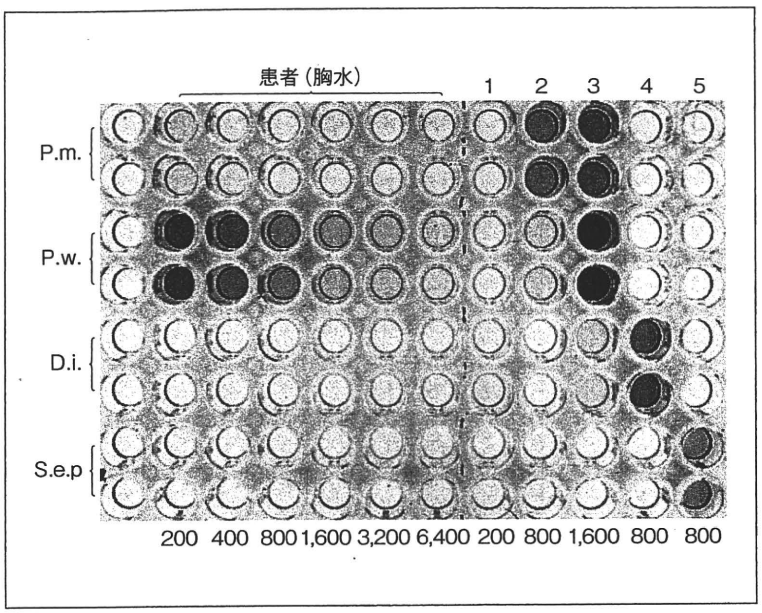

図1　酵素抗体法による寄生虫症のスクリーニング検査
患者胸水を用いて4種類の寄生虫抗原に対する特異的IgG抗体の検出を行ったところ，ウェステルマン肺吸虫抗原(P.w.)に対する抗体が検出された.
P.m.：宮崎肺吸虫抗原，P.w.：ウェステルマン肺吸虫抗原，D.i.：イヌ糸状虫抗原，S.e.p.：マンソン孤虫抗原.
1. 陰性対照血清，2. 宮崎肺吸虫症患者血清，3. ウェステルマン肺吸虫症患者血清，4. イヌ糸状虫症患者血清，5. マンソン孤虫症患者血清.
plate下の数字は胸水ならびに血清の希釈倍率.

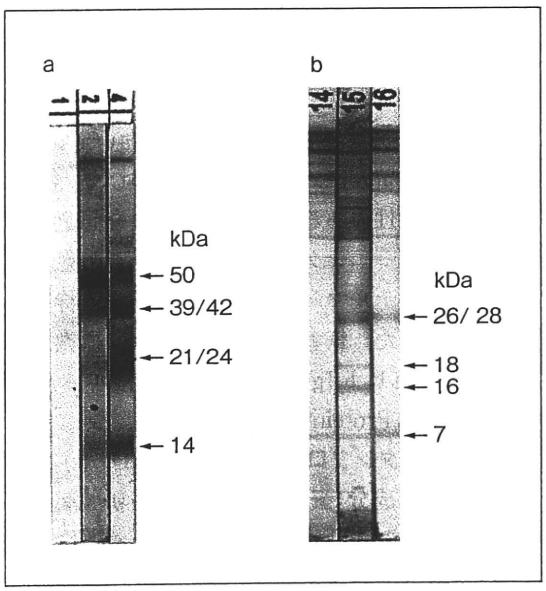

図2　ウェスタンブロットによる検査
a　有鉤嚢虫症，b　エキノコックス症
1, 14：陰性対照血清，2, 15：陽性対照血清，4, 16：被検患者血清.

したバンドとして可視化される．**図2**は市販の有鉤嚢虫症検査キット(Immunetics社)とエキノコックス症検査キット(LDBIO DIAGNOSTICS社)を用いた検査例を示した．それぞれの寄生虫に特異的な分子サイズの抗原に対するバンドが検出されるか否かで判定する．確定検査に適した方法である.

3.　イムノクロマト

抗原を吸着させたニトロセルロース膜を固相(担体)としてデバイスに内蔵し，小穴に被検者血清，展開液，標識二次抗体，発色基質を順次滴下すると，抗体が抗原部位まで移動してその部位が発色する．移動相が垂直式と水平式のものがあり，**図3**には垂直式のトキソカラ症検査キットを示した[2]．陽性であれば小穴の中心部に赤色スポットが出現する．国立感染症研究所寄生動物部(以下，感染研)でも，遺伝子組換え抗原を吸着させた水平式のトキソカラ症検査キットを開発中で