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Experimental Parasitology 109 (2005) 7–15
Experimental Parasitology
www.elsevier.com/locate/yexpr

Investigating serum factors promoting erythrocytic growth of *Plasmodium falciparum*

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Received 11 May 2004; received in revised form 16 October 2004; accepted 19 October 2004
Available online 10 December 2004

Abstract

The elucidation of factors inducing the growth of *Plasmodium falciparum* can provide critical information about the developmental mechanisms of this parasite and open the way to search for novel targets for malaria chemotherapy. The ability of components of a growth-promoting factor derived from bovine serum and various related substances to sustain growth of *P. falciparum* was characterized. A simple total lipid fraction (GFS-C) containing non-esterified fatty acids (NEFAs) as essential factors was noted to promote the parasite's growth. Various proteins from a variety of animals were tested, indicating the importance not only of GFS-C, but also of specific proteins, such as bovine and human albumin, in the parasite growth. Several combinations of the NEFAs tested sustained low parasite growth. Among various phospholipids and lysophospholipids tested, lysophosphatidylcholine containing C-18 unsaturated fatty acids was found to sustain the complete development of the parasite in the presence of bovine albumin. Several other lysophospholipids can partially support growth of *P. falciparum*.

Index Descriptors and Abbreviations: ALB, albumin; ANOVA, multifactorial analysis of variance; BSAF, NEFA-free ALB from bovine; C16:0, hexadecanoic acid; C16:1, *cis*-9-hexadecenoic acid; C18:0, octadecanoic acid; C18:1, *cis*-9-octadecenoic acid; C18:2, *cis*,*cis*-9,12-octadecadienoic acid; C18:3, *cis*,*cis*,*cis*-6,9,12-octadecatrienoic acid; C20:4, *cis*-5,8,11,14-eicosatetraenoic acid; C20:5, *cis*-5,8,11,14,17-eicosapentaenoic acid; C22:6, *cis*-4,7,10,13,16,19-docosahexaenoic acid; CDKs, cyclin-dependent protein kinases; CDPKs, calcium-dependent protein kinases; CHOL, cholesterol; CRPMI, basal medium; DGs, diglycerides; FBS, fetal bovine serum; GFS, a growth-promoting fraction derived from adult bovine serum; GFS-C, a total simple lipid fraction obtained from GFS; GFSRPMI, CRPMI containing 10% GFS; GFS-WM, a total complex lipid fraction obtained from GFS; Hepes, 4-(2-hydroxyethyl)-piperazine ethanesulfonic acid; HS, human serum; HSRPMI, CRPMI containing 10% HS; LPA, lysophosphatidic acid; 18:1 LPA, *cis*-9-oleoyl LPA; LPC, lysophosphatidylcholine; 12:0 LPC, lauroyl LPC; 14:0 LPC, myristoyl LPC; 16:0 LPC, palmitoyl LPC; 18:0 LPC, stearoyl LPC; 18:1 LPC, *cis*-9-oleoyl LPC; 18:2 LPC, *cis*-9-linoleoyl LPC; 18:U LPC, LPC containing primarily C-18 unsaturated fatty acids; LPC-brain, LPC from bovine brain; LPCRPMI, CRPMI containing 3 mg/ml BSAF and 40 μg/ml 18:U LPC; LPE, lysophosphatidyl ethanolamine; LPI, lysophosphatidyl inositol; LPS, lysophosphatidyl serine; Lyso PAF, γ-*O*-alkyl LPC; MAPK, mitogen-activated protein kinase; MEK, MAPK/extracellular signal-regulated kinase kinase; MGs, monoglycerides; NEFA, non-esterified fatty acid; OD_{650}, absorbance at 650 nm; 8:0 PA, dioctanoyl phosphatidic acid; 18:1 PA, *cis*-9-dioleoyl phosphatidic acid; PAF, β-acetyl-γ-*O*-alkyl PC; PC, phosphatidylcholine; PE, phosphatidyl ethanolamine; PI, phosphatidyl inositol; PI-bovine, PI from bovine liver; PI-soybean, PI from soybean; PKC, protein kinase C; PLs, phospholipids; pLDH, parasite lactate dehydrogenase; PS, phosphatidyl serine; PTK, protein tyrosine kinase; RBCs, red blood cells; SD, standard deviation; SM, sphingomyelin; S-1-P, sphingosine 1-phosphate; SPC, sphingosylphosphorylcholine; sphingosine, D- sphingosine

Keywords: Protozoan *Plasmodium falciparum*; Growth-promoting factor; Simple total lipids; Lysophospholipids

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0014-4894/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.exppara.2004.10.002

1. Introduction

Malaria remains a devastating disease, particularly in the Tropics. The estimated incidence of malaria in the world is of 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 (WHO, 1997). The efficacy of conventional antimalarial drugs and insecticides in controlling malaria outbreaks is declining, with increasing resistance of parasites and their vectors. This highlights the need to develop new chemotherapeutic approaches based on a better understanding of parasite biology and its interaction with the host.

The continuous in vitro cultivation of *P. falciparum* erythrocytic stages represented a significant advance in malaria research (Trager and Jenson, 1978). Despite considerable advances in understanding the molecular and genetic characteristics of this protozoan (PlasmoDB, http://www.plasmodb.org), the mechanisms responsible for *P. falciparum* growth remain largely unknown. It has been suggested that *P. falciparum* requires some factors present in human serum (HS), although the role of HS in the growth of this parasite is still unknown. When only dialyzable factors of HS (low-molecular-mass compounds) are present in the commonly employed basic medium, RPMI1640, the parasite fails to develop from rings to schizonts, but good growth is observed by adding non-dialyzable HS factors (Jensen, 1979).

We previously reported a growth-promoting fraction derived from adult bovine serum (GFS) that yields good intraerythrocytic *P. falciparum* growth (Asahi and Kanazawa, 1994). In addition, a low-molecular-weight factor that is of importance in serum-free media was clearly shown to be a purine precursor, such as hypoxanthine, adenine or adenosine (Asahi et al., 1996; Divo and Jensen, 1982). It is essential but not sufficient for the optimum parasite growth (Asahi et al., 1996). GFS is a 55–70% ammonium sulfate fraction of adult bovine serum, and contains lipid-rich albumin (ALB) as a major component (Kudo et al., 1987). Similarly, Cranmer et al. (1997) reported that commercially available lipid-enriched bovine ALB (Albumax II; Gibco-BRL Life Technology, USA) can replace HS for the continuous in vitro cultivation of *P. falciparum*. Consequently, data are still too crude to allow direct identification of the functional factors required for the growth of *P. falciparum*.

The replacement of HS in a culture medium with chemically or functionally defined substances would not only be advantageous for the culture of the parasite, but it also provides critical clues to a thorough understanding of the parasite's proliferation at the erythrocyte stage, and may lead to the identification of novel targets for malaria chemotherapy. Therefore, this study was undertaken to characterize the ability of the components of GFS and various related substances to sustain parasite growth.

2. Materials and methods

2.1. 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 routinely maintained by in vitro culture techniques using culture medium free of whole serum, consisting of basal medium (CRPMI) supplemented with 10% GFS (Wako Pure Chemical Industries, Japan, under the trade name Daigo's GF21), as previously reported (Asahi et al., 1996). This complete medium is termed GFSRPMI. CRPMI consists of RPMI1640 containing 2 mM glutamine, 25 mM 4-(2-hydroxyethyl)-piperazine ethanesulfonic acid (Hepes), and 24 mM $NaHCO_3$ (Gibco-BRL Life Technology, USA), 25 μg/ml gentamycin (Sigma Chemical, USA), and 150 μM hypoxanthine (Sigma). Briefly, red blood cells (RBCs), which were preserved in Alsever's solution (Asahi et al., 1996) for 3–30 days, were washed, dispensed in a 24-well culture plate at a hematocrit of 2% (1 ml of suspension/well), and cultured under a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 37 °C. For subculture, 4 days after inoculation, infected and uninfected RBCs were washed with CRPMI. Parasitemia was adjusted to 0.1% (for subcultures) or 0.4% (for growth tests) by adding uninfected RBCs, and hematocrit adjusted to 2% by adding the appropriate volume of culture medium, either GFSRPMI or the medium to be tested.

Cultures were occasionally synchronized by two successive exposures at 34–38-h intervals to 5% (w/v) D-sorbitol (Asahi and Kanazawa, 1994).

2.2. Growth-promoting activity experiments

The growth experiments were performed by replacing GFSRPMI with CRPMI supplemented with the substances to be tested. The following were tested for their growth-promoting activity: 10% (v/v) heat-inactivated HS (O-type) (HSRPMI); 2.5–10% heat-inactivated fetal bovine serum (FBS); 3 mg/ml non-esterified fatty acid (NEFA)-free ALB from bovine (BSAF), human, porcine, ovine, guinea pig or equine serum; and 3 mg/ml ovalbumin, lactoglobulin, lysozyme, and casein (Merck, Germany). CRPMI containing BSAF at a final concentration of 3 mg/ml, except when otherwise stated, was further supplemented with graded concentrations of: hexadecanoic acid (palmitic acid, C16:0); *cis*-9-hexadecenoic acid (palmitoleic acid, C16:1); octadecanoic acid (stearic acid, C18:0); *cis*-9-octadecenoic acid (oleic acid, C18:1); *cis*,*cis*-9,12-octadecadienoic acid (linoleic acid, C18:2); *cis*,*cis*,*cis*-