International Journal for Parasitology 42 (2012) 155–160

Contents lists available at SciVerse ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Cryopreserved *Plasmodium vivax* and cord blood reticulocytes can be used for invasion and short term culture

Céline Borlon [a,*], Bruce Russell [b], Kanlaya Sriprawat [c], Rossarin Suwanarusk [b], Annette Erhart [a], Laurent Renia [b], François Nosten [c,d,e], Umberto D'Alessandro [a,f]

[a] Department of Biomedical Sciences, Institute of Tropical Medicine, Antwerp, Belgium
[b] Laboratory of Malaria Immunobiology, Singapore Immunology Network, Agency for Science, Technology and Research, Biopolis, Singapore
[c] Shoklo Malaria Research Unit, Mae Sot, Thailand
[d] Mahidol Oxford Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand
[e] Centre for Tropical Medicine, Nuffield Department of Clinical Medicine, University of Oxford, CCVTM, Oxford OX3 7LJ, UK
[f] Disease Elimination Research Theme, Medical Research Council (MRC), Atlantic Road, Fajara, Gambia

ARTICLE INFO

Article history:
Received 26 July 2011
Received in revised form 7 October 2011
Accepted 29 October 2011
Available online 27 December 2011

Keywords:
Malaria
Plasmodium vivax
Reticulocyte
Cord blood
Invasion
Cryopreservation

ABSTRACT

The establishment of a *Plasmodium vivax in vitro* culture system is critical for the development of new vaccine, drugs and diagnostic tests. Although short-term cultures have been successfully set up, their reproducibility in laboratories without direct access to *P. vivax*-infected patients has been limited by the need for fresh parasite isolates. We explored the possibility of using parasite isolates and reticulocytes, both cryopreserved, to perform invasion and initiate short-term culture. Invasion results obtained with both cryopreserved isolates and reticulocytes were similar to those obtained with fresh samples. This method should be easily replicated in laboratories outside endemic areas and will substantially contribute to the development of a continuous *P. vivax* culture. In addition, this model could be used for testing vaccine candidates as well as for studying invasion-specific molecular mechanisms.

1. Introduction

Despite its public health importance in several Asian and South American countries (Baird, 2007), research on *Plasmodium vivax* biology lags behind that of *Plasmodium falciparum*, mainly because a continuous culture system has not yet been established. Indeed, *P. vivax* cannot be maintained in culture under the same conditions established to cultivate *P. falciparum*. One of the obstacles is that *P. vivax* preferentially invades young erythrocytes (reticulocytes), which are short-lived and represent only approximately 1% of the circulating red blood cells (RBCs) (Kitchen, 1938). Even in the presence of reticulocytes, the continuous culture of *P. vivax* remains elusive. To date, only short-term cultures have been set up using reticulocytes from either cord blood (Udomsangpetch et al., 2007), haemochromatosis patients' blood (Golenda et al., 1997), or reticulocytes derived from hematopoietic stem cells isolated from cord blood (Panichakul et al., 2007). Although none of these methods have been able to achieve continuous culture, they can be used for invasion inhibition assays (Russell et al., 2011) or for studying the biology of *P. vivax* to test vaccine candidates targeting merozoite invasion (Grimberg et al., 2007; Russell et al., 2011). However, a major limitation of all of these methods is the need for freshly isolated *P. vivax* from human (or primate) and cord blood isolates, restricting studies to laboratories located in endemic areas or those with easy access to primates. The possibility to use both cryopreserved *P. vivax* isolates and reticulocytes would allow other laboratories without access to the resources mentioned above to embark on *P. vivax* studies. Here, we compared paired fresh and frozen samples of both *P. vivax* isolates and concentrated reticulocytes in one of the most promising invasion assays (Russell et al., 2011), and then attempted short-term cultures.

2. Materials and methods

2.1. Sample collection

Plasmodium vivax isolates were collected from patients with acute *P. vivax* malaria (mono-infection with a density >1/1,000 RBCs) attending the clinics of the Shoklo Malaria Research Unit (SMRU), located in the Mae Sot region of Tak Province, northwest Thailand. After having explained the study purpose and procedures

* Corresponding author. Address: Department of Parasitology, Institute of Tropical Medicine, 155, Nationalestraat, 2000 Antwerpen, Belgium. Tel.: +32 3 247 64 88; fax: +32 3 247 63 59.
E-mail address: cborlon@itg.be (C. Borlon).

0020-7519 © 2012 Australian Society for Parasitology Inc. Published by Elsevier Ltd. Open access under CC BY-NC-ND license.
doi:10.1016/j.ijpara.2011.10.011

to each patient and obtaining written informed consent (OXTREC 027-025, University of Oxford, Centre for Clinical Vaccinology and Tropical Medicine, United Kingdom; MUTM 2008-215 from the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand), 5 ml of blood were collected by venipuncture in 5 ml lithium-heparinised tubes and sent to the SMRU laboratory within 5 h. Each patient's blood group was checked and the parasite stage confirmed by microscopic examination of Giemsa-stained thick and thin films. Samples with >80% parasites in the trophozoite stage were kept for direct use while those with >80% in the ring stage were frozen. Platelets and leucocytes were removed from the isolates using a CF11 column as previously described (Sriprawat et al., 2009).

2.2. Reticulocyte-enriched blood processing

Umbilical cord blood samples (15 ml each) from placentas of pregnant women delivering at the SMRU clinics were collected in 5 ml lithium-heparinised tubes and sent to the culture laboratory within 2–10 h. After checking the blood group, platelets and leucocytes were removed as previously described (Sriprawat et al., 2009). Reticulocytes were then concentrated by centrifugation (15 min at 400*g* without applying a brake) through 70% Percoll (Sigma, Belgium). The proportion of reticulocytes was checked after staining with New Methylene Blue (Reticulocyte stain, Sigma). A RBC scored as positive for the reticulocyte stage contained two or more blue-stained granules and samples with more than 40–50% reticulocytes were used for *P. vivax* invasion tests.

2.3. Parasite cultures

Plasmodium vivax isolates containing >80% early trophozoites were cultured in McCoy medium (Invitrogen, Belgium) supplemented with 25% human serum from normal AB group donors and 0.5% glucose. All cultures were flushed with gas (90% N_2, 5% O_2, 5% CO_2) and incubated at 37.5 °C for 20–24 h until schizont maturation, which was determined by microscopic examination of Giemsa-stained thick and thin films.

2.4. Parasite and reticulocyte freezing and thawing

Both *P. vivax* isolates with >80% early ring stage and concentrated reticulocytes were frozen in Glycerolyte 57 (Baxter, Belgium). The protocol of cryopreservation using Glycerolyte 57 was

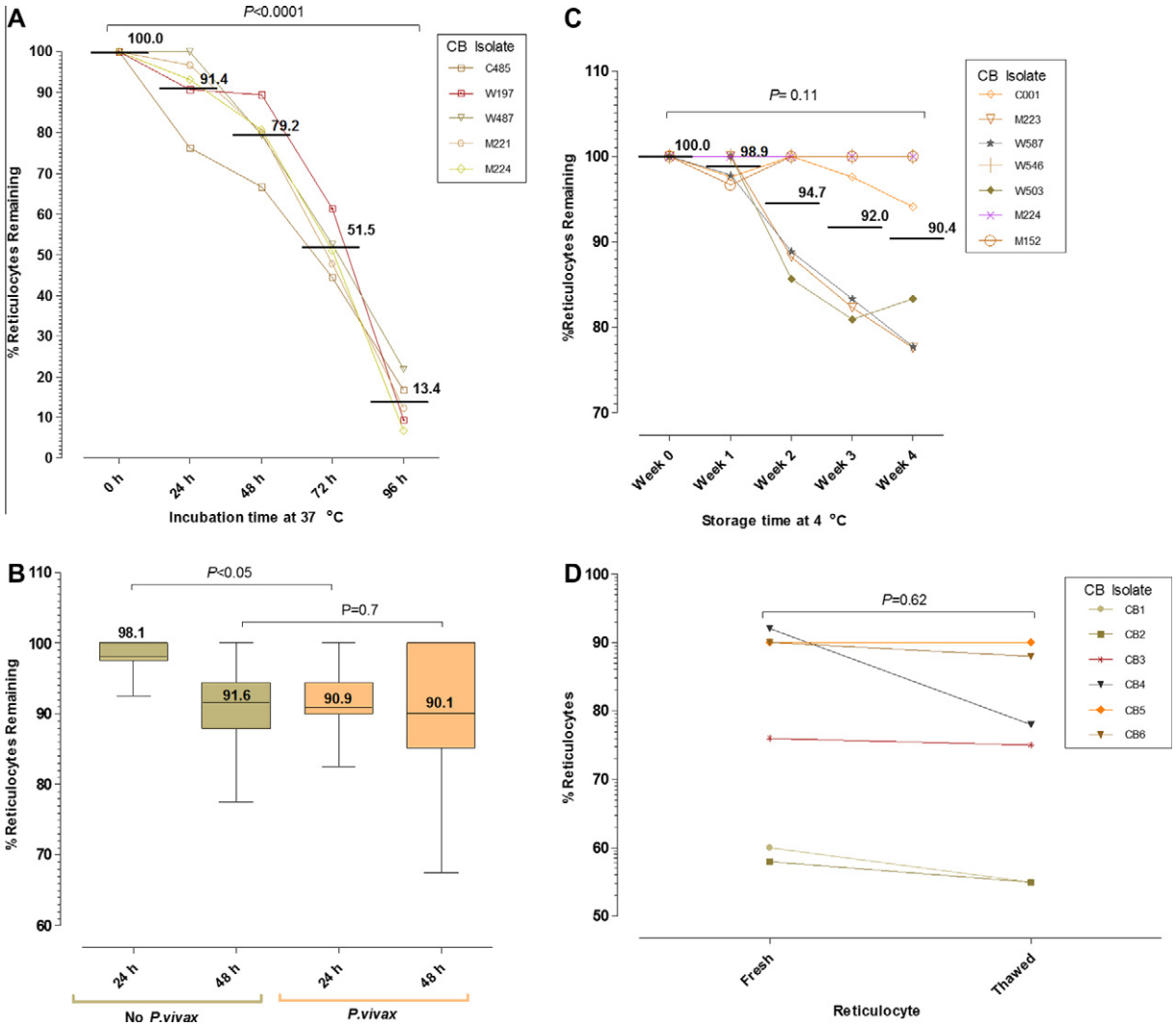

Fig. 1. Reticulocyte storage and maturation. Reticulocytes were identified by Blue Methylene staining. Results are expressed as a percentage of positive cells after staining. (A) Percentage of reticulocytes kept at 37 °C for 4 days (five independent experiments; horizontal lines represent mean values calculated at each time point). (B) Percentage of reticulocytes kept at 37 °C for 48 h with and without *Plasmodium vivax*. Values inside boxes represent the median from nine independent experiments. (C) Percentage of reticulocytes kept at 4 °C for 4 weeks (seven independent experiments, horizontal lines represent mean values calculated at each time point). (D) Comparison of the percentage of reticulocytes before and after a freezing cycle in liquid nitrogen (six independent experiments).

originally used to preserve RBCs (Mazur, 1970; Thomas and Bell, 1995; Scott et al., 2005), and is also widely used in the cryopreservation of *Plasmodium* spp. (Diggs et al., 1975; Christofinis and Miller, 1983; Rossan, 1985; Mathai and Singh, 1989). Briefly, two volume of Glycerolyte 57 to one volume of infected cell pellet were drawn up using an insulin syringe. First, 20% of the Glycerolyte 57 volume was added to the blood cell suspension drop by drop while continuously agitating the tube to mix the contents. This suspension was incubated for 5 min at room temperature before adding the rest of the Glycerolyte 57. The RBC–Glycerolyte mixture was then aliquoted into cryovials placed overnight in a −80 °C freezing container before being stored in a liquid nitrogen canister.

The thawing method used was a stepwise NaCl method (Blomqvist, 2008) which has been successfully used in previous *P. vivax* studies (Kosaisavee et al., 2006; Sharrock et al., 2008). Briefly, to thaw parasite isolates and reticulocytes, cryovials were removed from liquid nitrogen and allowed to warm for 1–3 min at 37 °C until completely thawed. The thawed content was measured and transferred to a 50 ml centrifuge tube. Using an 18 gauge needle and a syringe, 0.2 volume of 12% NaCl was first added drop by drop (slowly) and allowed to stand for 5 min, then 10 volume of 1.6% NaCl were added drop-wise (quickly). This suspension was centrifuged for 5 min at 200*g*. The supernatant was removed and 10 V of 0.9% NaCl were added drop-wise (quickly) followed by another centrifugation (200*g* for 5 min). The supernatant was removed, the pellet of reticulocytes re-suspended in RPMI 1640 media (Lonza, Belgium) and stored at 4 °C. For *P. vivax* isolates, the pellet was re-suspended in 37 °C McCoy's 5A containing 25% human serum (normal AB group donors) and 0.5% glucose and transferred to a culture flask, flushed with gas and placed in a 37 °C incubator for 36–44 h to allow maturation into schizonts.

2.5. Invasion test

Invasion tests were performed as previously described (Russell et al., 2011). Briefly, mature schizonts obtained after maturation of paired fresh and frozen samples were concentrated after centrifugation (15 min at 1,200*g*) through a 45% Percoll solution (Sigma). The purity of schizonts obtained was approximately 90% (Russell et al., 2011). Pure schizonts were added to cord blood concentrated reticulocytes in a 1:6 ratio, and cultivated in McCoy medium (Invitrogen) supplemented with 25% human serum (normal AB group donors) and 0.5% glucose for 24 h. Different ratios (1:2, 1:4 and 1:6) were tested. Little difference was observed in the invasion efficiency among those (data not shown), and the ratio 1:6 was selected as it allows the working material to be increased. Invasions were set up in 300 µl of medium in a 96 well plate (Russell et al., 2011). The parasite stage was determined by microscopic examination of the Giemsa-stained thin film.

2.6. Statistical analysis

The percentage of reticulocytes was defined as the proportion of positive scoring cells after staining and was determined, simultaneously with paired fresh/frozen samples, at different time points and temperatures. Invasion efficiency was defined as the percentage of RBCs newly invaded (containing *P. vivax* ring stages) per 3,000 target host cells, following a 24 h incubation period after invasion. Non-parametric comparisons between more than two unmatched observations utilised Kruskal–Wallis tests, and if repeated measures were compared, either a Wilcoxon signed rank test or a Friedmans test (Post-hoc analysis) were used. All post hoc analyses utilised a Dunn's post-test.

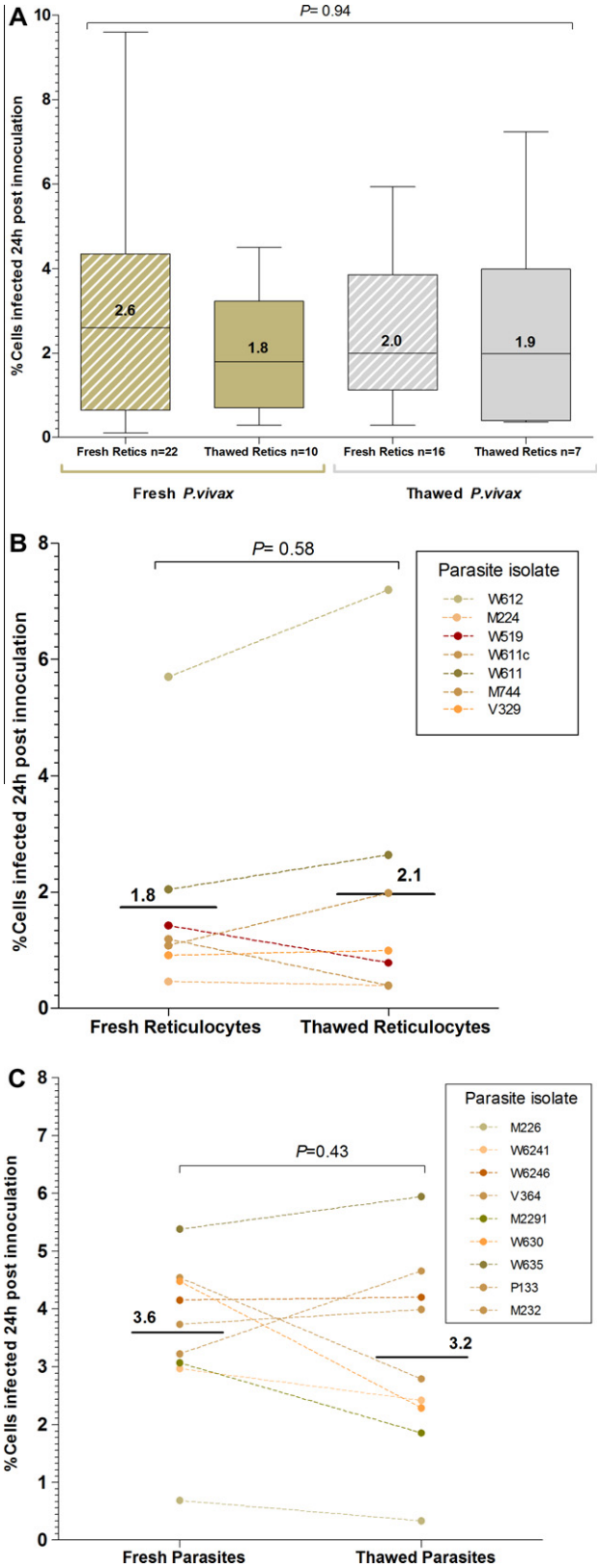

Fig. 2. Invasion of cord blood concentrated reticulocytes by *Plasmodium vivax* merozoites. Results are expressed as a percentage of parasitised red blood cells 24 h post invasion. (A) Percentage of invaded cells for all invasion tests performed with fresh and frozen *P. vivax* isolates on fresh and frozen reticulocytes. Values inside boxes represent the median. (B) Invasions of fresh *versus* frozen reticulocytes by fresh *P. vivax* isolates (horizontal lines represent means of the seven independent experiments). (C) Invasions of fresh reticulocytes by fresh *versus* frozen *P. vivax* isolates (horizontal lines represent means of the nine independent experiments).

3. Results

3.1. Reticulocyte conservation

Reticulocytes from a total of 99 cord blood samples were concentrated following the above described protocol. After concentration of fresh cord blood, samples contained up to 90% reticulocytes. Only cell populations with more than 40–50% reticulocytes (n = 58) were used for further work. At 37 °C, reticulocytes matured into RBCs within 4 days (Fig. 1A), i.e. their proportion significantly decreased to <15% of the initial amount. This process was also tested against the presence of *P. vivax* and results confirmed that at 37 °C, *P. vivax* did not alter the cell maturation process after 48 h. (Fig. 1B). Reticulocytes were stable at 4 °C as they could be maintained for up to 4 weeks in plain RPMI medium without any significant maturation of the cells (Fig. 1C). Moreover, a freezing cycle in liquid nitrogen did not significantly affect the proportion of reticulocytes nor did it induce any maturation (Fig. 1D). Therefore, samples could be stored either at 4 °C, for short-term use, or in liquid nitrogen for long term use, without any significant decrease in the proportion of reticulocytes.

3.2. Invasion test

Successful invasions were obtained with all 55 tested *P. vivax* isolates. Invasion could be performed with similar efficiency for any of the combination used (fresh/frozen reticulocytes and *P. vivax* isolates) (Fig. 2A). When comparing the same reticulocyte sample (Fig. 2B) and the same parasite isolate (Fig. 2C) (fresh *versus* frozen) the efficiency of invasion was similar regardless of the freezing cycle, indicating that differences between isolates were related to their intrinsic ability to invade.

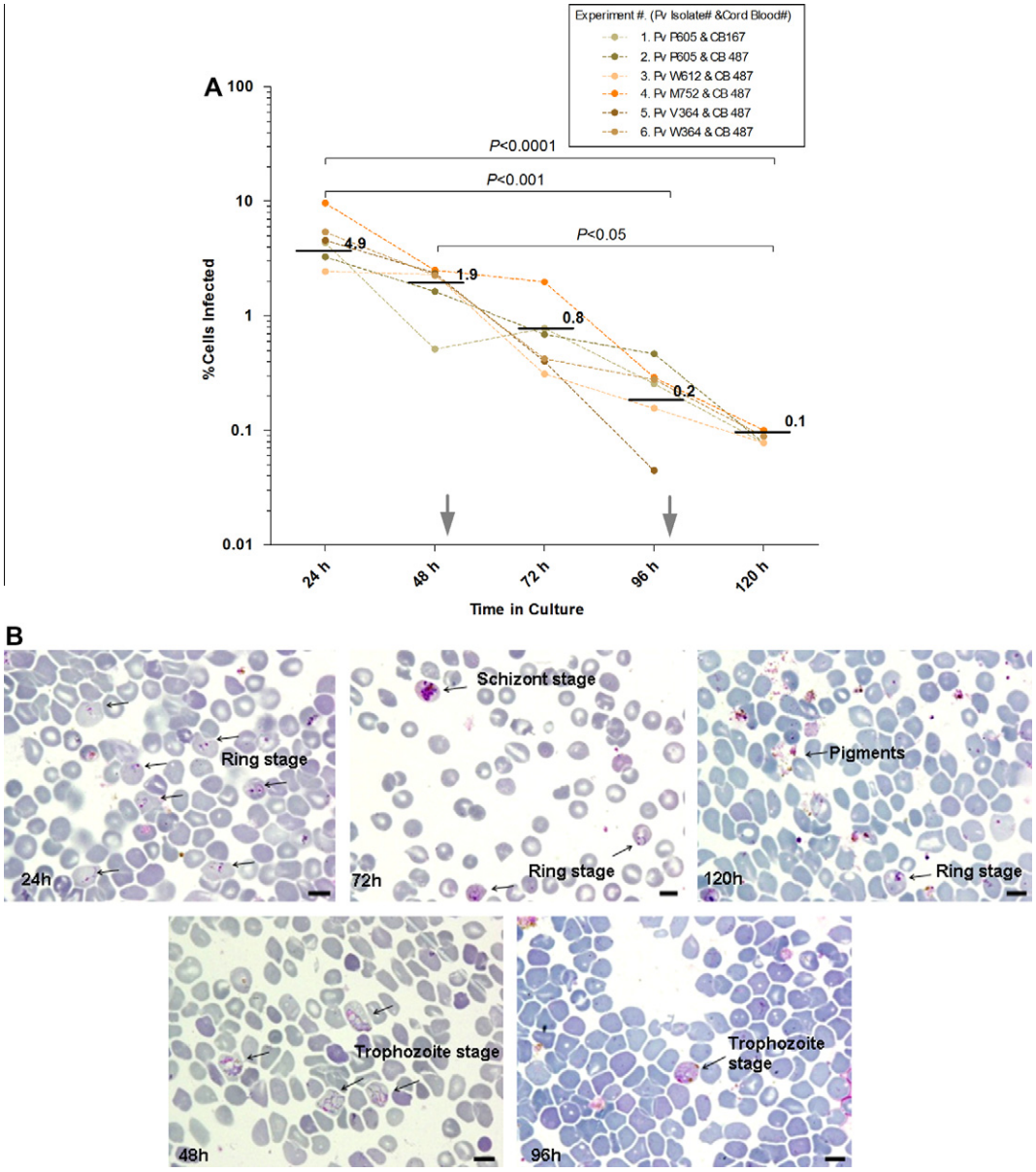

Fig. 3. Short term culture of *Plasmodium vivax* in concentrated reticulocytes from cord blood supplemented with reticulocytes every 48 h, following the parasite's life cycle. (A) Results are expressed as a percentage of parasitised red blood cells during 5 days of culture. Six cultures maintained for up to 5 days with a parasite density over 0.1% are shown. Arrowheads in the graph indicate the addition of fresh reticulocytes (horizontal lines represent means of the six independent experiments at each time point). (B) The arrows on the Giemsa-stained thin blood films indicate young ring stage (24 h), maturing trophozoite (48 h), multinucleated schizont and ring stage (72 h), maturing trophozoite (96 h), and young ring stage and pigment reflecting presence of dead parasites (120 h). Scale bars = 10 μm.

3.3. Culture of *P. vivax*

To explore the suitability of the invasion technique for the initiation of a continuous culture, freshly concentrated reticulocytes were periodically (every 48 h) added to the culture to provide a continuous supply of susceptible cells to be invaded by the merozoites. Six isolates were continuously cultured up to 10 days. A parasite density of <0.1% was observed at day 10 although this density had already been reached at day 5 (Fig. 3). The culture was stopped at day 10 as parasites did not grow well.

4. Discussion

Recently, freshly concentrated reticulocytes from cord blood have been successfully used for *P. vivax* invasion (Russell et al., 2011). Here, we show that in addition to fresh samples, both frozen reticulocytes and parasites can be used for this purpose, an important finding that will allow research on *P. vivax* culture to be performed outside endemic areas. Moreover, cryopreservation of large amounts of *P. vivax* isolates and reticulocytes will add substantial flexibility to this newly established invasion assay, which is an asset given the overall small number of invasion assays published to date (Russell et al., 2011). Similar to previously published studies (Skadberg et al., 2003), our results showed that reticulocytes isolated from cord blood matured in approximately 4 days at 37 °C and this process was not altered if they were in direct contact with *P. vivax* isolates. In addition, they could be kept at 4 °C for at least 3 weeks and a freezing/thawing cycle into liquid nitrogen did not interfere with their maturation. These methods of storing reticulocytes represent a major advantage as they will allow the storage of large amounts of reticulocytes for later use in *P. vivax* culture. Indeed, the cryopreserved concentrated reticulocytes were successfully invaded by *P. vivax* and could even support short-term culture for up to 10 days. Interestingly, the performance of invasion tests was similar when using frozen reticulocytes and parasites or when using those fresh. Although variations with the same paired parasite isolate (fresh *versus* frozen) were negligible, the performance of invasion tests varied substantially from one isolate to another. It has previously been shown that not all *P. vivax* isolates were able to adapt to any given animal or cellular *in vitro* model, and continued attempts are made to adapt new isolates to culture conditions (Stewart, 2003). Currently, gene expression profiles have shown that the expression of some parasite-specific proteins interacting with the immune system at the surface of the host cells are particularly important to the parasite's capacity for adaptation (Mackinnon et al., 2009). After an initial invasion, we attempted to maintain the culture by periodically adding reticulocytes. Parasite density decreased over the first 5 days to approximately 0.1% and did not increase significantly during the next 5 days. At day 10 of culture, parasites did not look healthy enough to support a new expansion of the culture, so the culture was arbitrarily terminated. Parasite multiplication was not limited by the number of reticulocytes as those were periodically added at high concentrations. Various reasons for decreased parasite densities have been reported, such as the apparent fragility of the infected cells, the rupture of schizonts before maturation and the inability of newly generated merozoites to invade reticulocytes (Mons et al., 1988; Lanners, 1992), reasons which did not seem to apply to our results as we could observe schizont maturation as well as second invasion during our attempts at short-term culture. Another hypothesis is that the weak parasite growth observed in our experiments could be due to the presence of foetal haemoglobin. It has been shown that *P. falciparum* can invade RBCs containing foetal haemoglobin but its growth is delayed (Pasvol et al., 1977; Wilson et al., 1977). Similarly, *P. vivax* can indifferently invade reticulocytes containing foetal or adult haemoglobin (Russell et al., 2011), however its growth, as for *P. falciparum*, may be impaired. This feature has previously been raised as a possible explanation for the relative protection against malaria in the first months of life (Pasvol et al., 1976). Since we used reticulocytes concentrated from cord blood, hence mainly containing foetal haemoglobin, this hypothesis cannot be ruled out and should be further investigated in order to confirm whether functional adult haemoglobin is a requirement to establish a continuous *P. vivax* line. The hypothesis of impaired parasite growth due to unsuitable haemoglobin can be further illustrated by the fact that other mechanisms underlying protection against malaria due to modifications or disorders in the RBC, i.e. haemoglobin-inherited disorders (e.g. thalassaemia), enzymopathies (e.g. G6PD deficiency) or erythrocyte polymorphisms (e.g. Duffy blood group), have already been described (Kwiatkowski, 2005; Lopez et al., 2010). Unlike previous studies using the cryopreserved Chesson parasite strain maintained in *Aotus* monkeys (Golenda et al., 1997), the advantage of our study is the use of fresh parasite isolates collected directly from patients and immediately frozen, thus avoiding any potential biological modification due to specific culture conditions. Also with a view to establishing the continuous culture of *P. vivax*, the use of frozen isolates in this newly described invasion assay (Russell et al., 2011) will allow more laboratories to test inhibitory activities induced by vaccines candidates, or to study drug resistance in *P. vivax* by performing proteomic and transcriptomic analyses.

In conclusion, our results showed that both reticulocytes and *P. vivax* isolates can be cryopreserved successfully without affecting the parasite's ability to invade cells. This technique will allow research in laboratories without direct access to fresh parasite isolates and thus will contribute to establishment of the continuous culture of *P. vivax*.

Acknowledgments

We would like to thank all patients who agreed to donate their blood within the framework of the present study. In addition, we would like to thank all Shoklo Malaria Research Unit (Mae Sot, Thailand) staff for their valuable contributions in the completion of this study. SMRU is sponsored by The Wellcome Trust of Great Britain, as part of the Oxford Tropical Medicine Research Programme of the Wellcome Trust-Mahidol University. This study was funded by the Secondary Research Funding (SOFI-B) project from the Institute of Tropical Medicine (ITM), Antwerp (Belgium), and additional funds were provided by the Agency for Science Technology and Research (A*STAR) (Singapore) and The Wellcome Trust (UK).

References

Baird, J.K., 2007. Neglect of *Plasmodium vivax* malaria. Trends Parasitol. 23, 533–539.

Blomqvist, K., 2008. Thawing of glycerolyte-frozen parasites with NaCl. In: Moll, K. et al. (Eds.), Methods in Malaria Research. MR4/ATCC, Manassas, p. 15.

Christofinis, G.J., Miller, H., 1983. A simplified method for cryopreservation of *Plasmodium falciparum* from continuous *in vitro* cultures. Ann. Trop. Med. Parasitol. 77, 123–126.

Diggs, C., Joseph, K., Flemmings, B., Snodgrass, R., Hines, F., 1975. Protein synthesis *in vitro* by cryopreserved *Plasmodium falciparum*. Am. J. Trop. Med. Hyg. 24, 760–763.

Golenda, C.F., Li, J., Rosenberg, R., 1997. Continuous *in vitro* propagation of the malaria parasite *Plasmodium vivax*. Proc. Natl Acad. Sci. USA 94, 6786–6791.

Grimberg, B.T., Udomsangpetch, R., Xainli, J., McHenry, A., Panichakul, T., Sattabongkot, J., Cui, L., Bockarie, M., Chitnis, C., Adams, J., Zimmerman, P.A., King, C.L., 2007. *Plasmodium vivax* invasion of human erythrocytes inhibited by antibodies directed against the Duffy binding protein. PLoS Med 4, e337.

Kitchen, S., 1938. The Infection of Reticulocytes by *Plasmodium vivax*. Am. J. Trop. Med. Hyg. s1–18 (4), 347–359.

Kosaisavee, V., Suwanarusk, R., Nosten, F., Kyle, D.E., Barrends, M., Jones, J., Price, R., Russell, B., Lek-Uthai, U., 2006. *Plasmodium vivax*: isotopic, PicoGreen, and microscopic assays for measuring chloroquine sensitivity in fresh and cryopreserved isolates. Exp. Parasitol. 114, 34–39.

Kwiatkowski, D.P., 2005. How malaria has affected the human genome and what human genetics can teach us about malaria. Am. J. Hum. Genet. 77, 171–192.
Lanners, H.N., 1992. Prolonged *in vitro* cultivation of *Plasmodium vivax* using Trager's continuous-flow method. Parasitol. Res. 78, 699–701.
Lopez, C., Saravia, C., Gomez, A., Hoebeke, J., Patarroyo, M.A., 2010. Mechanisms of genetically-based resistance to malaria. Gene 467, 1–12.
Mackinnon, M.J., Li, J., Mok, S., Kortok, M.M., Marsh, K., Preiser, P.R., Bozdech, Z., 2009. Comparative transcriptional and genomic analysis of *Plasmodium falciparum* field isolates. PLoS Pathog. 5, e1000644.
Mathai, E., Singh, B., 1989. A comparative study of two methods for the cryopreservation of *Plasmodium falciparum*. Trans. R. Soc. Trop. Med. Hyg. 83, 469–470.
Mazur, P., 1970. Cryobiology: the freezing of biological systems. Science 168, 939–949.
Mons, B., Collins, W.E., Skinner, J.C., van der Star, W., Croon, J.J., van der Kaay, H.J., 1988. *Plasmodium vivax*: *in vitro* growth and reinvasion in red blood cells of *Aotus nancymai*. Exp. Parasitol. 66, 183–188.
Panichakul, T., Sattabongkot, J., Chotivanich, K., Sirichaisinthop, J., Cui, L., Udomsangpetch, R., 2007. Production of erythropoietic cells *in vitro* for continuous culture of *Plasmodium vivax*. Int. J. Parasitol. 37, 1551–1557.
Pasvol, G., Weatherall, D.J., Wilson, R.J., 1977. Effects of foetal haemoglobin on susceptibility of red cells to *Plasmodium falciparum*. Nature 270, 171–173.
Pasvol, G., Weatherall, D.J., Wilson, R.J., Smith, D.H., Gilles, H.M., 1976. Fetal haemoglobin and malaria. Lancet 1, 1269–1272.
Rossan, R.N., 1985. Cryopreservation of the blood stages of *Plasmodium falciparum* and *Plasmodium vivax* for *in vivo* studies. Am. J. Trop. Med. Hyg. 34, 207–208.
Russell, B., Suwanarusk, R., Borlon, C., Costa, F., Chu, C., Rijken, M., Sriprawat, K., Warter, L., Koh, E., Malleret, B., Colin, Y., Bertrand, O., Adams, J., D'Alessandro, U., Snounou, G., Nosten, F., Renia, L., 2011. A reliable *ex vivo* invasion assay of human reticulocytes by *Plasmodium vivax*. Blood 118, e74–e81.
Scott, K.L., Lecak, J., Acker, J.P., 2005. Biopreservation of red blood cells: past, present, and future. Transfus. Med. Rev. 19, 127–142.
Sharrock, W.W., Suwanarusk, R., Lek-Uthai, U., Edstein, M.D., Kosaisavee, V., Travers, T., Jaidee, A., Sriprawat, K., Price, R.N., Nosten, F., Russell, B., 2008. *Plasmodium vivax* trophozoites insensitive to chloroquine. Malar. J. 7, 94.
Skadberg, O., Brun, A., Sandberg, S., 2003. Human reticulocytes isolated from peripheral blood: maturation time and hemoglobin synthesis. Lab. Hematol. 9, 198–206.
Sriprawat, K., Kaewpongsri, S., Suwanarusk, R., Leimanis, M.L., Lek-Uthai, U., Phyo, A.P., Snounou, G., Russell, B., Renia, L., Nosten, F., 2009. Effective and cheap removal of leukocytes and platelets from *Plasmodium vivax* infected blood. Malar. J. 8, 115.
Stewart, V.A., 2003. *Plasmodium vivax* under the microscope: the *Aotus* model. Trends Parasitol. 19, 589–594.
Thomas, M.J., Bell, S.H., 1995. Cryopreservation of human red blood cells. Methods Mol. Biol. 38, 235–250.
Udomsangpetch, R., Somsri, S., Panichakul, T., Chotivanich, K., Sirichaisinthop, J., Yang, Z., Cui, L., Sattabongkot, J., 2007. Short-term *in vitro* culture of field isolates of *Plasmodium vivax* using umbilical cord blood. Parasitol Int. 56, 65–69.
Wilson, R.J., Pasvol, G., Weatherall, D.J., 1977. Invasion and growth of *Plasmodium falciparum* in different types of human erythrocyte. Bull. World Health Organ. 55, 179–186.