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Intraerythrocytic *Plasmodium falciparum* Growth in Serum-Free Medium with an Emphasis on Growth-Promoting Factors

Hiroko Asahi
Department of Parasitology,
National Institute of Infectious Diseases,
Japan

1. Introduction

Malaria remains a devastating disease, particularly in the tropics. The annual incidence of malaria worldwide is estimated to be between 294 and 500 million clinical cases, while estimates of annual mortality from malaria, caused largely by the protozoan *Plasmodium falciparum*, range from 0.97 to 2.7 million worldwide (World Malaria Report 2010[1], World Health Organization; Snow et al., 2005). The emergence of resistance to conventional antimalarial drugs and insecticides means that new chemotherapeutic approaches with alternative targets are needed (Ridley, 2002). Better understandings of antimalarial drugs and the biology of the parasites are needed to allow the development of new medications.

A review of the impact of continuous cultures of *P. falciparum* underscores their significant contributions to malaria research (Trager & Jensen, 1997). The mechanisms responsible for the growth of the parasite, however, remain largely unknown. Culture media for *P. falciparum* require human serum, a growth-promoting fraction derived from adult bovine plasma (GFS), or lipid-enriched bovine albumin (Asahi & Kanazawa, 1994; Asahi et al., 1996; Cranmer et al. 1997; Jensen, 1979). Elucidation of the factors able to induce the growth of *P. falciparum* could be of help, not only for successful culture of the parasite, but also for providing critical clues to understanding the biology of parasite proliferation during the erythrocytic phase.

In order to identify the factors controlling parasite development, and the effects of growth-promoting factors on the parasite, we initially investigated growth-promoting substances to formulate a chemically defined culture medium (CDM) suitable for sustaining the complete development and intraerythrocytic growth of *P. falciparum*. We also developed a simple and sensitive flow-cytometry-based assay for following each developmental stage of the parasite's erythrocytic growth. The distinct roles of the growth-promoting factors in the growth of *P. falciparum* were then investigated.

[1]http://www.who.int/malaria/world_malaria_report_2010/en/index.htm

2. Parasites and culture

Cultures of the FCR3/FMG (ATCC Catalogue No. 30932, Gambia) strain of *P. falciparum* were used in all experiments. The parasites were routinely maintained using *in vitro* culture techniques. The culture medium was devoid of whole serum, and consisted of basal medium supplemented with 10% GFS (Daigo's GF21; Wako Pure Chemical Industries, Japan), as previously reported (Asahi, 2009; Asahi & Kanazawa, 1994; Asahi et al., 1996; Asahi et al., 2005; Asahi et al., 2011). This complete medium is referred to as GFSRPMI. The basal medium consisted of RPMI-1640 containing 2 mM glutamine, 25 mM 4-(2-hydroxylethyl)-piperazine ethanesulfonic acid, 24 mM $NaHCO_3$ (Invitrogen Ltd., USA), 25 μg/ml gentamycin (Sigma-Aldrich Corp., USA) and 150 μM hypoxanthine (Sigma-Aldrich). Briefly, erythrocytes (RBC) were preserved in Alsever's solution (Sigma-Aldrich; Asahi et al., 1996) for 3–30 days. They were then washed, dispensed into 24-well culture plates at a hematocrit (% of packed RBC in medium) 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, 3-4 days after inoculation, infected RBC and uninfected RBC were washed. Parasitemia (% of infected RBC in total RBC) 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. The cultures were synchronized at the ring stage by three successive exposures to 5% (w/v) D-sorbitol (Sigma-Aldrich) at 41- and 46-h intervals. After the third sorbitol treatment, residual schizonts and cell debris were removed by isopycnic density centrifugation on 63% Percoll PLUS (GE Healthcare Bio-Science Corp., USA). The current cultivation method remains essentially the same as initially described (Jensen, 2002), with a few refinements, particularly in terms of the culture medium. The growth experiments were performed by replacing GFSRPMI with test samples. Growth rate was estimated by dividing the parasitemia of the test sample after incubation by the initial parasitemia.

3. CDM for continuous intraerythrocytic growth of *P. falciparum* using lipids

The *P. falciparum* parasite develops through three distinct stages within RBC during its cycle of approximately 48 h: ring, trophozoite, and schizont (Bannister et al., 2000). However, the development of *P. falciparum* requires the presence of currently unknown factors present in human serum (Jensen, 1979). Although numerous studies have attempted to identify the factors and substances able to sustain parasite growth (Asahi & 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), the establishment of a fully-defined culture medium for the parasite has represented a major challenge. We previously reported that GFS supported intraerythrocytic growth of *P. falciparum* (Asahi and Kanazawa, 1994; Asahi et. al., 1996; Asahi et al., 2005). GFS contains lipid-rich albumin as a major component. Similarly, Cranmer et al. (1997) described a commercially available lipid-enriched bovine albumin (Albumax II; Invitrogen) that could replace human serum for the *in vitro* cultivation of *P. falciparum*. These serum substitutes are currently widely employed to maintain parasite cultures. However, there is still insufficient information on these indispensable additives to allow direct identification of the functional components required for the growth of *P. falciparum.* The replacement of human serum or GFS in culture medium with chemically- or functionally-defined substances could not only be advantageous for

parasite culture, but could also provide critical clues about the parasite's biology and its requirements for proliferation at the erythrocyte stage. To establish a CDM for continuous, intraerythrocytic growth of *P. falciparum*, we initially characterized the ability of the various components of GFS to sustain parasite growth (Asahi et al., 2005). Based on these results, we subsequently determined the ability of structurally defined chemicals to sustain parasite growth, and formulated a CDM for *P. falciparum* growth (Asahi, 2009).

3.1 The ability of GFS components to sustain parasite growth

We investigated the components of GFS and related substances that have shown an ability to sustain parasite growth (Asahi et al., 2005). A simple total lipid fraction of GFS obtained by lipid extraction has been shown to sustain complete parasite development. However, specific proteins such as bovine and human albumin, as well as the simple total lipid fraction of GFS, have also been shown to be important (Figure 1a). The simple total lipid fraction of GFS contained phospholipids, diacylglycerides (DAG), cholesterol, monoglycerides, nonesterified fatty acids (NEFA) and cholesteryl esters. The components of the NEFA fraction were mainly *cis*-9-octadecenoic acid (C18:1-*cis*-9, 43%), hexadecanoic acid

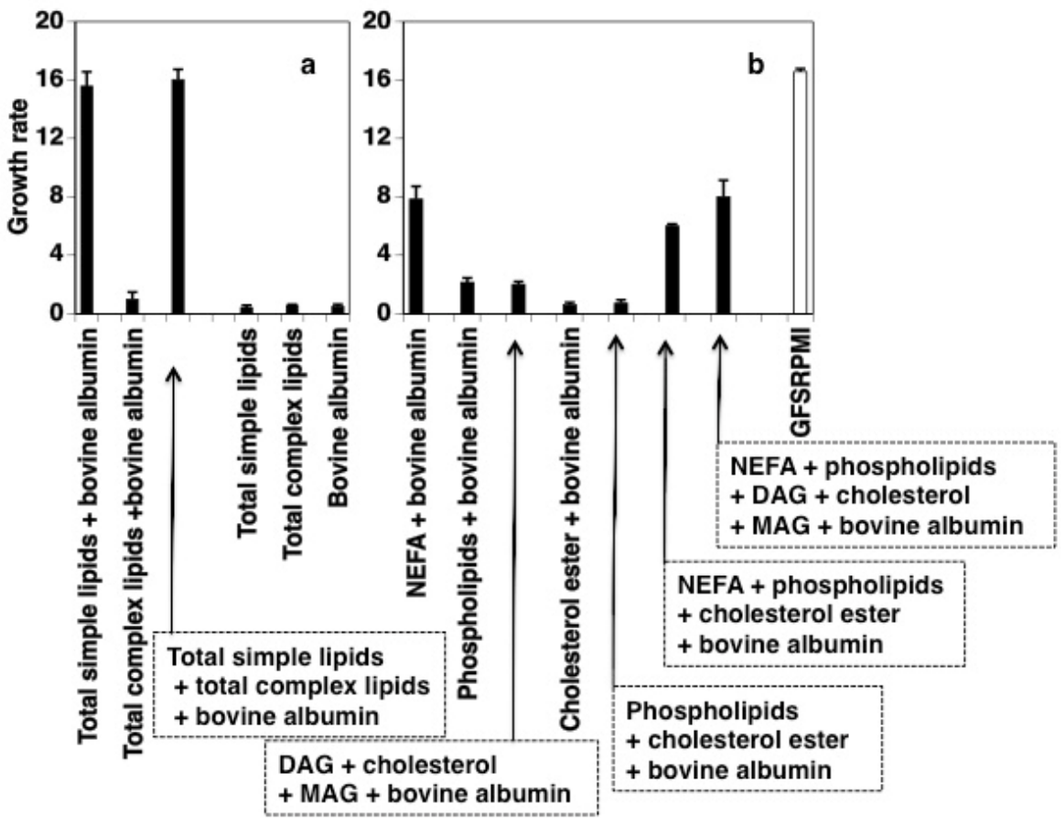


Fig. 1. Abilities of fractions derived from GFS (a) and a total simple lipid fraction of GFS (b) to sustain growth of *P. falciparum*. Growth rate was estimated 4 days after inoculation.

(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 NEFA enriched with bovine albumin (fatty-acid free) was tested for its ability to promote parasite growth. Mixtures of NEFA, but not individual NEFA, sustained parasite growth to a low extent (Figure 1b), but parasite growth in the presence of various combinations of NEFA was still lower than that achieved with a simple total lipid fraction of GFS, or with GFS- or human serum-containing medium. These results implied that, although the NEFA components of the simple total lipid fraction are functional factors in promoting parasite growth, other factor (s) must also contribute to the high growth-promoting activity of GFS.

3.2 CDM for intraerythrocytic growth of *P. falciparum*

Initial experiments designed to determine the factor (s) responsible for the high growth-promoting activity of GFS involved culture of *P. falciparum* with the lipid classes found in the simple total lipid fraction of GFS, and different concentrations of a mixture of the two most abundant NEFA, C18:1-*cis*-9 (0–60 g/ml [212.4 M]) and C16:0 (0–30 μg/ml [117.0 M]) 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 g/ml C18:1-*cis*-9 plus 15 g/ml C16:0, with declines at lower and higher concentrations. However, the growth rates were much lower than that obtained with a simple total lipid fraction of GFS or with GFSRPMI.

A mixture of all the constituents detected in a simple total lipid fraction of GFS sustained complete parasite growth. In an attempt to identify the factor (s) responsible for this growth-promoting effect, each lipid was omitted from the medium in turn. Parasite growth in the absence of phosphatidylcholine (PC) decreased to a level similar to that seen with the NEFA mixture (Figure 2a). Phospholipids were also tested for their possible efficacies in augmenting the ability of NEFA to promote parasite growth, by adding each phospholipid to cultures with NEFA. Phospholipids, even PC alone, markedly amplified the growth-promoting ability of the NEFA mixture (Figure 2b). These results indicate the critical importance of PC for amplifying the parasite-growth-promoting ability of NEFA mixtures. Phospholipids other than PC, such as phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidic acid (PA), were also beneficial for parasite growth, while the growth rates in the absence of phosphatidylinositol (PI), cholesterol, and cholesterol ester were significantly higher (Figure 2a), indicating these phospholipids were detrimental. DAG had no effect on the growth rate of the parasite at the concentrations tested.

The effects of various types of NEFA mixtures enriched with phospholipids were tested for their abilities to promote parasite growth. The growth rate was dependent on the ratios of the two NEFA; the highest growth rate occurred at 2:1 (C18:1-*cis*-9 to C16:0) at a total concentration of 45 g/ml. The growth rates with the best mixtures of NEFA in the presence of phospholipids were significantly higher than those with the same NEFA in the absence of phospholipids (Figure 3). The culture media were also reconstituted by mixing phospholipids with two types of NEFA (either C18:1-*cis*-9 plus a saturated NEFA or C16:0 plus an unsaturated NEFA). The best combination of NEFA was C18:1-*cis*-9 plus C16:0, followed by *cis*-11-octadecenoic acid (C18:1-*cis*-11) plus C16:0, C18:1-*cis*-9 plus pentadecanoic acid (C15:0), C18:1-*cis*-9 plus C18:0, and C18:1-*cis*-9 plus tetradecanoic acid (C14:0). The combinations of C16:1 plus C16:0, *cis*-6-octadecenoic acid (C18:1-*cis*-6) plus

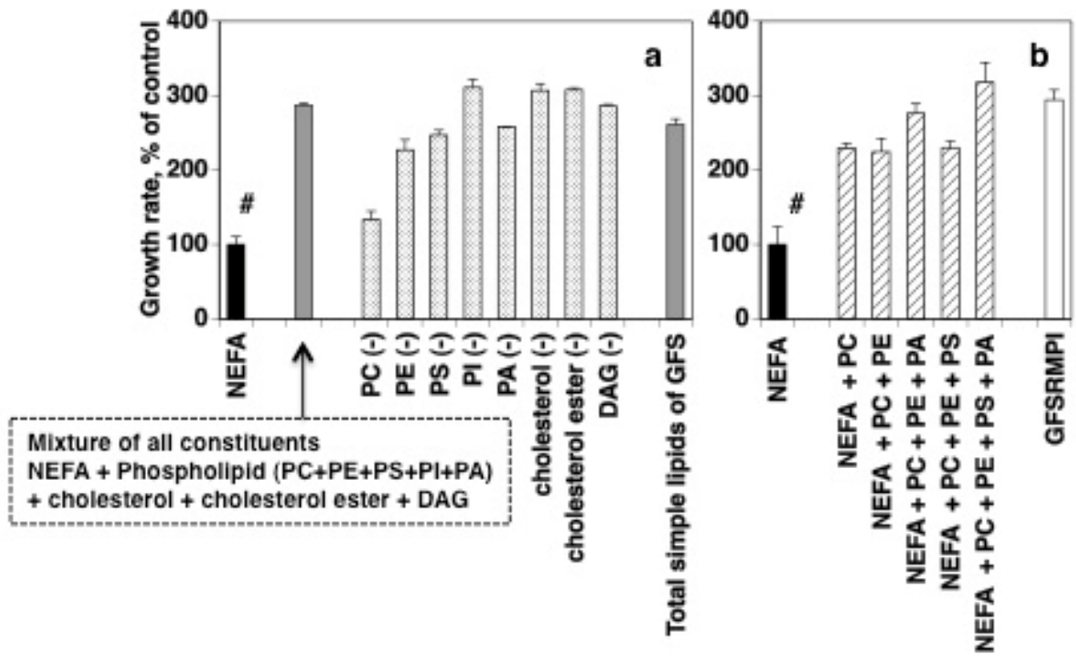


Fig. 2. Effects of various classes of lipids and combinations of phospholipids on abilities of NEFA to sustain growth of *P. falciparum*. The combination of 30 g/ml C18:1-*cis*-9 and 15 g/ml C16:0 served as a control. Growth rate was estimated 4 days after inoculation.

C16:0, *cis*-13-octadecenoic acid (C18:1-*cis*-13) plus C16:0, and C18:2 plus C16:0 showed similar growth-promoting effects to that seen with C18:1-cis-9 plus C16:0 in the absence of phospholipids. Combinations of C18:1-*cis*-9 plus dodecanoic acid (C12:0), C18:1-*cis*-9 plus docosanoic acid (C22:0), *cis,cis,cis*-6,9,12-octadecatrienoic acid (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 *trans*-9-octadecenoic acid (C18:1-*trans*-9) plus C16:0 also deterred parasite growth (Figure 4). The efficacies of NEFA in sustaining the growth of *P. falciparum* thus varied markedly, depending on the type, total amount, and combinations used; 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 the growth of *P. falciparum*. Higher degrees of unsaturation were also detrimental to parasite growth. The growth-promoting effects of NEFA with 18 carbons and one double bond are specific to the *cis*-form, and the position of the double bond in these NEFA influences their growth-promoting effects.

Various PC containing different fatty acid moieties, such as two of hexanoic acid, C12:0, C14:0, C16:0, C18:1, C18:1 in racemic form, C18:2, and C20:4, two different fatty acids of C18:1 and C16:0, and C20:4 and C16:0, and PC derived from soy beans and egg yolk, were tested at graded concentrations ranging from 20–320 M, for their abilities to augment the effects of the NEFA mixture on parasite growth. Among the 12 tested PC, 1,2-dioleoyl-sn-glycero-3-phosphocholine (PC-di18:1) markedly amplified the growth-promoting ability of

the NEFA mixture in a dose-dependent manner and over a wide range of concentrations, to a level similar to that seen with GFSRPMI (Figure 5). This was followed by 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (PC-di16:0) and 2-oleoyl-1-palmitoyl-sn-glycero-3-phosphocholine (PC-18:1/16:0). The addition of PC other than PC-di18:1 at certain concentrations also augmented the growth-promoting ability of the NEFA mixture to various extents, ranging from 0–270%.

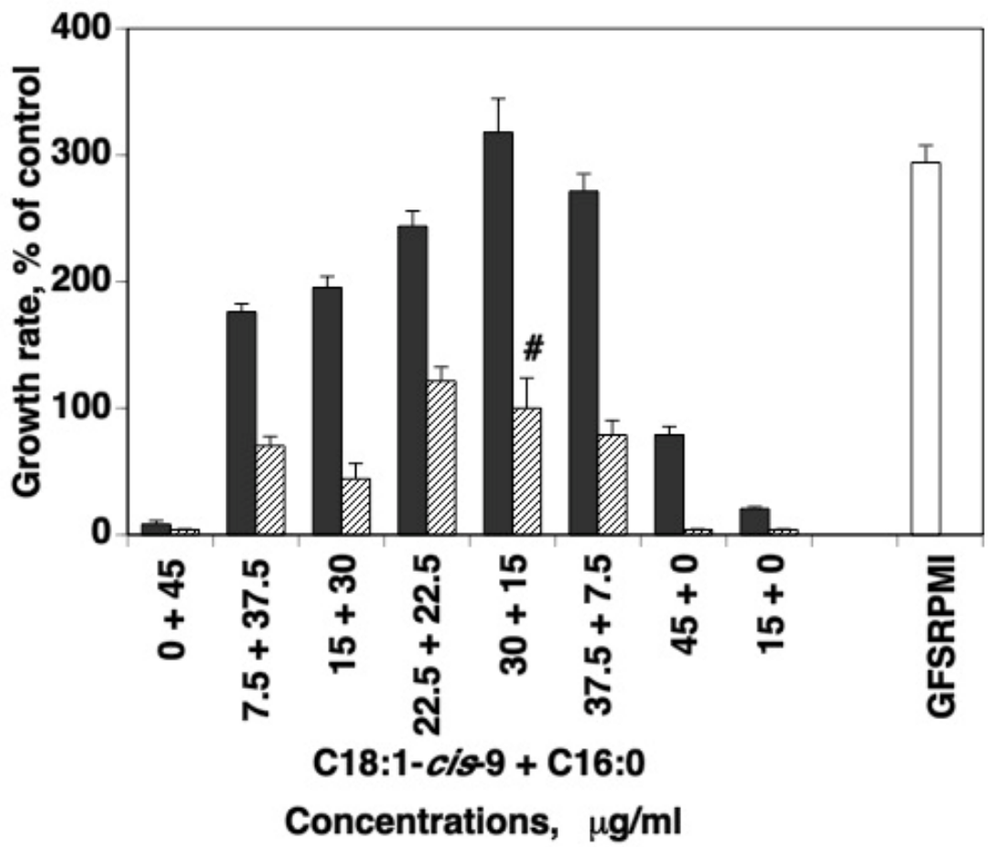


Fig. 3. Growth of *P. falciparum* with NEFA (C18:1-*cis*-9 plus C16:0) in the presence (■) or absence (▨) of phospholipids. Growth rate was estimated 4 days after inoculation. #, A paired NEFA of C18:1-*cis*-9 and C16:0 served as a control.

Specific proteins such as bovine and human albumin were shown to be required for *P. falciparum* growth in serum-free culture with lipids, as stated above. Recombinant human albumin could replace serum albumin for sustaining parasite growth in the presence of lipids (Figure 6).

All stages of *P. falciparum* cultured in the formulated CDM containing the best combination of two NEFA, phospholipids, and human, bovine, or recombinant albumin were morphologically indistinguishable from growth in complete medium (Figure 7).

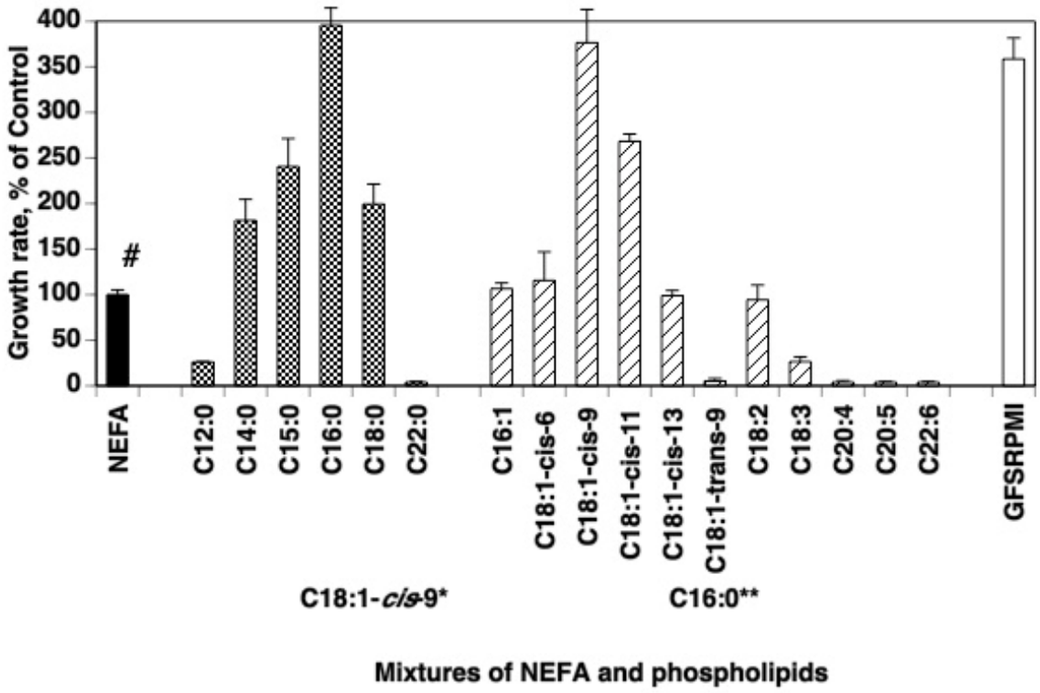


Fig. 4. Growth of *P. falciparum* in the presence of various combinations of paired NEFA. Each saturated NEFA was added at 15 g/ml in the presence of 30 g/ml C18:1-*cis*-9 (*) and each unsaturated NEFA at 30 g/ml in the presence of 15 g/ml C16:0 (**). These culture media contained phospholipids. #, NEFA (C18:1-*cis*-9 + C16:0) in the absence of phospholipids served as a control. Growth rate was estimated 4 days after inoculation.

4. Development of a measure of intraerythrocytic growth of *P. falciparum* using flow cytometry and SYBR Green I

Growth-promoting and antimalarial effects on plasmodia can be assessed both quantitatively and qualitatively by directly examining RBC smears from blood or cultures under a microscope; however, 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 of these 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 parasite DNA (Noedl et al., 2003; Webster et al., 1985; Yayon et al., 1983). These methods are relatively reliable and objective, but not sufficiently sensitive, and require the use of hazardous radioactive material. The assays are well suited for screening large numbers of compounds. Parasite lactate dehydrogenase levels have also been used to assess 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 or parasite lactate dehydrogenase, by double-

site sandwich ELISA (Druihe, et al., 2001; Noedl et al., 2002; Noedl et al., 2003). ELISA-based tests are rapid and easy to perform, and are also well suited for the screening of large number of drugs. These methods have been widely employed to detect and analyze the growth of parasites, although they are poorly suited for discriminating the developmental stages of the parasite in parasitized RBC. Flow cytometry using nucleic acid staining offers the possibility of studying the cell cycle and developmental stages of intraerythrocytic growth of malaria parasites. Flow cytometric analysis using intercalating dyes, such as acridine orange, thiazole orange, hydroethidine, propidium iodine, YOYO-1, and SYTO-16, has already been used successfully to test human and murine samples (Barkan et al., 2000; Janse and Van Vianen, 1994; Jimenez-Diaz et al., 2009; Jouin et al., 1995; Li et al., 2007; Nyakeriga, 2004; Persson et al., 2006). However, the use of flow cytometry has been limited by its lack of specificity and the complicated preparation required. We modified the flow cytometry system and introduced SYBR Green I as an intercalating dye, allowing the growth and development of *P. falciparum* to be analyzed with a high degree of accuracy (Izumiyama et al., 2009).

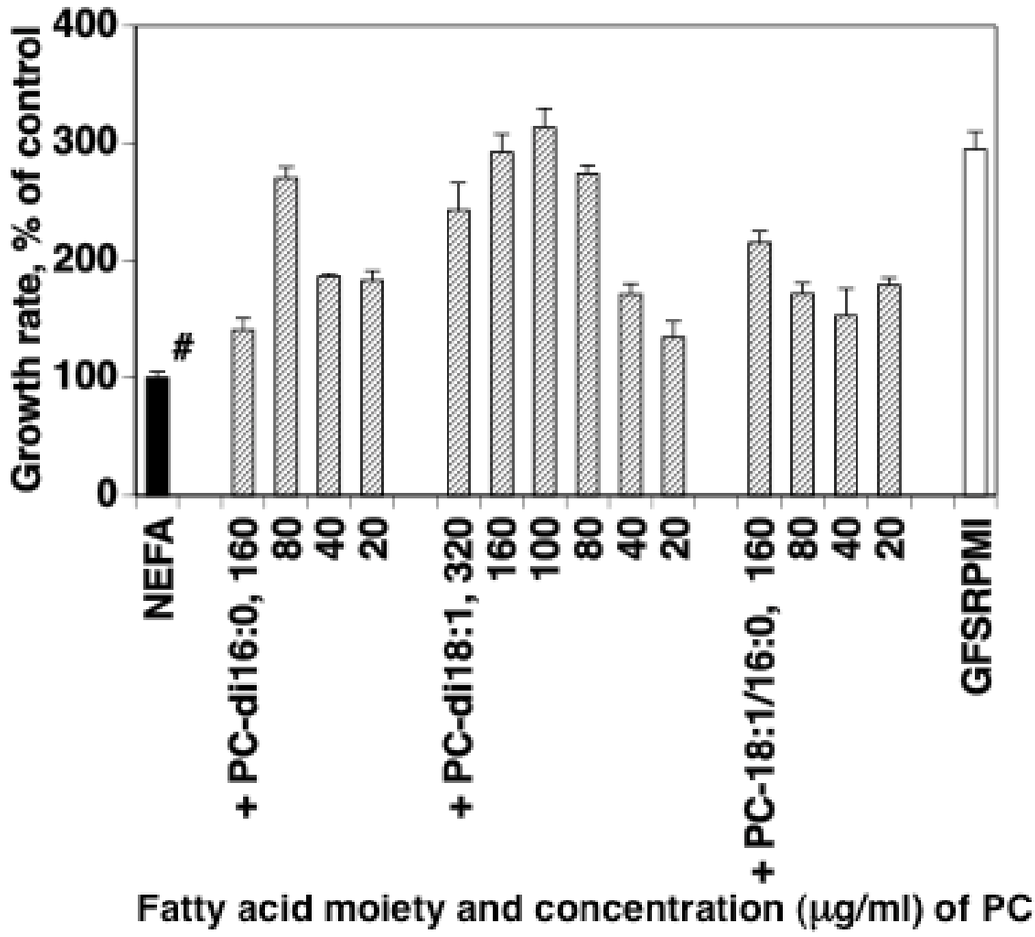


Fig. 5. Effects of various types of PC containing different fatty acid moieties on the abilities of NEFA to sustain growth of *P. falciparum*. Growth rate was estimated 4 days after inoculation. #, A paired NEFA of C18:1-*cis*-9 and C16:0 served as a control.

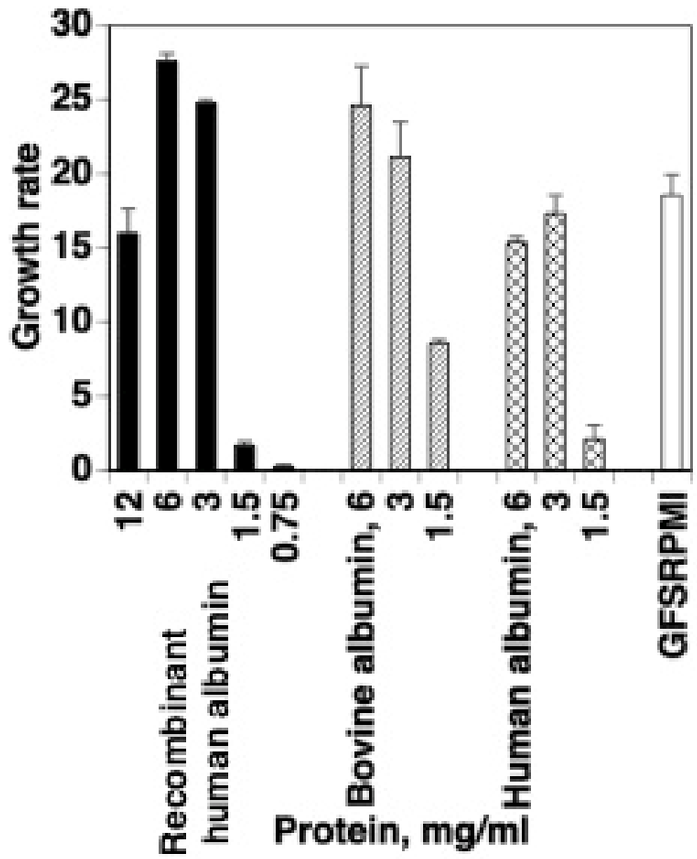


Fig. 6. Effects of various proteins on the ability of a mixture of NEFA and phospholipids to sustain growth of *P. falciparum.* Growth rate was estimated by dividing the parasitemia of the test sample 4 days after inoculation by the initial parasitemia.

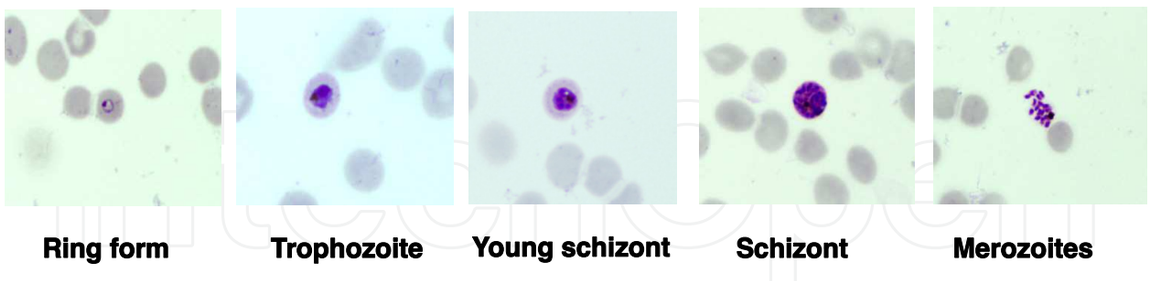


Fig. 7. Different stages of the parasite cultured in the formulated CDM, stained with Giemsa.

4.1 Optimization of flow cytometric measurement of infected RBC with SYBR Green I

The cytometer was equipped with a single argon-ion laser tuned to a fluorescence excitation of 488 nm for 15 mW output (PAS flow cytometer, Partec Co. Ltd., Germany). A FACSCalibur (Becton Dickinson Immunocytometry Systems, USA) was also used with a single fluorescence measurement (530 nm). Analysis was performed using FCS express software (De Novo Software Inc., Canada).

In order to distinguish infected RBC or merozoites from platelets, leukocytes, and RBC debris by flow cytometry, it is essential for them to retain their normal morphological characteristics and membrane integrity, without disruption. We evaluated the effects of (1) fixatives, (2) dilution buffer and concentration range of SYBR Green I, and (3) staining period on the intensity of fluorescence of infected RBC. (1) Infected RBC in culture were fixed using fixatives such as 1% paraformaldehyde or 1% glutaraldehyde in Tris-saline solution (20 mM Tris (hydroxymethyl) aminomethane hydrochloride at pH 7.2 and 138 mM NaCl), phosphate-buffered saline (PBS) (10 mM phosphate buffer at pH 7.2 and 138 mM NaCl), or Alsever's solution. Paraformaldehyde combined with Alsever's solution proved the most useful fixative, with no noticeable lysis or deformity of infected/uninfected RBC, such as often occurs when Tris-saline or PBS is used to dilute fixatives. (2) Concentration ranges and dilution buffers for SYBR Green I were tested to determine the optimal solution for producing clearly resolved peaks of fluorescence. Individual peaks in histograms corresponding to the development stages of infected RBC showed most clearly with SYBR Green I diluted at concentrations ranging from 0.00625–2 in Tris-saline at pH 8.8 (SYBR Green I-basic). Both fixed and unfixed infected RBC gave the best results at 1 dilution of SYBR Green I. (3) The dependence of fluorescence with time for fixed or unfixed infected RBC in SYBR Green I-basic at 1 dilution was evaluated over 30 min by time-curve analysis of *P. falciparum* cultures. The frequency distribution of fluorescence was similar after staining for 5 min or longer with SYBR Green I-basic, although fluorescence could be detected within seconds after addition of the fluorescent stain to fixed or unfixed infected RBC. SYBR Green I in Tris-saline at pH 6.8 and PBS resulted in inadequate signals for infected RBC stained for less than 30 min, and in deformation and hemolysis of infected RBC. Accordingly, the optimized protocol was fixation of infected/uninfected RBC by the addition of 1% paraformaldehyde combined with Alsever's solution, and staining the fixed RBC, at measure by flow cytometry, by adding into SYBR Green I-basic solution containing SYBR Green I at x1 dilution for 5 min.

4.2 Visualization of infected RBC populations with SYBR Green I-basic

Infected RBC populations were stained as described above. All developmental stages were clearly stained with SYBR Green I with no autofluorescence of RBC (Figure 8).

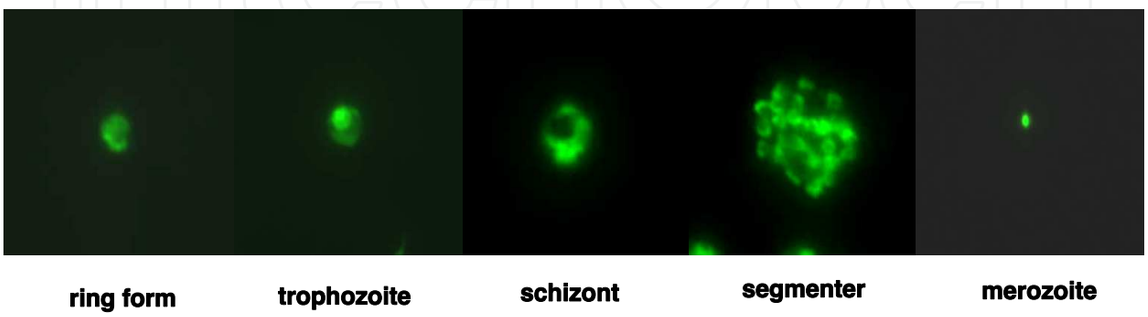


Fig. 8. Developmental stages of *P. falciparum* stained with SYBR Green I-basic.

Infected RBC were stained with SYBR Green I-basic by adding 8 x 10^5 cells into 1 ml staining solution, followed by analysis with flow cytometry. SYBR Green I-basic provided brilliant resolution of infected versus uninfected RBC and permitted visualization of infected RBC populations with high accuracy. Infected RBC were located as three clusters in two-parameter dot-plot presentations of infected/uninfected RBC from *P. falciparum* cultures: (1) cluster 1 (C1) contained predominantly ring forms with low DNA content (low fluorescence intensity); (2) cluster 2 (C2) contained predominantly late trophozoites and young schizonts with moderate DNA content (moderate fluorescence intensity); and (3) cluster 3 (C3) contained late schizonts and segmenters with high DNA content (high fluorescence intensity) (Figures 9a, b).

In regular cultures, schizonts burst spontaneously to release free merozoites, which then enter new RBC and increase the number of infected RBC. While the majority of released free merozoites remain in culture for a while, the merozoites after culture indicate the completion of schizogony. Parasites were synchronized at the ring form stage and cultured over 45 h under the pressure of various concentrations of the anti-schizogony drug chloroquine, to confirm the accuracy of counting the number of merozoites by flow cytometry. Merozoites released from mature schizonts were counted clearly and sensitively by flow cytometry (Figures 10a,b).

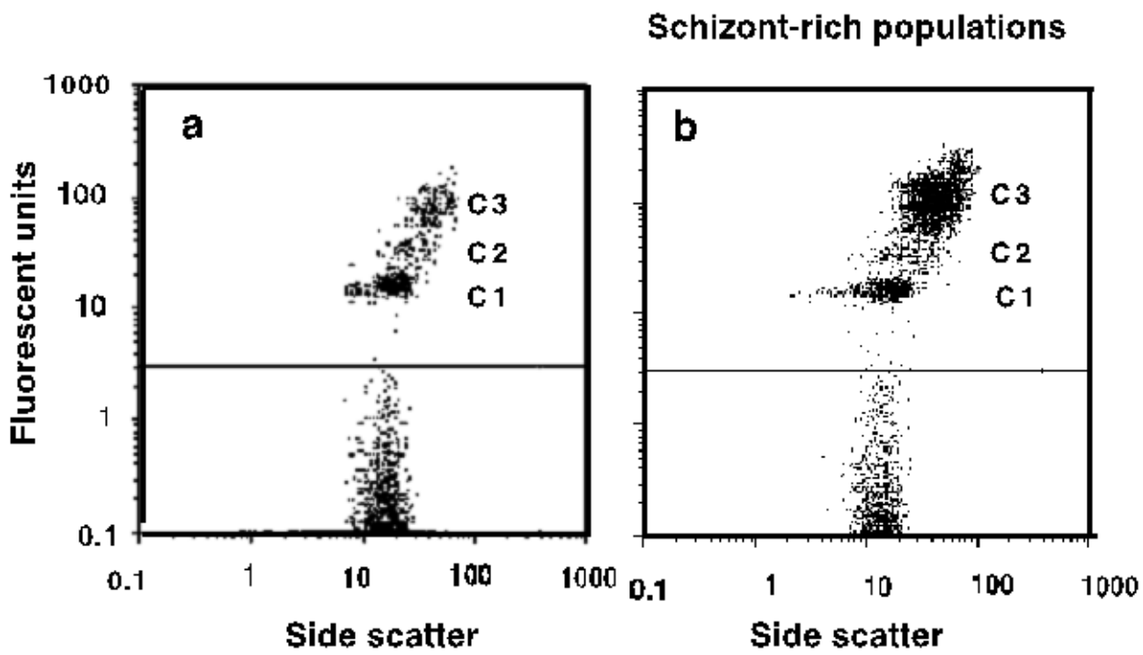


Fig. 9. Two-parameter dot-plot representation of fluorescent units and side scatter from asynchronous culture (a) of *P. falciparum* and schizont-rich populations (b) separated by Percoll sedimentation.

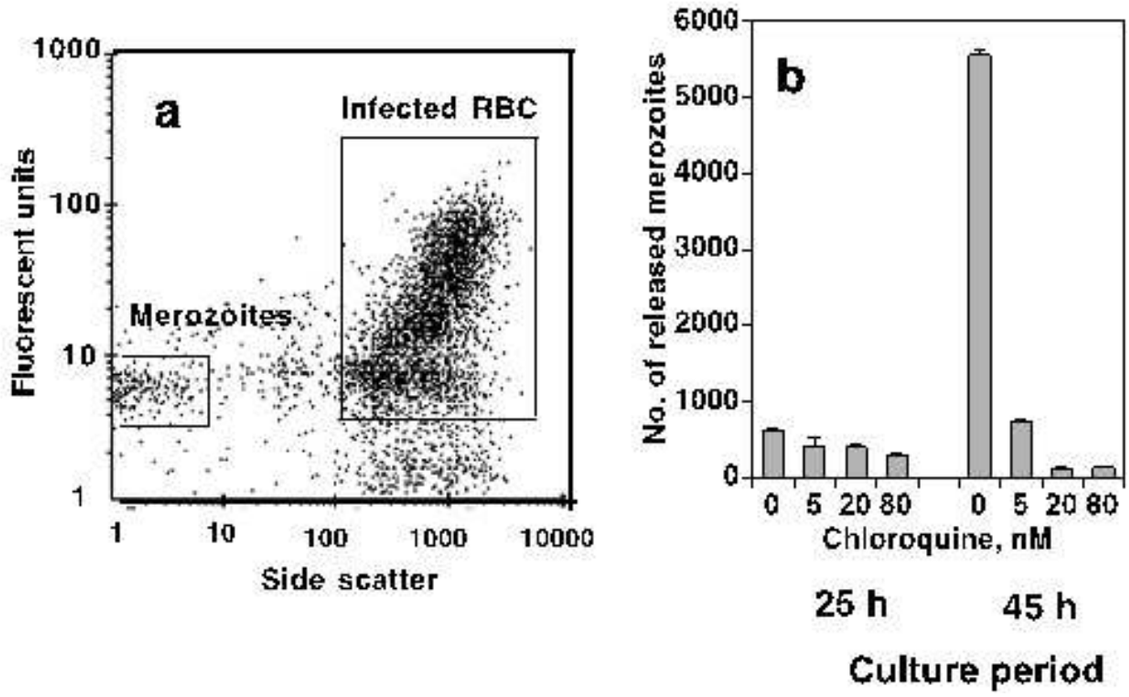


Fig. 10. Merozoites released into surrounding medium were counted (a) and the number of merozoites decreased under the pressure of graded concentrations of chloroquine (b). The numbers of merozoites are shown per 5,000 infected RBC (b).

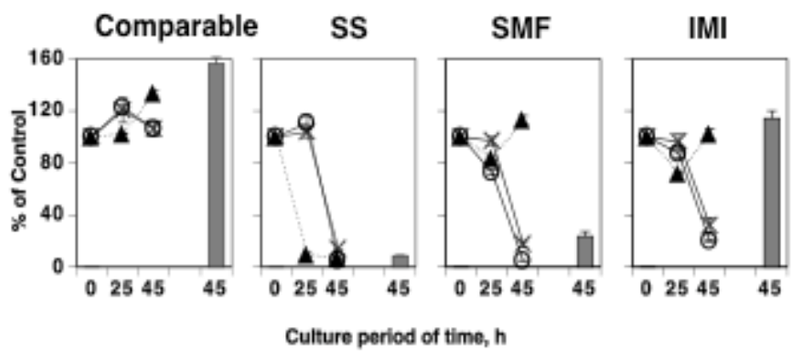
5. Differing effects of NEFA and phospholipids on intraerythrocytic growth of *P. falciparum* in serum-free medium

The efficacies of NEFA in sustaining general growth of *P. falciparum* varied markedly, depending on the type, total amount, and combinations. Certain structural characteristics of NEFA, such as carbon-chain length, degree and position of unsaturation, and isomerism were important. However, the mechanisms responsible for the different abilities of the various NEFA in the presence or absence of phospholipids, and of specific proteins such as bovine and human albumin for promoting parasite growth are unknown. Subsequent experiments therefore investigated the distinct effects of various NEFA on each developmental stage of *P. falciparum*, including schizogony, merozoite formation, and reinvasion of RBC, to provide clues to the mechanisms underlying the growth-promoting properties of NEFA.

5.1 Four typical growth patterns

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 phospholipids and bovine albumin, further supplemented with one or two NEFA. The distribution of the parasites among the different developmental stages was determined using flow cytometry with SYBR Green I-basic at 25 and 45 h during the first cycle of growth (Izumiyama et al., 2009). Late schizonts at 25 h (schizont-25h), released merozoites at 45 h (released merozoite-45h), new ring forms at 45 h (ring form-45h), and

parasitemia at 45 h (parasitemia-45h) were compared between parasites grown under test conditions and those grown in complete medium GFSRPMI. Different types and combinations of NEFA exerted markedly distinct effects on parasite growth in the presence/absence of phospholipids. Four typical growth patterns were defined: no inhibition (comparable to growth in complete medium); 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 (Figure 11).



Schizont-25h	High	Low	High	Moderate~High
Released merozoite-45h	High	Low	Low	High
Ring form-45h	High	Low	Low	Low
Parasitemia-45h	High	Low	Low	Low

Fig. 11. Representative modification of growth of *P. falciparum* cultured synchronously in the presence of various growth promoters, indicating comparable growth, SS, SMF, and IMI. Each developmental stage was compared with complete growth in GFSRPMI (control): ring forms (—○—), late schizonts (…▲…), parasitemia (—X—), and released merozoites (closed bars). Parasites at the ring stage (adjusted to 5.0% parasitemia) were maintained in different culture media.

5.2 Growth-rate-determining steps in development of *P. falciparum* cultured in various growth factors

All stages of the parasite cultured in medium supplemented with NEFA (C18:1-*cis*-9 plus C16:0) in the presence of phospholipids were comparable to those grown in GFSRPMI. Medium containing C18:1-*cis*-9 and C12:0 caused parasites to accumulate in clusters of 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, or C18:1-*trans*-9 plus C16:0. Partial SS (less suppressed) was detected when the mixture of C18:1-*cis*-13 plus C16:0 was 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 phospholipids, indicating that exogenous phospholipids were crucial for the development of complete merozoites. Adding C18:1-*cis*-13 plus C16:0 or C16:1 plus C16:0 to the media caused accumulation of the 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 at 45 h culture. These results indicate that different NEFA exert distinct roles in parasite development by arresting development at different stages (Figure 12).

NEFA	Growth-rate determining step	Growth level Ring --- Trophozoite --- Schizont --- Merozoite --- New ring
C18:1-cis-9	SMF	
C18:1-cis-9+C12:0	SS	
C18:1-cis-9+C14:0	partial IMI	
C18:1-cis-6+C16:0	partial IMI	
C18:1-cis-9+C16:0	comparable/ better	
C18:1-cis-11+C16:0	partial IMI	
C18:1-cis-13+C16:0	partial SS, IMI	
C18:1-cis-9+C18:0	partial IMI	
C18:1-cis-9+C22:0	SMF	
C16:0	SS	
C16:1+C16:0	IMI	
C18:2+C16:0	SS	
C20:4+C16:0	SS	
C18:1-trans-9+C16:0	SS	
C18:1-cis-9+C16:0, Phospholipids (-)*	SMF	
GFSRPMI	complete growth	

Fig. 12. Growth-rate-determining step and growth level in development of *P. falciparum* cultured in the presence of NEFA alone or in combination. Growth of the parasite was examined in the presence of optimal phospholipids, except for C18:1-*cis*-9 plus C16:0 in the absence of phospholipids (*) and GFSRPMI. Bovine albumin was added to the medium, except for GFSRPMI.

5.3 Microscopic examination of *P. falciparum* cultured with NEFA exerting IMI and SMF effects

Microscopic examination revealed that parasites cultured in medium containing NEFA (C18:1-*cis*-9 plus C16:0), phospholipids and bovine albumin closely resembled 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 phospholipids (SMF effect) for 45 h were found to be degenerate (Figure 13).

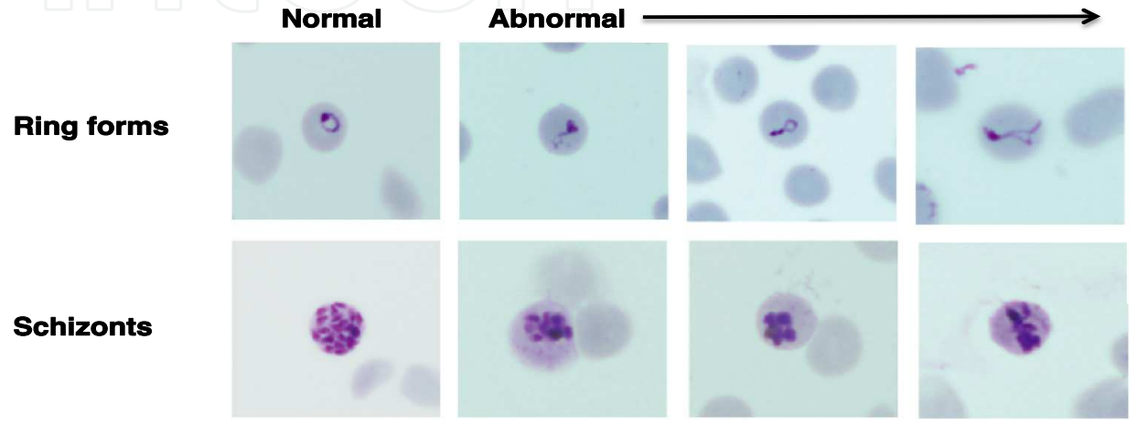


Fig. 13. Abnormal parasites (ring forms and schizonts) grown in non-optimal culture media.

6. Conclusions and future perspectives

In an attempt to elucidate the mechanisms responsible for growth of *P. falciparum*, growth-promoting factors were identified and a CDM suitable for complete growth of the parasite was established. The CDM consists of paired NEFA, phospholipids with specific fatty acid moieties, and specific proteins dissolved in basal medium RPMI1640 supplemented with hypoxanthine. The most effective combination of NEFA was C18:1-*cis*-9 and C16:0. The best phospholipid crucial for serum-free culture medium supplemented with NEFA was PC-di18:1 at concentrations of 80–320 g/ml. A simple protocol for flow cytometry with SYBR Green I was developed and used to analyze the various developmental stages of *P. falciparum*. Different stages of the parasite in RBC and released merozoites were quantified using this flow cytometry protocol. These techniques were applied to investigate the distinct roles of the identified growth-promoting factors in the development of the parasite, demonstrating that different combinations of NEFA and phospholipids exerted distinct roles in the growth of *P. falciparum* by sustaining development at different stages.

These findings can be usefully applied in diverse aspects malaria research, including drug resistance, vaccine development, genetics, parasite biochemistry, and studies of the relationship between the parasites and the host RBC. Culture in CDM produces similar results to those using the original culture method with human serum (Trager & Jensen, 1997), with the added advantage of avoiding the adverse effects caused by human serum. In particular, the methods reported here will allow the components crucial to each developmental stage of the parasite to be established. We have already performed a large-

scale analysis of the genome-wide gene expression in *P. falciparum* cultured in the various CDM established here, to investigate the relationship between gene expression regulation and parasite development at different stages. This comprehensive analysis identified a number of genes possibly involved in arresting parasite development. These results will be reported elsewhere. Interactions between growth-promoting factors and parasite components can provide critical clues to the understanding of the general biology of *P. falciparum,* and will provide the foundations for future drug and vaccine development efforts aimed at eradicating this disease.

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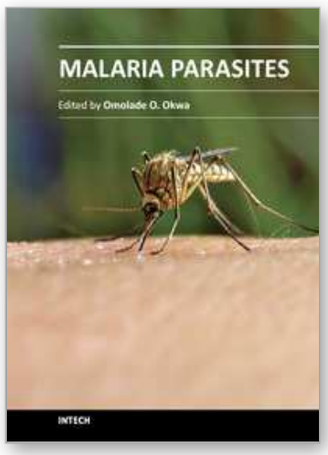

Malaria Parasites

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Malaria is a global disease in the world today but most common in the poorest countries of the world, with 90% of deaths occurring in sub-Saharan Africa. This book provides information on global efforts made by scientist which cuts across the continents of the world. Concerted efforts such as symbiont based malaria control; new applications in avian malaria studies; development of humanized mice to study P.falciparium (the most virulent species of malaria parasite); and current issues in laboratory diagnosis will support the prompt treatment of malaria. Research is ultimately gaining more grounds in the quest to provide vaccine for the prevention of malaria. The book features research aimed to bring a lasting solution to the malaria problem and what we should be doing now to face malaria, which is definitely useful for health policies in the twenty first century.

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