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International Journal for Parasitology 37 (2007) 1551–1557
www.elsevier.com/locate/ijpara

Production of erythropoietic cells in vitro for continuous culture of *Plasmodium vivax*

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Received 27 February 2007; received in revised form 11 April 2007; accepted 9 May 2007

Abstract

Plasmodium vivax cannot be maintained in a continuous culture. To overcome this major obstacle to *P. vivax* research, we have developed an in vitro method to produce susceptible red blood cell (RBC) precursors from freshly isolated human cord hematopoietic stem cells (HSCs), which were activated with erythropoietin to differentiate into erythroid cells. Differentiation and maturation of erythroid cells were monitored using cell surface markers (CD71, CD36, GPA and Fy6). Duffy$^+$ reticulocytes appeared after 10 days of erythroid cell culture and exponentially increased to high numbers on days 14–16. Beginning on day 10 these erythroid cells, referred to as growing RBCs (gRBCs), were co-cultured with *P. vivax*-infected blood directly isolated from patients. Parasite-infected gRBCs were detected by Giemsa staining and a *P. vivax*-specific immunofluorescence assay in 11 out of 14 *P. vivax* isolates. These *P. vivax* cultures were continuously maintained for more than 2 weeks by supplying fresh gRBCs; one was maintained for 85 days before discontinuing the culture. Our results demonstrate that gRBCs derived in vitro from HSCs can provide susceptible Duffy$^+$ reticulocytes for continuous culture of *P. vivax*. Of particular interest, we discovered that parasites were able to invade nucleated erythroid cells or erythroblasts that are normally in the bone marrow. The possibility that *P. vivax* causes erythroblast destruction and hence inflammation in the bone marrow needs to be addressed.
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Keywords: Malaria; *Plasmodium vivax*; Reticulocyte; Erythroblast; Growing red blood cells; Hematopoietic stem cells; Cord blood

1. Introduction

Plasmodium vivax malaria accounts for over half of malaria cases outside Africa and is a major public health problem in many regions of Asia and Latin America (Mendis et al., 2001). As an important factor of morbidity and an obstacle for economic development, the re-emergence and resurgence of *P. vivax* in many endemic areas and the appearance of drug-resistant parasite strains are a great concern. Despite this obvious significance, research on *P. vivax* has lagged far behind that on *Plasmodium falciparum*, largely due to the lack of a continuous culture technique for this parasite. The development of an in vitro culture technique was central to the advances in *P. falciparum* research (Trager and Jensen, 1976). However, culturing *P. vivax* has been difficult because of the stringent requirement for reticulocytes as the target cells.

Compared with *P. falciparum*, *P. vivax* has many distinct biological characteristics, including a hypnozoite stage that is responsible for relapses of the disease.

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doi:10.1016/j.ijpara.2007.05.009

Whereas *P. falciparum* has evolved multiple invasion pathways to infect red blood cells (RBCs) (Cowman and Crabb, 2006), invasion of *P. vivax* is dependent on interaction with the Duffy antigen/receptor for chemokines (DARC) (Miller et al., 1975; Horuk et al., 1993). Furthermore, *P. falciparum* can infect both normocytes and reticulocytes (Pasvol et al., 1980; Mitchell et al., 1986), but *P. vivax* can only infect reticulocytes, which are often below 1% in normal blood (Kitchen, 1938; Mitchell et al., 1986; Mons et al., 1988). Consequently, efforts for establishing short- and long-term *P. vivax* cultures have been undertaken to utilize blood sources enriched with reticulocytes. In an early attempt to culture *P. vivax*, Golenda et al. (1997) developed a sophisticated protocol that uses reticulocyte-enriched blood from patients treated for hemochromatosis. However, this technique received very limited application, probably due to the unavailability of this blood source in most *P. vivax*-endemic areas. In an earlier study, we developed a technique for short-term culturing of *P. vivax* fresh field isolates and demonstrated that the parasites can be studied ex vivo for one week without any requirement for additional reticulocytes (Chotivanich et al., 2001). This short-term culture system is suitable for studying drug resistance in field parasite isolates (Russell et al., 2003). To extend this short-term culture system, we have sought a more convenient source of reticulocytes, umbilical cord blood, and maintained *P. vivax* culture for over 1 month (Udomsangpetch et al., 2007).

Hematopoietic stem cells (HSCs) are of interest in both clinical medicine and basic developmental biology. All mature cells in the blood are derived from HSCs and all blood cell types can be generated from a single HSC (Osawa et al., 1996). HSCs can be obtained from bone marrow, peripheral blood and umbilical cord blood, and HSCs from all these sources can be cultured in vitro to generate fully mature RBCs (Giarratana et al., 2005). In recognizing HSCs as a potential reticulocyte source, we have developed a technique to culture RBCs from human cord blood stem cells and demonstrated that these growing RBCs (gRBCs) can be used for continuous in vitro culture of *P. vivax*.

2. Materials and methods

2.1. Collection and isolation of HSCs

Forty-seven umbilical cord blood samples were collected from normal, full-term deliveries for HSC isolation. The Ethical Committee of Research on Human Beings from Ramathibodi Hospital, Faculty of Medicine, Mahidol University (ID 04-45-16) approved this study. Mononuclear cells (MNCs), including HSCs, were separated from cord blood by using an IsoPrep solution (Robbins Scientific Corporation, CA, USA). HSCs were then isolated from the MNC fraction by using a CD133 isolation kit with magnetic microbead selection and Mini-MACS columns (Miltenyi Biotech, Germany) as described in the manufacturer's protocol. Viability of the cells was determined by trypan blue staining. The isolated HSCs/CD133$^+$ cells were cultured and used for producing gRBCs.

2.2. Production of RBCs

RBCs were produced from HSCs/CD133$^+$ cells using a modified procedure (Giarratana et al., 2005). HSCs/CD133$^+$ cells, 2×10^5 cells/ml, were cultured in StemlineII medium (Sigma–Aldrich Corporation, MO, USA) supplemented with 100 ng/ml stem cell factor (SCF) (PeproTech, Rocky Hill, NJ, USA), 5 ng/ml IL-3 (R&D Systems, Inc., MN, USA), 10 μM hydrocortisone (Sigma–Aldrich), 100 μg/ml transferrin (Sigma–Aldrich), 100 μg/ml Humulin® N (Lilly Pharma Fertigung UND Distribution, Giessen, Germany), 0.18 mg/ml ferrous sulfate (Sigma–Aldrich), 0.16 M monothioglycerol (Sigma–Aldrich) and 4 IU/ml erythropoietin (EPO; Cilag AG International, Zug, Switzerland). The first step of culture was cell expansion for 8 days and on day 4, cells were diluted in 4 volume of fresh medium. At the second step, cells were cultured for 4 days in StemlineII medium without SCF, IL-3 and hydrocortisone. At the last step (8 days), cells were cultured in StemlineII medium without cytokines and maintained in medium supplemented with 10% human AB serum (PromoCell®, Heidelberg, Germany). All cultures were incubated at 37 °C with 5% CO_2. One-week-old erythroid cells were kept in freezing medium at −80 °C or liquid nitrogen until use.

2.3. Determination of maturation of erythroid cells

Surface membrane markers were analyzed to confirm cell types of HSCs and derived erythroid cells. Cell markers were detected using mouse antibodies against CD133 (Miltonic Biotech), CD34 (Becton Dickinson Biosciences, CA, USA), CD38, CD71, CD45 (Celtic Laboratories, CA, USA), CD36 (Serotec Inc., NC, USA), glycophorin A (Beckman Coulter®, Inc., FL, USA) and Fy6 (kindly provided by Dr. C. King, Center for Global Health and Disease, Case Western Reserve University School of Medicine, Cleveland, OH, USA) that were conjugated with fluorescein isothiocyanate or phycoerythrin and analyzed by flow cytometry (FACScan, Becton Dickinson). Cells were also stained with Giemsa and Brilliant cresyl blue to examine the morphology of RBCs and reticulocytes, respectively. Enucleated cells were monitored for standard hematological variables, including the MCV (fl), MCHC (g/dl) and MCH (pg/cell) using a Technicon H3 RTX/RTC™ automat (Bayer Corporation, NY, USA).

Heterogeneous gRBC populations were separated by Percoll using 30–60% discontinuous gradients (Sigma–Aldrich). The gRBCs in suspension were layered on top of the Percoll gradients and centrifuged at 1200*g* for 20 min at 20 °C. Each cell fraction was separately collected for analysis of the cell markers and morphology. Separated cells from two fractions of 50% and 60% Percoll were used for parasite cultivation.

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