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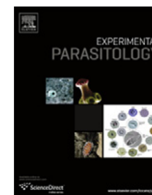

Plasmodium falciparum: Differing effects of non-esterified fatty acids and phospholipids on intraerythrocytic growth in serum-free medium

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ABSTRACT

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

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1. Introduction

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

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

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

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

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

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

2. Materials and methods

2.1. Parasites, culture and synchronization

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

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

2.2. Growth-promoting activity experiments

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

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

2.3. Assessment of parasite growth

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

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

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

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