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.

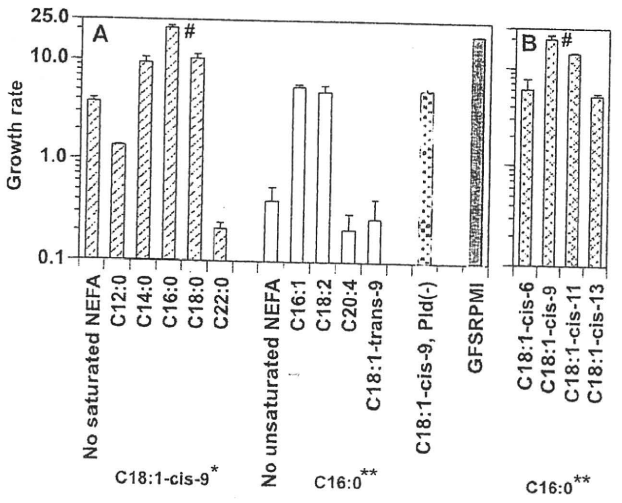

Fig. 1. Growth of *P. falciparum* in the presence of various NEFA (A) and isoforms of C18:1-cis-9 (B) added alone or in combination. Each saturated NEFA was added in the presence of 100 μM C18:1-cis-9 (*) and each unsaturated NEFA in the presence of 60 μM C16:0 (**). The culture media contained Pld and 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

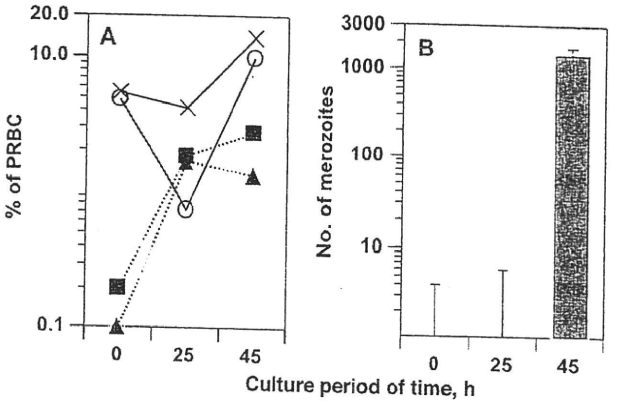

Fig. 2. Growth of *P. falciparum* in GFSRPMI. *P. falciparum* parasites were characterized by FCM at 25 and 45 h of synchronized culture: ring forms (—O—), late trophozoites and young schizonts (...■...), late schizonts (...▲...), and parasitemia (—X—) (A), and released merozoites (closed bars) (B).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

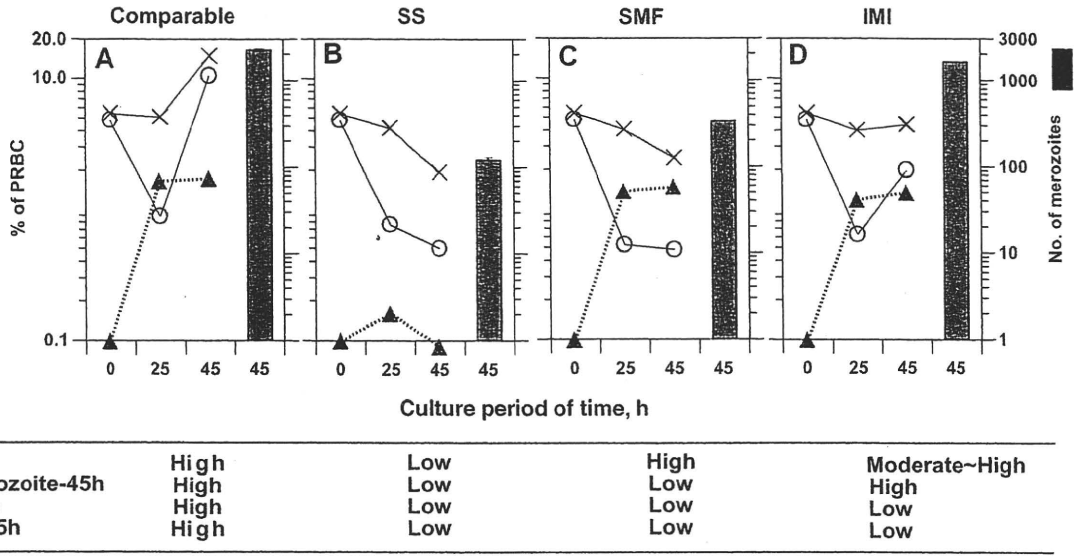

Fig. 3. Representative modification of growth of *P. falciparum* cultured in the presence of various growth promoters, indicating comparable growth (A), SS (B), SMF (C), and IMI (D). Parasites in synchronized culture were characterized by FCM and compared with complete growth in GFSRPMI: ring forms (—O—),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

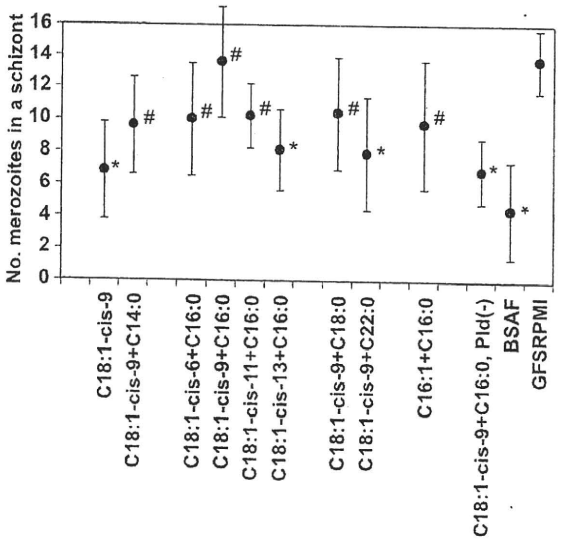

Fig. 4. Difference in numbers of merozoites in each mature schizont/segmenter. Parasites were cultured synchronously for 40–45 h in different media. All culture media, except for GFSRPMI, contained Pld and BSAF. Merozoites in schizonts cultured in NEFA (C18:1-cis-9 plus C16:0) + BSAF in the absence of 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).

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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.

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Plasmodium falciparum: Chemically defined medium for continuous intraerythrocytic growth using lipids and recombinant albumin

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ABSTRACT

Dioleoylphosphatidylcholine and other phosphatidylcholines containing different fatty acid moieties were found to increase the ability of nonesterified fatty acids (NEFA) to sustain continuous intraerythrocytic growth of *Plasmodium falciparum* in the presence of specific proteins. Other phospholipids, including phosphatidylethanolamine, phosphatidylserine, and phosphatidic acid, were beneficial to parasite growth. Different combinations and concentrations of NEFA tested in the presence of phospholipids and bovine albumin had variable effects on parasite growth. The most effective combination for promoting parasite growth consisted of 30 μg/ml *cis*-9-octadecenoic acid (oleic acid) plus 15 μg/ml hexadecanoic acid (palmitic acid). Recombinant human albumin could replace bovine or human albumin in culture media enriched with structurally defined lipids. This study therefore established a chemically defined culture medium suitable for sustaining the growth of *P. falciparum*.

1. Introduction

Malaria remains a devastating disease, particularly in the Tropics. The estimated incidence of malaria worldwide is in the order of 300–500 million clinical cases annually. The annual estimates of malaria mortality, particularly those caused by the protozoan *Plasmodium falciparum*, vary from 1.5 to 2.7 million worldwide (World Malaria Report 2005, WHO, http://rbm.who.int/wmr2005/; Snow et al., 2005). Because of emerging resistance to conventional antimalarial drugs and insecticides, there is an increasing need for new drugs with alternative targets (Ridley, 2002). It is therefore necessary to gain a better understanding of malarial parasite biology, and the mechanisms of action of growth-promoting host factors and antimalarial drugs.

It has been suggested that *P. falciparum* requires some factors present in human serum (HS)[1] in order to develop, although the role of HS in the growth of this parasite is still unknown. We previously described a growth-promoting fraction derived from adult bovine plasma (GFS), which supported intraerythrocytic growth of the parasite (Asahi and Kanazawa, 1994). GFS is a 55–70% ammonium sulfate fraction of adult bovine plasma and contains lipid-rich albumin (ALB) as a major component (Asahi and Kanazawa, 1994; Asahi et al., 2005). Similarly, Cranmer et al. (1997) described a commercially available lipid-enriched bovine ALB (Albumax II; Invitrogen Ltd., USA) that could be used to replace HS for in vitro cultivation of *P. falciparum*. Although these serum substitutes have often been used to maintain parasite cultures, data are still insufficient to allow the direct identification of the functional components required for the growth of *P. falciparum*. The replacement of HS or GFS in culture medium with chemically- or functionally-defined substances is not only advantageous for the culture of the parasite, but will also provide critical clues concerning the parasite's requirements for proliferation at the erythrocyte stage. We previously investigated the components of GFS and related substances which have the ability to sustain parasite growth (Asahi et al., 2005). A simple total lipid fraction (GFS-C), which was obtained after lipid extraction of GFS, has been shown to sustain the complete development of the parasite. The importance of, not only GFS-C, but also specific proteins such as bovine and human ALB, has also been indicated (Asahi et al., 2005). GFS-C has been shown to contain phospholipids (Pld), diacylglycerides, cholesterol (CHOL), monoglycerides, nonesterified fatty acids (NEFA) and cholesteryl esters (CE) (Asahi et al., 2005). The components of the NEFA fraction of GFS-C have been shown to contain mainly *cis*-9-octadecenoic acid (C18:1[C18:1-cis-9], 43%),

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[1] *Abbreviations used:* ALB, albumin; ANOVA, multifactorial analysis of variance; BSAF, NEFA-free bovine serum ALB; CE, cholesteryl ester; CHOL, cholesterol; DAG, 1,2-dioleoyl-sn-glycerol; CRPMI, basal medium; GFS, a growth-promoting fraction derived from adult bovine plasma; GFS-C, a total simple lipid fraction obtained from GFS; GFSRPMI, CRPMI containing 10% GFS; HS, human serum; HSAF, NEFA-free human ALB; NEFA, nonesterified fatty acids; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PfLDH, *Plasmodium falciparum* lactate dehydrogenase; PI, phosphatidylinositol; Pld, phospholipids; PRBC, RBC infected with *Plasmodium falciparum*; PS, phosphatidylserine; RBC, red blood cell; recHA, recombinant human ALB.

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hexadecanoic acid (C16:0, 21%), octadecanoic acid (C18:0, 14%), *cis,cis*-9,12-octadecadienoic acid (C18:2), *cis*-9-hexadecenoic acid (C16:1), *cis*-5,8,11,14-eicosatetraenoic acid (C20:4), *cis*-5,8,11,14,17-eicosapentaenoic acid (C20:5), and *cis*-4,7,10,13,16,19-docosahexaenoic acid (C22:6). Each of the NEFA enriched with BSAF has been tested for the ability to promote parasite growth. Mixtures of NEFA, but not individual NEFA, have sustained parasite growth to a low extent (Asahi et al., 2005). However, parasite growth in the presence of several combinations of NEFA is much less than that with GFS-C-, GFS- or HS-containing medium. These results have implied that while the NEFA components of GFS-C are functional factors in promoting parasite growth, other factor(s) must also contribute to the high growth-promoting activity of GFS.

This study was undertaken to determine the ability of structurally defined chemicals to sustain parasite growth and to formulate a chemically defined medium for intraerythrocytic growth of the parasite using chemicals and recombinant human ALB (recHA).

2. Materials and methods

2.1. Parasite and culture

Cultures of the FCR3/FMG (ATCC Catalogue No. 30932, Gambia) strain of *P. falciparum* were used in the experiments. The parasites were routinely maintained by in vitro culture techniques using culture medium devoid of whole serum. It 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., 1996). This complete medium was termed GFSRPMI. CRPMI consisted of RPMI1640 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, red blood cells (RBC), which had been preserved in Alsever's solution (Asahi et al., 1996) for 3–30 days, were 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. For subculture, 4 days after inoculation, infected RBC (PRBC) and uninfected RBC were washed with CRPMI. Parasitemia was adjusted to 0.1% (for subculture) or 0.4% (for growth tests), by adding uninfected RBC, and the hematocrit was adjusted to 2% by adding the appropriate volume of either GFSRPMI or the test medium.

2.2. Growth-promoting activity experiments

The growth experiments were performed by replacing GFSRPMI with CRPMI supplemented with the test substances. The following substances were tested for their growth-promoting activities: CRPMI containing NEFA-free bovine serum-(BSAF) or HS-ALB (HSAF), or recHA (albucult™; Novozymes Delta Ltd., Denmark) at a final concentration of 3 mg/ml, except when otherwise stated, was further supplemented with different concentrations of dodecanoic acid (C12:0), tetradecanoic acid (C14:0), pentadecanoic acid (C15:0), C16:0, C16:1, C18:0, *cis*-6-octadecenoic acid (C18:1-cis-6), C18:1, *cis*-11-octadecenoic acid (C18:1-cis-11), *cis*-13-octadecenoic acid (C18:1-cis-13), *trans*-9-octadecenoic acid (C18:1-trans-9), C18:2, *cis,cis,cis*-6,9,12-octadecatrienoic acid (C18:3), C20:4, C20:5, docosanoic acid (C22:0), C22:6, cholesteryl ester oleoyl (CE-18:1), CHOL, 1,2-dioleoyl-sn-glycerol (DAG), GFS-C; 1,2-dioleoyl phosphatidic acid sodium salt (PA-di18:1), 1,2-dihexanoyl-sn-glycero-3-phosphocholine (PC-di6:0), 1,2-dilauroyl-sn-glycero-3-phosphocholine (PC-di12:0), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (PC-di14:0), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (PC-di16:0), 1,2-dipalmitoyl-rac-glycero-3-phosphocholine (PC-rac-di16:0), 1,2-distearoyl-sn-glycero-3-phosphocholine (PC-di18:0), 1,2-dioleoyl-sn-glycero-3-phosphocholine (PC-di18:1), 2-oleoyl-1-palmitoyl-sn-glycero-3-phosphocholine (PC-18:1/16:0), 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (PC-di18:2), 1,2-diarachidoyl-sn-glycero-3-phosphocholine (PC-di20:4), 2-arachidonoyl-1-palmitoyl-sn-glycero-3-phosphocholine (PC-20:4/16:0), PC from egg yolk (PC-EY), PC from soybean (PC-SB), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (PE-di18:1) (Fluka Biochemica, Switzerland), PE from soybean (PE-SB), 1,2-diacyl-sn-glycero-3-phosphoinositol from soybean (PI-SB), and 1,2-dioleoyl-sn-glycero-3-phosphoserine sodium salt (PS-di18:1). Unless otherwise stated, all the compounds were obtained from Sigma. The parasites were cultured for 4 days after inoculation (two cycles of complete growth), except when otherwise stated.

For the reconstitution of lipids, dried lipid precipitates were prepared, added with culture media, and sterilized, as previously described (Asahi et al., 2005).

2.3. Assessment of parasite growth

Samples were taken at the times indicated, and thin smears were made and stained with Giemsa. More than 10,000 RBC were examined to determine the percentages of PRBC (parasitemia). The growth rate was first estimated by dividing the parasitemia of the test sample 4 days after inoculation by the initial parasitemia, except when otherwise stated. Measurement of growth was also performed using the lactate dehydrogenase of *P. falciparum* (PfLDH) assay (Asahi et al., 2005; Makler and Hinrichs, 1993). The Malstat reagent (Flow Inc., USA) was used, and the PfLDH assay was performed according to the manufacturer's instruction. Briefly, PRBC/RBC in cultures was hemolyzed by three freeze-thaw cycles, and a 15-μl aliquot was transferred to each well of a 96-well microtiter plate. Then, 100 μl of the Malstat reagent, 10 μl of 1 mg/ml nitroblue tetrazolium (Wako) and 10 μl of 1 mg/ml diaphorase (Wako) were added to each well. The plate was allowed to stand for 40 min at 37 °C, and the reaction was stopped by the addition of acetic acid. The absorbance at 655 nm was determined and the initial value was subtracted from the final reading. For each experiment, PRBC were divided into identical aliquots, and different treatments were performed simultaneously. To make the results comparable across experiments, untreated control wells, cultured in GFSRPMI, were set up each time. All experiments were repeated two to four times.

2.4. Separation of lipids

Known amounts of GFS were extracted using the method of Bligh and Dyer (Asahi et al., 2005). The GFS-C was evaporated and resuspended in the original volume of culture medium used for the assay.

2.5. Statistical analysis

Statistical significance of differences between means was evaluated using multifactorial analysis of variance (ANOVA). All the 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. For the graphical representation of the data, *y*-axis error bars were added to indicate the standard deviation for each point.

3. Results

3.1. Factors in GFS responsible for amplifying growth-promoting activity of NEFA

For the initial experiments designed to determine the factor(s) responsible for the high growth-promoting activity of GFS,

P. falciparum was cultured with the lipid classes found in GFS-C and with various chemically defined lipids, in the presence of BSAF and different concentrations of a mixture of the two most abundant NEFA found in GFS-C, C18:1 (0–60 μg/ml [212.4 μM]) and C16:0 (0–30 μg/ml [117.0 μM]) at a ratio of 2:1. The growth rate was dependent on the concentrations of the NEFA in the mixture: the maximum effect was obtained with 30 μg/ml C18:1 plus 15 μg/ml C16:0 (mean ± standard deviation, 7.59 ± 1.06), with a decline at 15 μg/ml C18:1 plus 7.5 μg/ml C16:0 (6.53 ± 0.35) and at 60 μg/ml C18:1 plus 30 μg/ml C16:0 (2.32 ± 0.15). These growth rates were, however, much lower than those with GFS-C plus BSAF (22.03 ± 4.50) and GFSRPMI (18.41 ± 1.24).

It was unexpectedly found that, when a lipid mixture containing Pld such as phosphatidylcholine (PC) at a high concentration, phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidic acid (PA), CHOL, CE, and DAG was added to the culture media containing NEFA, the parasite growth was increased to an extent similar to or greater than that seen with GFS-C + BSAF and GFSRPMI (Table 1). In an attempt to identify the factor(s) involved in this amplified growth-promoting effect on the parasite, each lipid was omitted from the medium in turn. In the absence of PC, parasite growth decreased to a level similar to that seen with NEFA plus BSAF (Table 1). Omission of PE, PS, or PA also resulted in a decrease in parasitemia, but to a lesser extent (Table 1). On the other hand, in the absence of PI, CHOL and CE, the growth rate was significantly higher than that in their presence. These results indicate the critical importance of PC for parasite growth in culture medium. PE, PS, and PA were beneficial to parasite growth, whereas PI, CHOL and CE were detrimental. DAG had no effect on the growth rate of the parasite at the concentration tested.

3.2. Effect of Pld on ability of NEFA to promote parasite growth

Graded concentrations of various PC-containing fatty acid moieties were tested for their abilities to augment the effects of the NEFA mixture on parasite growth, in the presence of other Pld (PE+PS+PA) and BSAF. Among 12 PC tested, PC-di18:1 was found to markedly amplify the growth-promoting ability of the NEFA mixture in a dose-dependent manner and at a wide range of concentrations, to a level similar to that seen with GFSRPMI (Table 2).

Table 1
Effect of various classes of lipid on ability of NEFA to sustain growth of *P. falciparum*

Constituents	Growth rate % of control ± SD
NEFA(C18:1+C16:0)* + BSAF	100.0 ± 10.5
Mixture of all constituents	287.1 ± 3.0#,§
[NEFA(C18:1+C16:0) + Pld(PC+PE+PS+PI+PA) + CHOL + CE + DAG + BSAF]**	
PC (−)***	133.5 ± 11.2##,¶
PE (−)	227.1 ± 13.6#,¶
PS (−)	246.9 ± 6.7#,¶
PI (−)	310.9 ± 10.8#,¶
PA (−)	258.4 ± 0.2#,¶
CHOL (−)	306.9 ± 9.1#,¶
CE (−)	307.8 ± 2.4#,¶
DAG (−)	286.2 ± 1.9#,§
GFS-C + BSAF	261.0 ± 7.6#,¶
GFSRPMI	214.9 ± 7.9#,¶

The culture media contained BSAF, except for GFSRPMI. *"The concentrations were 30 μg/ml C18:1, 15 μg/ml C16:0, 165 μg/ml PC-di18:1, 20 μg/ml PE-SB, 10 μg/ml PS-di18:1, 10 μg/ml PI-SB, 10 μg/ml PA-di18:1, 10 μg/ml CHOL, 10 μg/ml CE-18:1, 10 μg/ml DAG. **"Each lipid was omitted from the mixture of all constituents. The growth in the presence of BSAF alone (3.7% ± 7.9%) and of a mixture of all constituents depleted of NEFA (33.0% ± 2.9%) was also tested for comparison. #Significant differences ($P < 0.001$) and ##($P < 0.05$) versus the growth in the presence of NEFA (C18:1+C16:0) + BSAF. ¶Significant difference ($P < 0.001$) versus the growth in a mixture of all constituents. §No significant difference.

The addition of PC-di12:0, PC-di16:0, and PC-18:1/16:0 also increased the growth rate to >200% at certain concentrations (Table 2). The addition of PC-di20:4 and PC-20:4/16:0 was also beneficial to parasite growth to a lesser extent. The addition of PC-di6:0, PC-di14:0, PC-rac-di16:0, PC-di18:2, PC-EY, and PC-SB to the medium had no marked effect on, or was detrimental to, parasite growth.

Although Pld other than PC were not critical for optimal growth of the parasite cultured in the presence of high concentrations of PC and NEFA associated with BSAF, PE, PS, and PA were tested for their possible efficacy in augmenting the ability of paired NEFA to promote parasite growth. The addition of PE, PS, or PA failed to increase the growth-promoting efficacy of paired NEFA, indicating that PE, PS, and PA could not substitute for PC (Table 3, Experiment A). However, the addition of mixtures of Pld (PE, PS, and PA) to the medium was beneficial to parasite growth to a lesser extent (Table 3, Experiment B).

3.3. Effects of various types of NEFA on parasite growth in the presence of Pld

NEFA mixtures of C18:1 and C16:0 enriched with BSAF and Pld, were tested for their ability to promote parasite growth. The growth rate was significantly higher than that with corresponding concentrations of NEFA mixtures in the absence of Pld (Fig. 1). The growth rate was dependent on the ratio of the two NEFA, ranging from 1:5 to 5:1 (C18:1 to C16:0), at a total concentration of 45 μg/ml. The highest growth rate was obtained using C18:1 (30 μg/ml, 106.2 μM) plus C16:0 (15 μg/ml, 58.5 μM) (Fig. 1).

The culture media were reconstituted by mixing Pld and BSAF with two NEFA (either C18:1 plus a saturated one or C16:0 plus an unsaturated one). The best combination of NEFA was found to

Table 2
Effect of various types of PC on ability of NEFA to sustain growth of *P. falciparum*

Constituents	Concentration (μg/ml)	Growth rate % of control ± SD
NEFA(C18:1+C16:0)* + BSAF		100 ± 4.9
*[NEFA(C18:1+C16:0) + Pld(PE+PS+PA) + BSAF]***		
+PC-di12:0***	160	Hemolyzed
	80	104.4 ± 21.6
	40	245.9 ± 24.7#
+PC-di16:0	160	140.8 ± 10.0
	80	270.3 ± 9.4#
	40	185.8 ± 2.8#
+PC-di18:1	320	242.6 ± 23.8#
	160	292.0 ± 15.3#,§
	100	312.7 ± 16.9#,§
	80	273.9 ± 6.9#,§
	40	170.9 ± 8.8#
+PC-18:1/16:0	160	215.2 ± 9.7#
	80	171.3 ± 9.3#
	40	152.9 ± 22.8#
+PC-di20:4	160	156.5 ± 23.5#
	80	166.7 ± 13.8#
	40	157.1 ± 15.3#
+PC-20:4/16:0	160	75.4 ± 11.1#
	80	157.1 ± 17.2#
	40	169.0 ± 16.9#
GFSRPMI		294.4 ± 15#,§

The culture media contained BSAF, except for GFSRPMI. *"The concentrations were 30 μg/ml C18:1, 15 μg/ml C16:0, 20 μg/ml PE-di18:1, 10 μg/ml PS-di18:1, 10 μg/ml PA-di18:1. **"Each PC was added to the mixture of NEFA + Pld (PE+PS+PA) + BSAF. The growth in the presence of BSAF alone (4.7% ± 0.6% and of a mixture of Pld (PC+PE+PS+PA) + BSAF (31.8% ± 0.7% were also tested for comparison. #Significant difference ($P < 0.001$) versus the growth in the presence of NEFA (C18:1+C16:0) + BSAF. §No significant difference. Others are significantly different ($P < 0.001$–0.05) versus the growth in GFSRPMI.

Table 3
Effects of Pld at graded concentrations (A) and combinations of (B) on ability of NEFA to sustain growth of *P. falciparum*

Constituents	Concentration (μg/ml)	Growth rate % of Control ± SD
Experiment A		
NEFA(C18:1+C16:0)*		100.0 ± 8.0
+PE-SB**	80	115.8 ± 8.6
	40	109.2 ± 4.0
	20	102.0 ± 8.0
+PE-di18:1**	80	133.3 ± 7.2##
	40	105.5 ± 5.2
	20	96.3 ± 11.8
+PS-di18:1**	80	111.2 ± 8.3
	40	117.2 ± 6.6
	20	107.5 ± 3.2
+PA-di18:1**	80	108.0 ± 6.0
	40	116.7 ± 13.2
	20	128.4 ± 19.3##
NEFA(C18:1+C16:0)* + Pld(PC+PE+PS+PA)***		272.7 ± 20.7#
GFSRPMI		258.0 ± 9.8#
Experiment B		
NEFA(C18:1+C16:0)*		100.0 ± 23.9
NEFA(C18:1+C16:0)*		
+Pld(PC)		229.4 ± 6.4#
+Pld(PC+PE)		224.5 ± 17.9#
+Pld(PC+PE+PA)		276.1 ± 13.2#
+Pld(PC+PE+PS)		230.0 ± 8.8#
+Pld(PC+PE+PS+PA)***		317.9 ± 26.7#,§
GFSRPMI		294.2 ± 13.5#,§

The culture media contained BSAF, except for GFSRPMI. *,***The concentrations were 30 μg/ml C18:1, 15 μg/ml C16:0, 100 μg/ml PC-di18:1, 20 μg/ml PE-di18:1, 10 μg/ml PS-di18:1, and 10 μg/ml PA-di18:1. **PE-SB and PE-di18:1 in the presence of NEFA + Pld (PS+PA), PS-di18:1 in the presence of NEFA + Pld (PE+PA) and PA-di18:1 in the presence of NEFA + Pld (PE+PS) was tested for the ability to promote growth of the parasite. #Significant differences ($P < 0.001$) and ##($P < 0.01$) versus the growth in the presence of NEFA (C18:1+C16:0) + BSAF. §No significant difference.

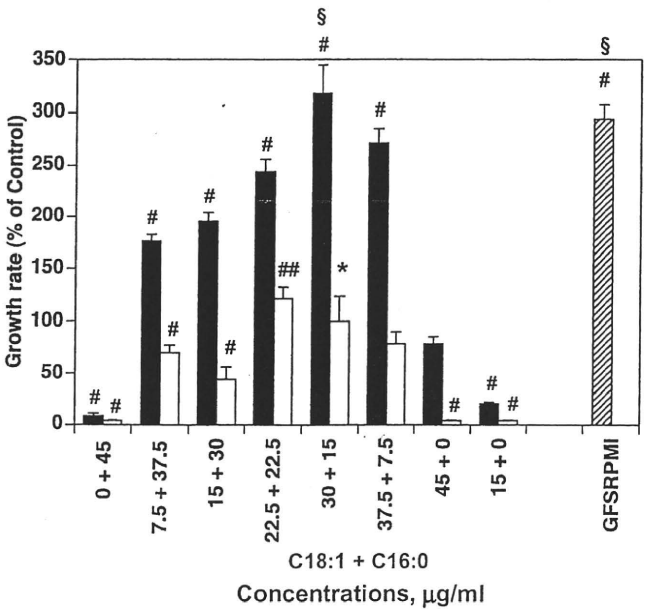

Fig. 1. Growth of *P. falciparum* in the presence of two NEFA at various ratios. The parasite was cultured either in the presence (■) or in the absence (□) of Pld. *The paired NEFA (30 μg/ml C18:1 and 15 μg/ml C16:0) added to media in the absence of Pld served as a control. The Pld concentrations were 100 μg/ml PC-di18:1, 20 μg/ml PE-di18:1, 10 μg/ml PS-di18:1, and 10 μg/ml PA-di18:1. The culture media contained BSAF, except for GFSRPMI. The growth in BSAF alone (4.0% ± 0.5%) and Pld (PC+PE+PS+PA) + BSAF (19.8% ± 3.8%) were also tested for comparison. #Significant differences ($P < 0.001$) and ##($P < 0.05$) versus the growth in the presence of NEFA (*). §No significant difference.

be C18:1 (C18:1-cis-9) plus C16:0, followed by the combinations of C18:1-cis-11 plus C16:0, C18:1 plus C15:0, C18:1 plus C18:0, and C18:1 plus C14:0 (Fig. 2). The combinations of C16:1 plus C16:0, C18:1-cis-6 plus C16:0, C18:1-cis-13 plus C16:0, and C18:2 plus C16:0 had growth-promoting effects at a level similar to that seen with C18:1 plus C16:0 in the absence of Pld. Combinations of C18:1 plus C12:0, C18:1 plus C22:0, C18:3 plus C16:0, C20:4 plus C16:0, C20:5 plus C16:0, and C22:6 plus C16:0 were detrimental to parasite growth. The combination of C18:1-trans-9 plus C16:0 also deterred parasite growth, indicating that the growth-promoting effect of C18:1 on the parasite is specific to the *cis*-form (Fig. 2).

3.4. Chemically defined medium for parasite growth with the use of recHA

To determine if chemically defined proteins could sustain parasite growth, *P. falciparum* was cultured with recHA, paired NEFA, and Pld. Parasite growth in culture medium enriched with recHA was similar to, or better than, that in media supplemented with BSAF or HSAF (Fig. 3). These results indicate that recHA can replace BSAF or HSAF for promoting and sustaining parasite growth in the presence of lipids (NEFA and Pld). These results provide a chemically defined culture medium suitable for sustaining the growth of *P. falciparum*.

3.5. Growth of parasites cultured in chemically defined media containing various growth promoters

PRBC were maintained for 2–5 days in culture media containing recHA or BSAF with various mixtures of lipid growth promoters. Parasite development in the presence of recHA was similar to that in the presence of BSAF and in GFSRPMI (Fig. 4). The parasites could be maintained in medium containing NEFA, Pld (PC+PE+PS+PA) and either recHA or BSAF for ⩾6 weeks (12 subcultures), without any decrease in growth rate.

4. Discussion

The ability of lipids to sustain the growth of *P. falciparum* was determined. It was found that high concentrations of PC-di18:1 was sufficient for the complete augmentation of the poor parasite growth-promoting efficacy of NEFA in the presence of specific proteins. Several other PC, including PC-di12:0, PC-di16:0, PC-18:1/16:0, PC-di20:4, and PC-20:4/16:0, were also beneficial to parasite growth in the presence of NEFA, but to a lesser extent. Although Pld other than PC, such as PE, PS and PA, were not critical for optimal parasite growth in the presence of NEFA and PC, their addition to the medium was beneficial to a small extent. Different combinations and concentrations of paired NEFA had differing effects on parasite growth, with the best combination being C18:1 plus C16:0, in the presence of Pld and BSAF. recHA could replace BSAF or HSAF in culture media enriched with structurally defined lipids, to produce a chemically defined medium suitable for parasite growth.

The maximum efficacy of NEFA mixtures for sustaining parasite growth was much lower than that of GFS-C, and the addition of PC-containing a specific fatty acid moiety amplified the poor growth-promoting efficacy of NEFA to an extent similar to that seen with GFS-C and GFSRPMI. Nevertheless, NEFA could be the dominant factors involved in growth promotion, because Pld plus BSAF alone had no growth-promoting ability. Malarial parasites were long considered to be unable to synthesize fatty acids or Pld via de novo biosynthesis; instead, they were thought to be dependent on fatty acids scavenged from the host plasma and RBC for the synthesis of membrane lipids (Holz, 1977; Vial and Ancelin, 1998). On the basis of our current understanding, however, type II fatty acid synthetic

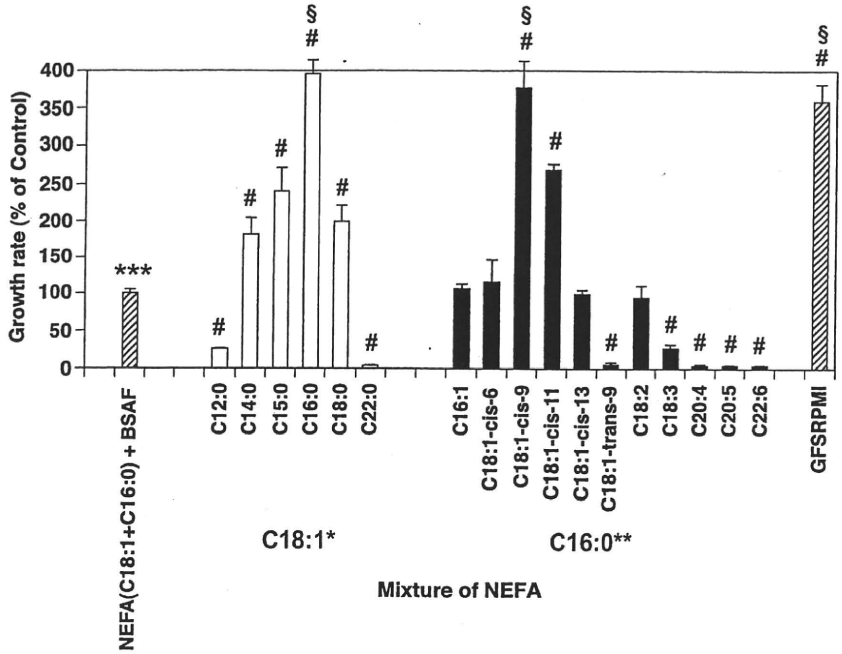

Fig. 2. Growth of *P. falciparum* in the presence of various combinations of paired NEFA. Each saturated NEFA was added at 15 μg/ml in the presence of 30 μg/ml C18:1 (*) and each unsaturated NEFA at 30 μg/ml in the presence of 15 μg/ml C16:0 (**). These culture media contained Pld (100 μg/ml PC-di18:1, 20 μg/ml PE-di18:1, 10 μg/ml PS-di18:1, and 10 μg/ml PA-di18:1) and BSAF. ***NEFA(C18:1+C16:0) + BSAF in the absence of Pld served as a control (100%). The growth in BSAF alone (6.3% ± 0.8%) and Pld (PC+PE+PS+PA) + BSAF (32.8% ± 1.7%) were also tested for comparison. #Significant difference ($P < 0.001$) versus the growth in the presence of NEFA (C18:1+C16:0) + BSAF. §No significant difference.

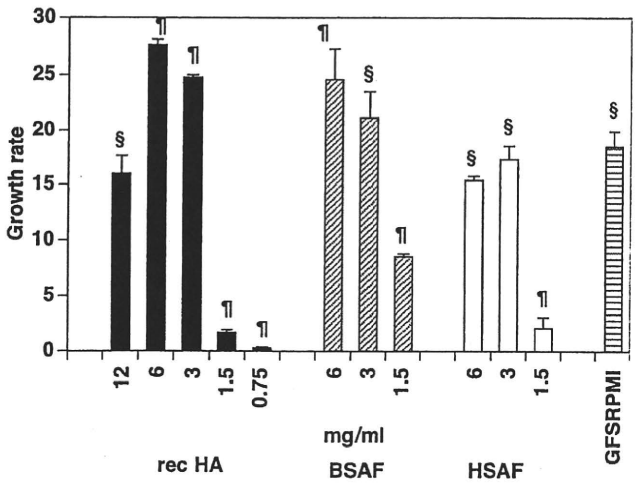

Fig. 3. Effect of various proteins on the ability of a mixture of NEFA and Pld to sustain growth of *P. falciparum*. The culture media contained NEFA (30 μg/ml C18:1 + 15 μg/ml C16:0) and Pld (100 μg/ml PC-di18:1 + 20 μg/ml PE-di18:1 + 10 μg/ml PS-di18:1 + 10 μg/ml PA-di18:1). recHA alone, BSAF alone, and HSAF alone failed to show a growth-promoting effect on the parasite. ¶Significant difference ($P < 0.001$) versus the growth in GFSRPMI. §No significant difference.

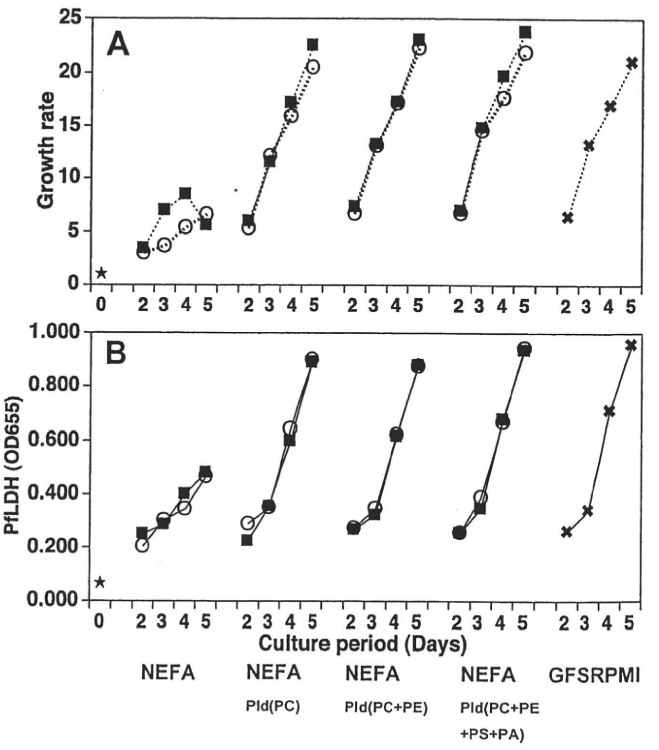

Fig. 4. Growth of *P. falciparum* cultured in the presence of various growth promoters and proteins. The media contained either BSAF (■) at 3 mg/ml or recHA (○) at 4 mg/ml, except for GFSRPMI. Culture media were CRPMI enriched with NEFA alone, NEFA + Pld (PC), NEFA + Pld (PC+PE), or NEFA + Pld (PC+PE+PS+PA). The paired NEFA were added to media at 30 μg/ml C18:1 and 15 μg/ml C16:0. The Pld concentrations were 100 μg/ml PC-di18:1, 20 μg/ml PE-di18:1, 10 μg/ml PS-di18:1, and 10 μg/ml PA-di18:1. The parasite growth was assessed in Giemsa-stained smears (A) and by PfLDH-based Malstat assay (B). The growth of the second subculture is shown, except for the growth of the first subculture seen in CRPMI supplemented with NEFA and either BSAF or recHA. *Start levels of the cultures.

machinery does exist in *P. falciparum* the parasite has been demonstrated to synthesize fatty acids (Surolia and Surolia, 2001; Waller et al., 2003; Yeh and Altman, 2006). Thus, *P. falciparum* may 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 data presented here show that *P. falciparum* predominantly scavenges NEFA from the external milieu for growth promotion, although it is unclear whether the NEFA are modified in any way. In particular, the NEFA involved in the growth promotion of *P. falciparum* have to be in specific pairs. Furthermore, the type and total amount of

NEFA markedly influenced parasite growth in the presence of Pld and BSAF, with the best combination being that of the two most abundant NEFA in GFS and HS. There is increasing evidence showing the involvement of unsaturated NEFA in numerous biological processes, including the activation of protein kinases, cell proliferation, differentiation and cell death (Malhi et al., 2006; Murakami et al., 1986). It has been reported that C16:0 increased oxidative stress, activation of stress-associated protein kinases, and apoptosis of myocyte cells, and that low concentrations of C18:1 completely prevented C16:0-induced cytotoxic stress (Hardy et al., 2000; Miller et al., 2005). Further study is necessary to determine the mechanisms underlying the actions of NEFA, in combination with Pld and proteins, in *P. falciparum*.

Pld metabolism is absent from normal mature human RBC, but in PRBC, the marked increase in membrane content is associated with a considerable increase in the total lipid content (Holz, 1977; Vial and Ancelin, 1998). It has been considered that malarial parasites satisfy their own requirements for nutrition and membrane-building using these Pld (Maguire and Sherman, 1990; Vial and Ancelin, 1998). In addition to the de novo synthesis of Pld, it has been well-demonstrated that RBC infected with *P. falciparum* or *P. knowlesi* readily take up intact Pld from exogenous sources (Grellier et al., 1991; Haldar, 1992; Moll et al., 1988; Simoes et al., 1991, 1992). Among the various PC tested here, PC-di18:1 markedly increased the ability of the NEFA mixture to promote parasite growth, while other PC exerted different effects on parasite growth: the addition of PC-di12:0, PC-di16:0, PC-18:1/16:0, PC-di20:4, and PC20:4/16:0 effectively augmented the growth-promoting effect of the NEFA mixture, but to a lesser extent. The addition of PC-di6:0, PC-di14:0, PC-rac-di16:0, PC-di18:2, PC-EY, and PC-SB either failed to alter the activity of the NEFA mixture significantly, or were detrimental. This suggests that certain structural parameters of not only NEFA, but also PC and proteins, are important for the growth-promoting activity of NEFA. Further studies are necessary to determine the mechanism(s) underlying the actions of Pld in association with NEFA mixtures.

The replacement of HS in culture medium for *P. falciparum* with chemically- or functionally-defined substances is not only advantageous for the culture of the parasite, but will also provide critical clues to the parasite's requirements for proliferation at the erythrocytic stage. Considerable efforts have been made to identify factors and substances with the ability to sustain parasite growth (Asahi and Kanazawa, 1994; Asahi et al., 1996, 2005; Cranmer et al., 1997; Divo and Jensen, 1982; Lingnau et al., 1994; Mi-Ichi et al., 2006; Nivet et al., 1983; Ofulla et al., 1993; Willet and Canfield, 1984). Nevertheless, the inclusion in the culture medium of specific proteins such as bovine and human ALB are essential for parasite growth, indicating that all the serum-free media described so far are only chemically semi-defined. The establishment of a fully-defined culture medium for the parasite still represents a major challenge. Recently, recHA has become commercially available and has been safely used for drug delivery and cell culture applications, with various benefits (Bosse et al., 2005). In this study, recHA could be used for the continuous culture of *P. falciparum*, as a substitute for bovine and human ALB in culture media enriched with structurally defined lipids. This indicates that we have established a chemically defined medium for *P. falciparum*. Further, in the growth-promoting activity experiment, parasites were cultured for 4 days after inoculation, without renewal of the medium, to avoid fluctuation of culture conditions. The rate of parasite growth might, however, be further improved by frequent renewal of the medium, particularly for continuous culture of the parasite.

We are currently attempting to characterize the parasite factors that interact at the molecular level with the growth-promoting agents detected here, with the hope that clarification of the mechanisms underlying the growth promotion of the parasite may lead to the development of novel antimalarial strategies.

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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.2008.09.009.

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Plasmodium falciparum: Development and validation of a measure of intraerythrocytic growth using SYBR Green I in a flow cytometer

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ABSTRACT

Reliable analytical techniques to test growth-promoting and antimalarial efficacy on plasmodia are very important. Flow cytometry (FCM) offers the possibility to study developmental stages of intraerythrocytic growth of malaria parasites using nucleic acid staining. To analyze the growth of *Plasmodium falciparum* SYBR Green I was introduced as an intercalating dye with FCM for the 488 nm line of an argon laser. Procedures employing FCM, including fixatives, dye concentrations, dilution buffer, and staining period, were optimized to simplify the method. FCM as described here allows parasitemia and parasites of different stages to be quantified according to the DNA content. The proportion of parasitized erythrocytes estimated by FCM and the Giemsa method agreed with determination by parasite lactate dehydrogenase. The protocol was extended to merozoite counting as a sensitive assay of growth inhibition of the parasite.

1. Introduction

Malaria is still a devastating disease, particularly in the Tropics. The annual incidence of malaria worldwide is estimated between 300 and 500 million clinical cases. Estimated annual malaria mortality, caused particularly by the protozoan *Plasmodium falciparum*, is from 1.5 to 2.7 million worldwide (World Malaria report 2005, WHO, http://rbm.who.int/wmr2005/; Snow et al., 2005).

Resistance has emerged to conventional antimalarial drugs and insecticides, so there is increasing need for new antimalarial drugs with alternate targets (Ridley, 2002). To create new medications we need to better understand the biology of parasites and interactions with host factors that promote growth and antimalarial drugs.

The effects of drugs and chemicals can be assessed both quantitatively and qualitatively by directly examining red blood cell (RBC) smears from blood or cultures with a microscope, although this method is tedious and subjective. Numerous novel *in vitro* assays have been introduced that are more objective, faster, more sensitive, and designed to be easier to handle. The most common include isotopic, enzymatic, and enzyme-linked immunosorbent assays (ELISA) (Noedl et al., 2003). Isotopic assays rely on the incorporation of radioactive ^{3}H-hypoxanthine into the DNA of the parasite (Yayon et al., 1983; Webster et al., 1985; Noedl et al., 2003). These methods are relatively reliable and objective, but not sufficiently sensitive. In addition, the required radioactive material is hazardous. The assays are suited well to screen large numbers of compounds.

The activity of parasite lactate dehydrogenase (pLDH) also has been used as an assay of the growth of malarial parasites (Asahi et al., 2005; Makler and Hinrichs, 1993; Noedl et al., 2003). ELISA-based assays can provide measures of parasite growth by quantifying biomolecules produced during parasite development, such as histidine-rich protein 2 (HRP2) or pLDH, by double-site sandwich ELISA (Druilhe et al., 2001; Noedl et al., 2002, 2003). The ELISA-based tests are easy and rapid to perform, and are also well suited for the screening of large number of drugs. These methods have been employed widely to detect and analyze the growth of parasites, although they are poorly suited to discriminate the developmental stages of the parasite in parasitized RBC (PRBC). Flow cytometry (FCM) using nucleic acid staining offers the possibility to study the cell cycle or developmental stages of intraerythrocytic growth of malaria parasites.

Abbreviations: Als, Alsever's solution; ANOVA, multifactrial analysis of variance; AO, acridine orange; CQ, chloroquine; CRPMI, basal medium; EC50, concentrations inhibiting schizont formation by 50%; FCM, flow cytometry; FSC, forward scatter; GFS, a growth-promoting fraction derived from adult bovine serum; ELISA, enzyme-linked immunosorbent assay; GIC50, concentration required to inhibit growth of the parasites (parasitemia) by 50%; GFSRPMI, CRPMI containing 10% GFS; HRP2, histidine-rich protein 2; H-FL, high intensity of RFU; L-FL, low intensity of RFU; M-FL, moderate intensity of RFU; OD_{655}, absorbance at 655 nm; PBS, phosphate buffered saline; PFA, paraformaldehyde; PFA/Als, combination of PFA and Als; pLDH, parasite lactate dehydrogenase; PRBC, parasitized RBC; RBC, red blood cell; RFU, relative fluorescent units; SD, standard deviation; SEM, standard error of mean; SSC, side scatter; SYBR Green I-acidic, SYBR Green I in TRIS–SALINE at pH 6.8; SYBR Green I-basic, SYBR Green I in TRIS–SALINE at pH 8.8; TRIS, Tris (hydroxymethyl) aminomethan hydrochloride; TRIS–SALINE, TRIS buffered saline.

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Analysis by FCM using different fluorescent dyes has also proven to be useful in analyzing the blood stage of malaria parasites (Janse and Van Vianen, 1994; Jouin et al., 1995). However, FCM has not become popular, probably because it lacks specificity and requires complicated preparation. FCM requires highly sophisticated, expensive laboratory equipment that may not be accessible to researchers in malaria endemic countries.

We sought to elaborate the FCM system to assess growth of *P. falciparum*. We used SYBR Green I as a fluorescent dye and a flow cytometer equipped with a single 488 nm laser to visualize PRBC with high accuracy and follow development of the parasite.

2. Materials and methods

2.1. Buffers

We prepared TRIS–SALINE solutions (20 mM Tris (hydroxymethyl) aminomethane hydrochloride (TRIS) at pH 6.8, pH 7.2 or pH 8.8 in 138 mM NaCl) and phosphate buffered saline (PBS) (10 mM phosphate buffer at pH 7.2 in 138 mM NaCl) as dilution buffers for fixatives or dyes.

2.2. Parasite, culture, and synchronization

Cultures of the FCR/FMG (ATCC Catalogue No. 30932, Gambia) strain of *P. falciparum* were used in the experiments. Parasites were maintained routinely by *in vitro* culture techniques using a culture medium free of whole serum that consisted of basal medium (CRPMI) supplemented with a 10% growth-promoting fraction derived from adult bovine serum (GFS, Wako Pure Chemical Industries, Japan, under the trade name Daigo's GF21), as reported previously (Asahi et al., 1996, 2005). This complete medium was termed GFSRPMI. CRPMI consisted of RPMI1640 that contained 2 mM glutamine, 25 mM 4-(2-hydroxylethyl)-piperazine ethanesulfonic acid, and 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 (Als) (Asahi et al., 1996) for 3–30 d, washed, then dispensed in a 24-well culture plate at a hematocrit of 2% (1 ml suspension/well), and then cultured under a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 37 °C. At 4 d after inoculation PRBC and uninfected RBC for subculture were washed with CRPMI. Parasitemia was adjusted to 0.1%, except where specified otherwise, by adding uninfected RBC, and 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 44 and 50 h intervals (Asahi and Kanazawa, 1994). After the third treatment with sorbitol, 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 were maintained for a further 28 h and schizonts were purified from the cultures by isopycnic, density centrifugation on 63% Percoll PLUS.

2.3. Assessment of parasite growth

For FCM the PRBC were stained with acridine orange (AO, 3 μg/ml, Sigma–Aldrich), Hoechst 33258 (20 μg/ml, Sigma–Aldrich), Hoechst 33342 (20 μg/ml, Sigma–Aldrich), SYBR Green I (×0.00625–×2 dilution, Invitrogen), or SYTO 9 (2.5 μM, Invitrogen), and then analyzed on a PAS flow cytometer (Partec Co. Ltd., Germany). This cytometer is equipped with an argon-ion laser tuned to a fluorescence excitation of 488 nm for 15 mW output. For analysis it is associated with the FCS express software (De Novo Software Inc., Canada). To assay the number of parasites a 16 μl aliquot (8×10^5 cells) of each well that contained 0.5% PRBC/RBC suspension was added to 1 ml staining solutions, except where otherwise specified. Stained PRBC/RBC were gated on the basis of forward scatter (FSC) and side scatter (SSC) signals and on relative fluorescent units (RFU). More than 10,000 stained PRBC/RBC were measured to determine the levels of nucleic acid synthesis by PRBC. Merozoites released from PRBC into surrounding medium also were measured. More than 5000 PRBC (S2, Fig. 2) were measured to count the number of released merozoites (S1, Fig. 2). Parasite growth assessed from the number of merozoites released after culture was termed the "Merozoite test".

For the Giemsa method samples were taken at the times indicated, thin smears were made and then stained with Giemsa (E. Merck, Germany). The number of nuclei of each PRBC was counted under a light microscope to monitor the development of parasites from ring forms to schizonts. PRBC were classified into three groups as less than 3 nuclei (<3N-G), three to six nuclei (3–6N-G), and more than six nuclei (>6N-G). More than 10,000 RBC were examined to determine the percentage of PRBC (parasitemia) and the three groups of developmental stages.

Growth was measured using a pLDH assay (Asahi et al., 2005; Makler and Hinrichs, 1993). The Malstat reagent (Flow Inc., USA) indicated viability, and the pLDH assay was performed according to the manufacturer's instructions. Briefly, at the indicated incubation times PRBC/RBC in culture were hemolyzed by three freeze–thaw cycles, and 15 μl aliquots were transferred to each well of a 96-well microtiter plate. Then 100 μl of the Malstat reagent, 10 μl of 1 mg/ml nitroblue tetrazolium (Wako), and 10 μl of 1 mg/ml diaphorase (Wako) were added to each well. The plated contents were allowed to stand for 40 min at 37 °C and the reaction was stopped by adding 50 μl of 5% (V/V) of acetic acid. The absorbance at 655 nm (OD_{655}) was read on a plate reader. The initial OD_{655} value measured when assay reagents were first added was subtracted from the endpoint reading. For each experiment the PRBC were divided into identical aliquots, and different treatments were performed simultaneously. In all the experiments the culture wells were run in triplicate or quadruplicate.

2.4. Determination of growth inhibition concentration (GIC50) and schizogony inhibition concentration (EC50)

Assays of susceptibility to chloroquine (CQ) were compared by measuring two different parameters: parasitemia/nucleic acid synthesis estimated by FCM and parasitemia/number of nuclei of PRBC observed by the Geimsa method. GIC50 (CQ concentration required to inhibit growth of the parasites (parasitemia) by 50% compared to CQ-free controls) and EC50 (CQ concentrations that inhibited schizont formation by 50% compared to CQ-free controls) were extrapolated from the concentration–response curves.

2.5. Statistical analysis

Statistical significance for differences was evaluated using multifactorial analysis of variance (ANOVA). All calculations were performed with GraphPad PRISM 4 (GraphPad Software, USA). The *P* value for significance was 0.05, and pairwise comparisons were made post hoc with Bonferroni's test. In graphical representation of the data the *y*-axis error bars indicate the standard deviation (SD) of the data for each point.

3. Results

3.1. Detection of PRBC by FCM using different fluorescent dyes

Several fluorescent dyes, including AO, Hoechst 33258, SYBR Green I, and SYTO 9, were tested for their ability to discriminate PRBC from uninfected RBC and to monitor nucleic acid synthesis

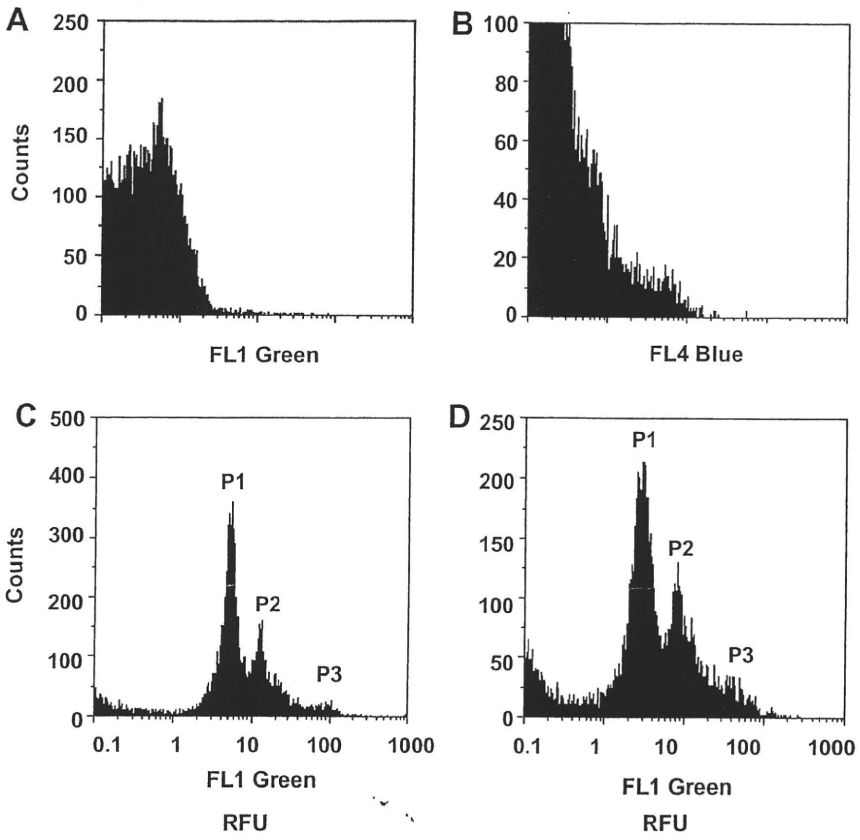

Fig. 1. Fluorescence distribution of unfixed PRBC from asynchronous control cultures (parasitemia, 5.77%) stained with AO (A), Hoechst 33258 (B), SYBR Green I (C) and SYTO 9 (D). Histograms depict cell counts against RFU (log scale) on the *x*-axis. PBS was used tentatively as a dissolving buffer. FL1 Green, green fluorescence (405 nm); FL4 Blue, blue fluorescence (341 nm).

by FCM. As shown in Fig. 1, only SYBR Green I and SYTO 9 stains separated fluorescent PRBC populations (right peaks) from uninfected RBC (left peak) after FCM. After FCM with SYBR Green I and SYTO 9 three peaks of increasing intensity of fluorescence emission (RFU) were clearly defined: P1 (ring forms and young trophozoits), P2 (late trophozoites and young schizonts) and P3 (mature schizonts and segmenters). A proportion of each peak was examined by fluorescence microscopy and microscopy on smears stained with Giemsa. Uninfected RBC exhibited no green fluorescence upon excitation by blue or green light.

3.2. Optimization of flow cytometric measurement of PRBC with SYBR Green I

PRBC or merozoites must retain their normal morphological characteristics and membrane integrity without disruption for FCM to distinguish them from platelets, leukocytes, and RBC debris. We evaluated how the intensity of fluorescence of PRBC depended on fixatives, dilution buffer, staining period and the concentration range of SYBR Green I. After the overlying supernatant of cultures was removed PRBC in culture were fixed by adding fixatives such as 1% paraformaldehyde (PFA) or 1% glutaraldehyde in TRIS–SALINE (pH 7.2), PBS or Als. PFA combined with Als (PFA/Als) was the most useful fixative. PFA/Als did not induce significant lysis or deformity of PRBC. These problems are observed frequently when, for example, TRIS–SALINE or PBS are used to dilute fixatives. PRBC can be stored without deterioration in the PFA/Als fixative at 4 °C for several months before processing (data not shown).

SYBR Green I concentration and a diluting buffer that provided brilliant resolution of peaks of fluorescence were tested. Each peak showed most clearly with SYBR Green I at diluted concentrations ranging from 0.00625 to 2× in TRIS–SALINE at pH 8.8 (SYBR Green I-basic). Both fixed and unfixed PRBC give the best results at 1× dilution (data not shown). Higher concentrations of the dye increased the fluorescent intensity of uninfected RBC.

The dependence of RFU with time for fixed or unfixed PRBC at 1× dilution of SYBR Green I-basic was evaluated over 30 min by time-curse analysis of *P. falciparum* cultures. The frequency distribution of RFU was similar after staining for 5 min or longer with SYBR Green I-basic, although fluorescence could be detected within seconds after the fluorescent stain was added to fixed and unfixed PRBC (data not shown). SYBR Green I in TRIS–SALINE (pH 6.8) (SYBR Green I-acidic) and PBS did not provide enough signal on PRBC before 30 min of staining, and deformed and hemolyzed PRBC (data not shown).

3.3. Two-parameter dot plot presentation of PRBC/RBC from P. falciparum culture

PRBC were stained with SYBR Green I-basic and analyzed by FCM. PRBC were gated based on SSC and RFU. SYBR Green I-basic provided brilliant resolution of PRBC versus uninfected RBC that permitted visualization of PRBC populations with high accuracy (Fig. 2). PRBC are located as three clusters (C1–C3) from 10 to 300 of the range of RFU (Fig. 2A and B). All stages were included in the populations having high (C3, H-FL, a high DNA content), intermediate (C2, M-FL, a moderate DNA content), or low (C1, L-FL, a low DNA content) RFU intensities. Free parasites released from ruptured *P. falciparum* segmenters were also fixed by PFA/Als and counted by FCM (Fig. 2C).

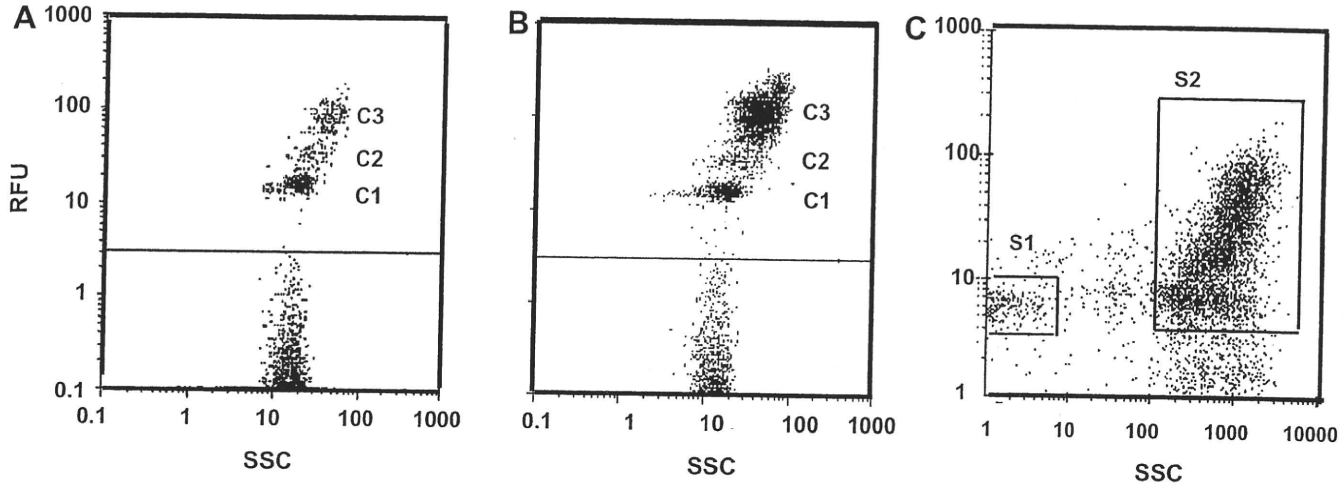

Fig. 2. Two-parameter dot plot representation of RFU and SSC from cultures of *P. falciparum*. Asynchronous culture of *P. falciparum* (parasitemia, 6.34%) (A) and PRBC predominantly containing schizonts (parasitemia, 20.17%) that were separated from the culture by sedimentation on percoll PLUS, as described in Section 2 (B), were measured by FCM. After 45 h of incubation synchronized cultures contained released merozoites (S1) and PRBC that showed H-FL, M-FL, and L-FL (S2) (C).

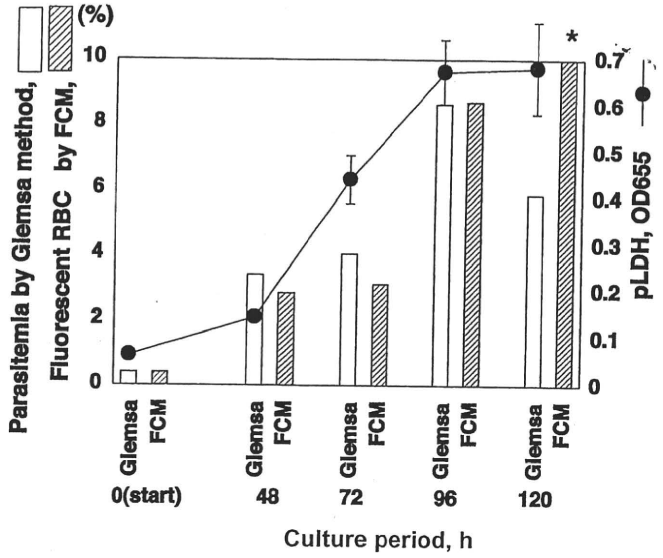

Fig. 3. Relationships among growth of the parasite measured by FCM, the Giemsa method and pLDH levels. Parallel culture of asynchronous parasites were initiated at a parasitemia of 0.4% and analyzed at 48–120 h. OD_{655} is shown after subtraction of pLDH levels for uninfected RBC. $^{*}P < 0.001$ versus Giemsa method after 120 h of culture.

3.4. Comparison of percent fluorescent RBC with PRBC determined by Giemsa method and pLDH estimation

Time course measurements with PRBC by FCM, microscopic counting and pLDH estimation were compared. The proportion of PRBC estimated by the three methods was quite similar at all time points examined, except at 120 h of culture when the % of PRBC (fluorescent RBC) detected by FCM was significantly higher (Fig. 3).

3.5. Distribution of different parasite stages determined by FCM

FCM using SYBR Green I-basic was believed to enable not only visualization of PRBC with high accuracy but also the ability to follow parasite development during its life cycle. The relative distribution of the parasite at different developmental stages was determined by FCM with SYBR Green I-basic after synchronized *P. falciparum* was cultured for several periods. The proportion of parasites at each stage as estimated by the FCM and Giemsa method were quite similar at all time points (Fig. 4). In normal growth of the parasite in RBC the increase in the number of nuclei determined by the Giemsa method was proportional to the increase in fluorescence intensity of schizonts stained with SYBR Green I-basic. Therefore, the frequency distribution of the fluorescence intensities at different time points can represent the development and degree of nucleic acid synthesis of the parasite.

3.6. Application of FCM to tests of CQ susceptibility

The frequency distribution of the fluorescence intensities, as stated above, may be useful in determining the effect of antimalarial drugs on the schizogony and nucleic acid synthesis of parasites. Changes in the percentages of schizonts after culture has been used often assess susceptibility to antimalarial drugs. The effects of CQ on schizogony, merozoite formation and reinvasion of *P. falciparum* were tested by FCM. Fig. 5A and B show representative results of CQ susceptibility determined for *P. falciparum* of synchronized cultures by FCM compared with results by microscopic examination. Reading a number of H-FL in 25 h cultures by FCM gave comparable results to microscopic examination of slides stained with Giemsa at 25 h of culture, except for the change in the number of M-FL and L-FL at 25 h of culture.

In regular cultures schizonts burst spontaneously to release free merozoites, which enter new RBC and increase the number of PRBC. While the majority of released free merozoites remain in culture for a while, the number of merozoites after culture was used assess susceptibility to CQ. Parasites were synchronized at ring forms or young trophozoites and cultured for 45 h in the presence of different concentrations of CQ. Fig. 6 shows results representative of the susceptibility of *P. falciparum* to CQ determined by counting the number of released merozoites with FCM (Merozoite test) (Fig. 6A) that are compared with the standard Giemsa method (Fig. 6B). The merozoite test showed the effect of CQ on the growth of the parasite at 45 h of culture clearly and sensitively.

3.7. Comparison of the growth inhibiting effect of CQ on the parasite as determined by different methods

The dose–response experiments GIC50 of CQ after 45 and 96 h of culture and EC50 of CQ after 16–25 h culturing were compared. Table 1 shows that GIC50/EC50 determined by the different meth-

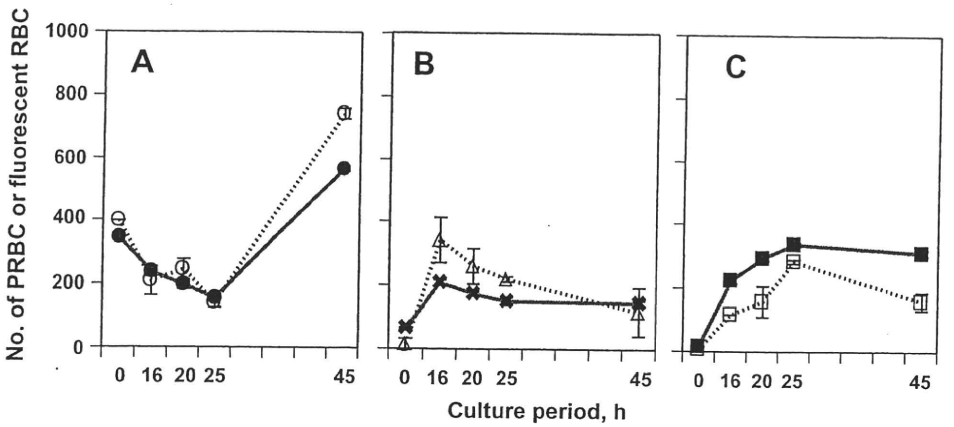

Fig. 4. Evaluation of RFU at different parasite stages. Parasites were characterized by microscopy of thin smears stained with Giemsa or by FCM: <3N-G (...○...) and L-FL (—●—) (A), 3-6N-G (...△...) and M-FL (—X—) (B), >6N-G (...□...) and H-FL (—■—) (C). The cells were fixed with PFA/Als before being stained with SYBR Green I-basic. The numbers of PRBC or fluorescent RBC per 10,000 RBC are shown.

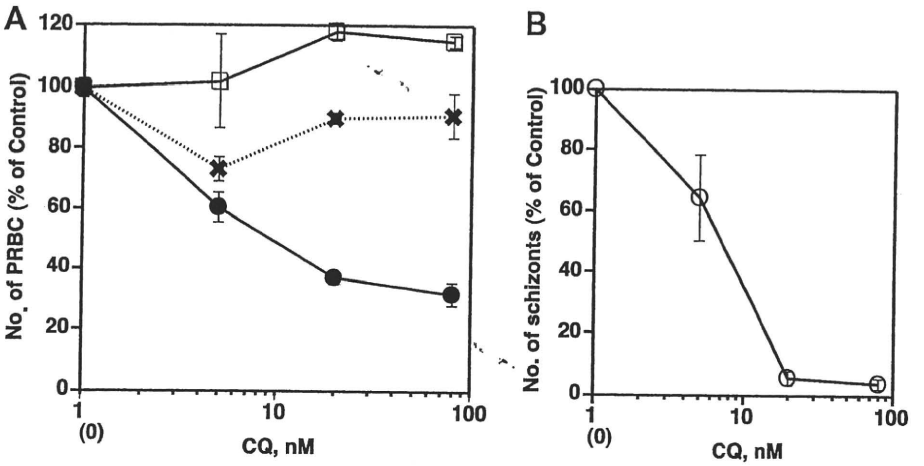

Fig. 5. CQ susceptibility of *P. falciparum* determined by FCM (A) and the Giemsa method (B). At 25 h of synchronized culture of *P. falciparum* in the presence of graded concentrations of CQ, the number of PRBC of H-FL (—●—), M-FL (—□—) and L-FL (...X...) were read by FCM, and 3-6N-G plus >6N-G was examined by microscopy of slides stained with Giemsa (—○—). The number of PRBC or schizonts in the absence of CQ served as 100% controls.

determined by the Merozoite test was significantly lower than GIC50 and EC50 assessed by other methods.

4. Discussion

FCM offers the potential to study the cell cycle or developmental stages of growth of malaria parasites within the erythrocyte by staining nucleic acid. To analyze the growth of *P. falciparum* SYBR Green I was introduced as an intercalating dye for FCM, and procedures on PRBC with SYBR Green I, such as fixatives, concentrations of the dye, dilution buffer, its pH and staining period, were optimized to give the best results with 1% PFA/Als as a fixative and 1× SYBR Green 1 in TRIS–SALINE at pH 8.8 (SYBR Green 1-basic) as a stain. This method allowed parasitemia and parasites in different stages of differentiation to be quantified according to their DNA content. The proportion of PRBC estimated by FCM and Giemsa method accorded with pLDH levels between 24 and 96 h in culture. PRBC of H-FL at 25 h culture gave results similar to those examined by Giemsa method when the CQ susceptibility of *P. falciparum* was determined by FCM, but PRBC of M-FL and L-FL did not. The protocol also was useful for Merozoite test and provided a sensitive assay to test growth inhibition with CQ.

Affordable and reliable analytical techniques to test growth-promoting and antimalarial efficacy on plasmodia are needed to improve tracking of malaria parasites. FCM has particular advantage in determining populations of dividing stages (schizogony). FCM that uses different intercalating dyes, such as AO, thiazole orange, hydroethidine, and YOYO-1, has already been used successfully to test human and murine samples (Janse and Van Vianen, 1994; Jouin et al., 1995; Barkan et al., 2000; Nyakeriga, 2004; Persson et al., 2006; Li et al., 2007). Each dye has advantages and disadvantages. Some of the dyes lack sufficient sensitivity and/or require complicated preparation procedures that makes their use problematic. To overcome these limitations we selected SYBR Green I as a DNA intercalating dye to take advantage of the brighter signal of SYBR Green I and to optimize FCM procedures for PRBC. The excitation and emission spectra of DNA-intercalated SYBR Green I are broad and provide considerable versatility in the choice of detection wavelengths (Bennett et al., 2004; Smilkstein et al., 2004). Indeed, by fluorescent microscopy and FCM at the 488 nm line of an argon laser (an illumination at convenient visible wavelengths) SYBR Green I provided brilliant resolution of PRBC versus uninfected RBC from asynchronous and synchronous cultures. The fluorescence intensity of SYBR Green I was proportional to the number of nuclei and the amount of nucleic acid. These properties made FCM with SYBR Green I [illegible]

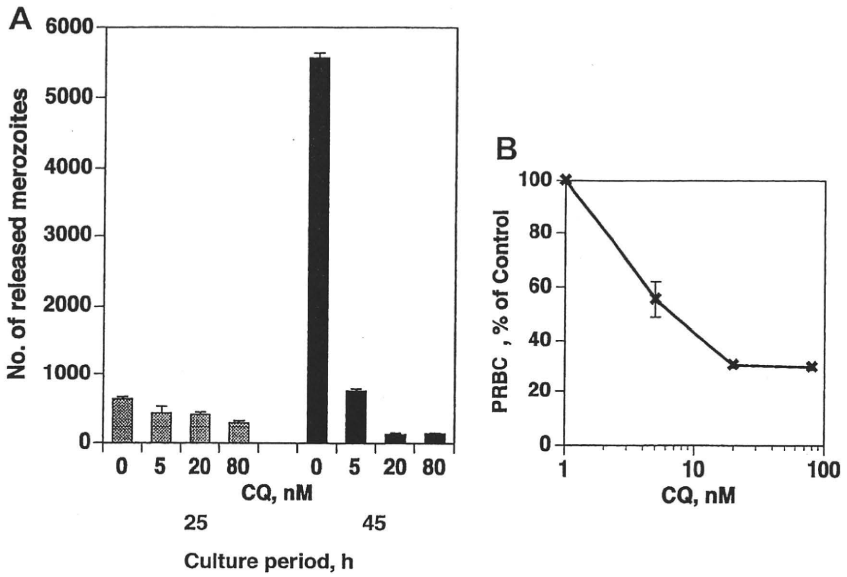

Fig. 6. Growth inhibition effect of CQ on the parasite as assessed by the Merozoite test (A). The number of merozoites is shown per 5000 PRBC counted in the field S2 in Fig. 2. Also shown for comparison is the growth of the parasite (parasitemia) in the presence of CQ after 45 h of culture determined by the Giemsa method (B). The number of background merozoites at a starting point in culture was subtracted in FCM.

Table 1
Comparison of GIC50 and EC50 of CQ determined by FCM, the Giemsa method and the Merozoit test. PRBC of H-FL and 3-6N-G plus >6N-G were counted to determine EC50.

Culture period (h)	GIC50 of CQ, Giemsa method	nM ± SEM FCM	Merozoite test	EC50 of CQ, Giemsa method	nM ± SEM FCM
16	ND	ND	ND	11.52 ± 0.29	7.47 ± 4.27
20	ND	ND	ND	11.02 ± 2.10	7.15 ± 1.49
25	ND	ND	ND	7.98 ± 4.07	11.55 ± 1.51
45	7.38 ± 3.93	7.47 ± 0.62	2.91 ± 0.01**	ND	ND
96*	12.25 ± 2.15	14.50 ± 0.02	ND	ND	ND

* Asynchronous culture; ND, not determined; SEM, standard error of mean.
** $P < 0.001$ versus other GIC50 and EC50.

valuable in following the dividing stages of *P. falciparum*. After analyzing many samples of *P. falciparum* in experiments of development and susceptibility to drugs using this technique we found this procedure to be robust and reproducible.

In the present study we used FCM with SYBR Green I stain to investigate the frequency distribution of fluorescence intensities at different parasitic stages in synchronized *P. falciparum* cultures with and without CQ pressure. In normal growth of parasites in RBC the increase in the number of nuclei determined by the Giemsa method was proportional to the increase in the fluorescence intensity of shizonts stained with SYBR Green I-basic. This implied that the frequency distribution of fluorescence can represent development of the parasite. However, readings for a number of H-FL under CQ pressure, but not M-FL and L-FL, at 25 h of culture gave results comparable to those of microscopic examination of slides stained with Giemsa. This suggested a discrepancy between nucleic acid synthesis and morphological change in schizogony processes in RBC under conditions inhibited growth, such as the presence of CQ. The discrepancy observed here should be taken into account for drug susceptibility tests by FCM, since changes in the percentages of schizonts after culture often have been used to assess susceptibility to antimalarial drugs.

The protocol described here can be extended to the merozoite counting method (Merozoite test) for a sensitive assay of pharmacokinetic effects of drugs and chemicals on the parasite. Representative experiments with CQ that measure free merozoites released into and remaining in culture medium for 45 h after synchronization can be used to examine the antimalarial effect on *P. falciparum*. Several researches have tried to detect parasites by FCM that were freed after hemolysing blood samples (Janse and Van Vianen, 1994; Saito-Ito et al., 2001). They used AO or Hoechst 33258 as the fluorescent dye. The freed parasites, however, were not separated clearly from the erythrocyte ghosts, or residual bodies of nuclei and platelets in the scattergrams obtained by their FCM methods. In our FCM technique merozoites released from ruptured PRBC at the segmenter stage could be detected by fixation with PFA/Als followed by staining with SYBR Green I-basic. This allowed precise counting of merozoites released into surrounding medium, as compared with microscopic examination of PRBC, which often misses merozoites. Assays of parasite susceptibility to CQ obtained by the Merozoite test detected effects clearly and sensitively: GIC50 values measured for CQ in Merozoite test were significantly lower than GIC50 and EC50 by other methods. This condition is enumerated in Table 1. However, this Merozoite test may have been limited in assessing parasite growth for the case of some antimalarial agents, such as antifolates, that begin acting at later stages in parasite development, while parasites under the influence of these drugs tend to produce relatively high numbers of merozoites which then will stop growing before reaching the next cycle, including invasion into new RBC. The correlations among microscopy, the SYBR Green I-basic method, and the pLDH method were excellent. However, small discrepancies between the three methods were observed after 120 h of incubation. The variation may have been because microscopy is a subjective technique and many PRBC that do not show a healthy morphology may be discarded during counting, whereas the FCM and pLDH method detect PRBC irrespective of their status. In high parasitemia and after culture for long periods without renewal of the medium the frequency of altered parasites observed by microscopy increases, affecting the data to a greater degree. Thus, the FCM and pLDH methods may show slightly higher values than microscopy after 120 h of culture, as shown in Fig. 3. This also means that FCM does not necessarily determine the viability of parasites, but the presence of nucleic acids. This should be taken into account in drug susceptibility tests by determining parasitemia by FCM. Particularly when a number of degrading parasites would be expected to form.

In summary, we reported a simple FCM procedure that allows a fast, sensitive, and precise assessment of parasitemia [illegible]

stages, and released merozoites of *P. falciparum* without ultraviolet laser and complicated preparation protocols.

Acknowledgments

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PLoS NEGLECTED TROPICAL DISEASES

Serological Studies of Neurologic Helminthic Infections in Rural Areas of Southwest Cameroon: Toxocariasis, Cysticercosis and Paragonimiasis

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Abstract

Background: Both epilepsy and paragonimiasis had been known to be endemic in Southwest Cameroon. A total of 188 people (168 and 20 with and without symptoms confirmed by clinicians, respectively, 84.6% under 20 years old) were selected on a voluntary basis. Among 14 people (8.3%) with history of epilepsy, only one suffered from paragonimiasis. Therefore, we challenged to check antibody responses to highly specific diagnostic recombinant antigens for two other helminthic diseases, cysticercosis and toxocariasis, expected to be involved in neurological diseases. Soil-transmitted helminthic infections were also examined.

Methodology/Principal Findings: Fecal samples were collected exclusively from the 168 people. Eggs of *Ascaris lumbricoides*, *Trichuris trichiura* and hookworms were found from 56 (33.3%), 72 (42.8%), and 19 (11.3%) persons, respectively. Serology revealed that 61 (36.3%), 25 (14.9%) and 2 (1.2%) of 168 persons showed specific antibody responses to toxocariasis, paragonimiasis and cysticercosis, respectively. By contrast, 20 people without any symptoms as well as additional 20 people from Japan showed no antibody responses. Among the 14 persons with epilepsy, 5 persons were seropositive to the antigen specific to *Toxocara*, and one of them was simultaneously positive to the antigens of *Paragonimus*. The fact that 2 children with no history of epilepsy were serologically confirmed to have cysticercosis strongly suggests that serological survey for cysticercosis in children is expected to be useful for early detection of asymptomatic cysticercosis in endemic areas.

Conclusions/Significance: Among persons surveyed, toxocariasis was more common than paragonimiasis, but cysticercosis was very rare. However, the fact that 2 children were serologically confirmed to have cysticercosis was very important, since it strongly suggests that serology for cysticercosis is useful and feasible for detection of asymptomatic cysticercotic children in endemic areas for the early treatment.

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Introduction

Parasitic infections are serious public health problems in many developing countries [1,2]. These diseases can affect various tissues and organs including the brain leading to neurological dysfunction. Cysticercosis caused by *Taenia solium* metacestodes has been assumed to be the most common parasitic infection of the brain worldwide including Cameroon [3–5]. As cysticercosis is one of the major causative agents of the late-onset of epilepsy, the major work on cysticercosis has been carried out for adults but not for children in endemic areas, and other causative agents of epilepsy still remain unclear. Therefore, we were lead to obtain more information on the causative agents of epilepsy in developing countries, since many helminthic diseases including toxocariasis, paragonimiasis, onchocerciasis etc., and also protozoan diseases including malaria, toxoplasmosis and others may cause epilepsy [4–6]. Among these neglected helminthic diseases, toxocariasis is expected to have cosmopolitan distribution, since dogs and cats

Author Summary

A total of 188 people (168 and 20 with and without symptoms confirmed by clinicians, respectively, 84.6% under 20 years old) were selected on a voluntary basis in Cameroon. Soil transmitted helminthic infections were prevalent among persons surveyed as is common in developing countries, since eggs of *Ascaris lumbricoides*, *Trichuris trichiura* and hookworms were found from 56 (33.3%), 72 (42.8%) and 19 (11.3%) persons, respectively. Serological analyses revealed that 61 (36.3%), 25 (14.9%) and 2 (1.2%) persons were positive to the diagnostic antigens specific for toxocariasis, paragonimiasis and cysticercosis, respectively. Among 14 people with epilepsy, 5 persons were seropositive to the antigen of *Toxocara* and one of them was simultaneously positive to the antigens of *Paragonimus*. Serological confirmation of cysticercosis in two children is very important, and we suggest that further serologic surveys of cysticercosis be carried out in both children and adults in this area for the promotion of a better quality of life including control and early treatment.

are companion animals with close contact with people in the world [7,8]. Although there are no data on the prevalence of human toxocariasis in Cameroon, its prevalence in dogs in Cameroon is high [9]. Simultaneously, there is poor information on cysticercosis in children in Cameroon, although it seems to be rather common in the adult population [4,5].

Tombel health district in South West Province in Cameroon (Figure 1) is known as an endemic focus of epilepsy and is also highly endemic for paragonimiasis [10,11]. Our previous report in this area showed that 8.3% of enrolled people (14/168) suffered from epilepsy but only one of the epileptic patients simultaneously suffered from paragonimiasis [11]. Therefore, we concluded that paragonimiasis was not the major cause of epilepsy in children in this area.

In this study, we used the same 188 samples examined for paragonimiasis [11] and additional 20 samples from Japan, where cysticercosis and paragonimiasis have long been eradicated and toxocariasis is very rare [12], as healthy controls. We performed serosurveys using highly specific recombinant antigens for toxocariasis and cysticercosis, and simultaneously analyzed the unpublished data on microscopic observation of soil-transmitted helminthic (STH) infections. Serological data on paragonimiasis for this study were modified from published data [11]. Although onchocerciasis was known to be endemic in Cameroon and might be involved in neurological disorder, we could not examine simply because the lack of serological tools [13,14].

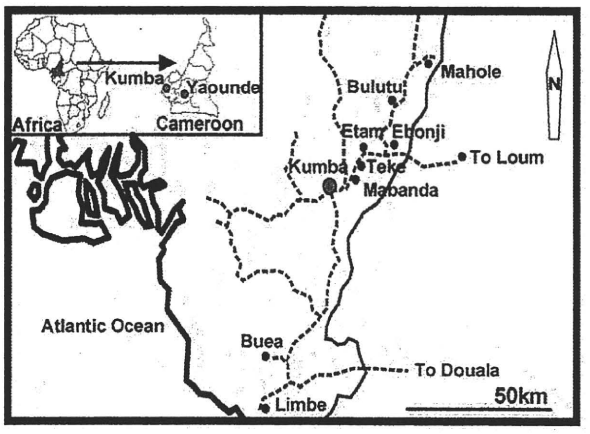

Figure 1. Locations of Bulutu, Ebonji, Etam and Teke in Tombel sub-Division, Southwest Province, Cameroon.
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Materials and Methods

Study sites

Four villages in rural areas, Bulutu, Ebonji, Etam and Teke, were selected for this study. They are located in the Tombel Health District (50,000–100,000 inhabitants) in the rain forest zone about 40 km northwest of Kumba, Manengouba Department, South West Province of Cameroon (4°3′N, 9°3′W). The annual average temperature is 24°C and the relative humidity varies from 52% to 74%. Agriculture is the principal economic activity; hunting and fishing are also practiced (Figure 1).

Ethical statement

The survey, approved by the National Ethics Committee of Cameroon, was conducted in the general population in January 2004 and February 2006 in villages mentioned above.

Human samples

The chief of each village was informed about the study and participants or parents/guardians were asked to give informed consent for participation. A total of 188 people ranged in age from 4 to 78 years (14.9±7.8 years in males and 13.1±6.1 years in females) were examined by clinicians and were asked whether they had experienced symptoms such as cough, haemoptysis, headache, epilepsy, chest pain, and eye disorder and whether they consumed raw and/or undercooked fresh water crabs or pork. Our study population with symptoms ranged from 0–10 years (80 persons), 11–20 years (63 persons), and >21 years (25 persons). Following the questionnaire, serum, sputum and fecal samples were collected from 168 people who accepted to participate to the study voluntarily (28, 52, 55 and 33 from Bulutu, Ebonji, Etam, and Teke villages, respectively). By contrast, 20 healthy persons [5 persons from each village including 11 females and 9 males ranged from 6 to 34 years (13.0±3.7 in males and 15.1±7.5 in females)] confirmed by clinicians donated serum samples exclusively; these serum samples were used as expected healthy controls. An additional 20 serum samples from students at Asahikawa Medical College (AMC), Japan, were used as confirmed healthy controls. Sputum was examined for eggs of *P. africanus* [11]. Fecal samples were examined by flotation techniques for the presence of eggs to provide a diagnosis of helminthic infections.

Serology

A total of 208 serum samples were examined by ELISA. A recombinant antigen of *T. canis* second-stage larvae (0.5 μg/ml) was used for toxocaraisis [15]. Glycoproteins (GPs) (1.0 μg/ml) from *T. solium* cyst fluid purified by preparative isoelectric focusing (pH 9.2–9.6) were used for screening of cysticercosis by ELISA [16]. Immunoblot using a recombinant chimeric antigen, 100% specific to cysticercosis (0.5 μg/mini gel) was applied for serological confirmation of cysticercosis [16–19]. Somatic antigens of *P. africanus* adult worms (5μg/ml), which showed few cross reactivity with other parasitic infections were used for paragonimiasis [11]. Briefly, 96-well microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with each of the antigens described above in PBS and incubated at 4°C overnight. The