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Transfection of *Plasmodium knowlesi* in baboon (*Papio anubis*) provides a new system for analysis of parasites expressed transgenes and host-parasite interface

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Abstract

Despite a lot of efforts to control malaria, it still remains a major health problem. With the new development of transfection technology, it is now possible to determine the structure-function relationship of vaccine candidates. The aim of this study was to develop a baboon (*Papio anubis*) model for transfection and analysis of host-parasite interface of transfected *P. knowlesi* parasites. One baboon was infected with wild-type *P. knowlesi* parasites for generation of parasites to be transfected. At peak parasitaemia, the baboon was anaesthetized bled and blood stream parasites were harvested, transfected with DNA plasmid constructs containing pyrimethamine resistant form of *dihydrofolate reductase thymydylate synthase (dhfr-ts)* gene from *Toxoplasma gondii* as selectable marker and monkey interferon gamma (IFN-γ) gene as the transgene. Both the selectable marker and the transgene were engineered for expression under control of *P. berghei* DNA regulatory sequences. Equal volumes of electroporated parasites were injected into two baboons, followed by a daily oral administration of pyrimethamine. Transfected parasites were detected in peripheral blood at day 10 post-transfection. At day 15 post-transfection, blood was collected from the baboons, subjected to Plasmodipur filtration to remove leucocytes and used for DNA isolation. Analysis of isolated DNA by PCR showed presence of *T. gondii dhfr-ts* and *IFN-γ* genes in transfected parasites. Enzyme Linked Immunosorbent Assay for IFN-γ showed release of significant levels of IFN-γ by transfected parasites. These studies have developed a *P. knowlesi* transfection protocol, which involves *in vitro* gene insertion and subsequent selection of transfected parasites in a baboon system. This opens new possibilities for using the *P. knowlesi*-baboon model in vaccine development using cutting edge technology.

Keywords: Transfection, *P. Knowlesi*, Baboon (*P. anubis)* and Interferon gamma

INTRODUCTION

Malaria is a major public health problem in over 90 countries where 40% of the world population lives (WHO 1998). There are 300-500 million cases each year and up to approximately 2.7 million deaths occur. Global campaign to eradicate malaria has not been fully successful due to parasite, mosquito, environmental and human related factors (Sachs and Melanie, 2002). Malaria parasites have evolved mechanisms such as antigenic variation (Smith *et al.,* 1995) for evading host immune responses, subsequently complicating vaccine development (Richie and Saul, 2002). Resistance of mosquito to insecticides due to emergence of new species makes it hard to control the spread of malaria. (Chandre *et al.,* 1999; Torre *et al.,* 2002).

New methods to combat malaria have to be intensified (Gwadz and Green, 1998), otherwise the number of malaria cases may double by the

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year 2020 (Breman, 2001). As a result, a deeper understanding of the biology of malaria is important in order to facilitate the development of an effective vaccine. Animal models such as non-human primate are being used to study the biology of malaria parasites. The animal models offer the only means to experimentally investigate host-parasite interactions *in vivo* (Ozwara *et al.,* 2003b). Rodent malaria parasites have been used to study parasites biology (Cox, 1988). However, these parasites are phylogenetically distant from human *Plasmodia* (Escalante *et al.,* 1998), and do not easily allow investigation of natural host-parasites interactions. Avian malaria parasites such as *Plasmodium gallinaceum* and *P. lophurae* are closely related to *P. falciparum* but their development in nucleated cells and the wide phylogenetic distance between birds and humans limits their applicability to answer important questions on host-parasite interactions relevant to human malaria.

Simian *Plasmodia* such as *Plasmodium knowlesi,* which is a natural parasite of macaques, have a comparable phylogeny and host-parasite relationship to human malaria parasites (Coatney *et al.,* 1971; Escalante *et al.,* 1998). The parasites can be used to identify, develop and evaluate vaccine and drug candidates (Wengelnik *et al.,* 2002; Deans *et al.,* 1984; Kocken *et al.,* 1999) and characterize host responses (Deans *et al.,* 1988; Rogers *et al.,* 2001; Gwadz and koontz, 1984). The non-human primate malaria, caused by *Plasmodium knowlesi* is a natural parasite of *Macaca fascicularis*. In addition, *P. knowlesi* malaria infection has been experimentally induced in a number of other non-human primates such as *M. mulatta, M. radiata, M. assamensis, Presbytis entellus Callithrix jaccchus, Aotus trivigatus* and *Saimiri sciureus* (Garnham, 1966; Coatney *et al.,* 1971; Collins *et al.,* 1978; Langhorne and Cohen, 1979). The parasite is phylogenetically close to *P. vivax* (Escalante *et al.,* 1995), and many genes identified in *P. vivax* have homologues in *P. knowlesi* and this allows determination of function by analogy.

Non-human primates are widely used in malaria drug and vaccine development (Butcher, 1996; Stower *et al.,* 2001; Wengelnik *et al.,* 2002). The baboon is attractive because it is well characterized and is frequently used in biomedical research (King *et al.,* 1988). Studies have shown that *P. anubis* infected with *P. knowlesi* displays various clinical characteristics which are also seen in human malaria including cerebral involvement (Ozwara *et al.,* 2003a). Recently, protocols for long term *in vitro* culture and genetic modification of *P. knowlesi* have been developed (van der Wel *et al.,* 1997; Kocken *et al.,* 2002). These are powerful tools for understanding parasite biology, especially gene function. In the current study characterization of genetically modified *P. knowlesi* that expresses host interferon gamma (IFN-γ) in baboon was carried out. The expression of IFN-γ is immunologically important because it is a key effector cytokine in protection against malaria particularly during the liver stages (Hoffman *et al*., 1997). Studies in human and in animal models looking at endogenously produced and exogenously administered IFN-γ have shown that the cytokine is also required for protection against blood stage infection (Su and Stevenson, 2000; Yoneto *et al*., 1999). Based on this observation it is postulated that *in vivo* expression of host cytokine by malaria parasites can manipulate host-parasites interaction and generate protective host responses. *In vitro* expression and bioactivity of *P. knowlesi* has been characterised (Ozwara *et al*., 2003c). The study described here report for the first time transfection technology used to characterise genetically modified *P. knowlesi* that expresses rhesus INF-γ in baboon.

In this study *P. knowlesi*, a natural malaria parasite of macaque monkey (Butcher, 1996) and an experimental system for human malaria, was transfected by electroporation using DNA plasmids constructs containing pyrimethamine resistant form of *dihydrofolate reductase thymydylate synthase* (*dhfr-ts*) gene from *Toxoplasm gondii* and monkey IFN-γ gene under control of *P. berghei* promoters and the transfected parasites selected *in vivo.* Baboon monkey (*Papio anubis*) was used for the first time for *in vivo* development and selection of the transfected *P. knowlesi* parasites by use of oral dosing of pyrimethamine. The *in vivo* selected genetically modified *P. knowlesi* parasites were characterized, showing for the first time that the transfected parasites expressed monkey IFN- γ in baboon.

MATERIALS AND METHODS

Parasites and animals

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Plasmodium knowlesi H strain (Chin *et al*., 1965) cryopreserved stocks were used to initiate blood-stage infection in baboon donor monkey. The parasites were cultured overnight and used to inoculate the donor monkey by intravenous inoculation of $1x10^6$ parasites.

Adult baboons (*Papio anubis*) of either sex, each weighing over 10 kilos were used in these studies. Each animal was housed individually in single cages to avoid blood contact that could lead to cross infection. The Institutional Animal Care and Use and the Scientific Review committees of the Institute of Primate Research approved the baboon experiments. All monkey experiments were performed in a biocontainment facility.

Transfection constructs and procedures

Engineering of the transfection construct $pD_B.D_{TM}.D_B/AB.\ \gamma_{MM}.D_B$ used in these studies is described in (Ozwara *et al*., 2003b). In brief, monkey IFN-γ gene was isolated from IFN-γ cloning vector (a gift from F. Villinger) by *Xba*I and *Spe*I restriction digestion and cloned into the blunted *Bam*HI site of plamid $pD_B.D_{TM}.D_B.\text{-}.D_B$ (Kocken *et al.,* 1998)) to obtain the transfection construct. The construct was used for episomal transfection of *P. knowlesi* in the baboon. The transfection construct contained pyrimethamine resistant form of *dihydrofolate reductase thymydylate synthase* (*dhfr-ts*) gene from *Toxoplasma gondii* as selectable marker and monkey IFN-γ gene as the transgene. Both the selectable marker and the transgene were engineered for expression under control of *P. berghei* DNA regulatory sequences.

One baboon (PAN 2574) was infected with wild-type *P. knowlesi* parasites for generation of parasites to be transfected. To determine parasitaemia, at least 2000 RBC'S on a thin smear were counted. This was done by counting erythrocytes from a quarter of each field and multiplying by four. The number of infected erythrocytes was also counted out of approximately 2000 erythrocytes. Percentage parasitaemia = total parasites counted divided by number of erythrocytes counted multiplied by 100. Parasitaemia development in the animal was observed on a daily basis and at peak parasitaemia (day 9 post-infection), the baboon was anaesthetized and bled. The blood was spun at 1800 rpm (Sorval) and top layer (brown layer) parasites were harvested and used for transfection by electroporation. Over 90% of the brown layer parasites were schizonts. 6.5×10^8 schizonts were resuspended in 300µl of cytomix and mixed with 100µl of Qiagen column (Qiagen) purified maxiprep transfection DNA at a concentration of 500 ng per µg. Electroporation condition and time constants are shown in Table-1. The electroporated parasites were pooled and equal volumes injected into two baboons (PAN 2838 and PAN 2851).

Table 1: Transfection parameters

	1st Transfection	2nd Transfection	3rd Transfection
Schizonts	800µL	800µL	800µL
Voltage	2.5KV	2.5KV	2.5KV
Capacitance	25µF	25µF	25µF
Resistance	200Ω	200Ω	200Ω
DNA Constructs	50µL	50µL	50µL
Time constants	8ms	9ms	9ms

In vivo selection of transfected parasites and parasitaemia observation

The two baboons PAN 2838 and PAN 2851 previously inoculated with electroporated parasites were each orally given 1mg/kg body weight of pyrimethamine per day starting 24 hours post infection. This was done during the entire period of the experiment. The baboons were supplemented once per week with 3.5mg folinic acid to counteract the bone marrow suppression caused by pyrimethamine (Schoondermark-van der ven *et al*., 1995). Parasitaemia observation was done on a daily basis. At day 15 post infection, the two baboons were bled and the blood was subjected to Plasmodipur (Eurodiagnostica) filtration to remove leucocytes. Some of the blood was used to prepare serum for *ex vivo* assay of IFN-γ cytokine. The rest of the blood was used for parasite DNA-isolation.

DNA analysis

Total parasite DNA was isolated directly from Plasmodipur filtered blood using whole blood DNA isolation kit (Gentra systems inc.,

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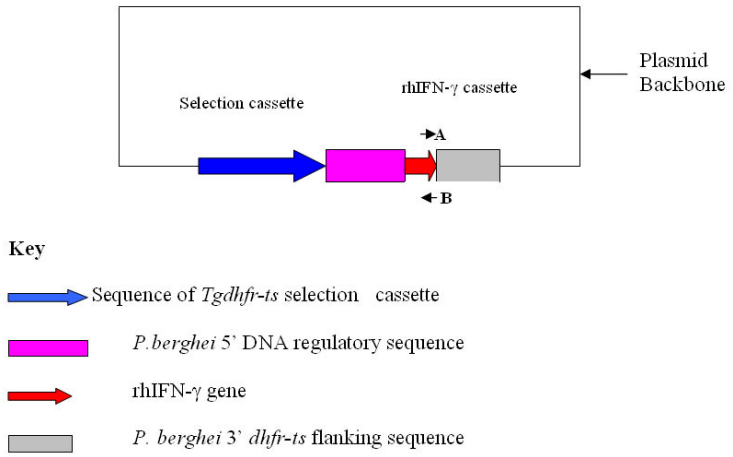

Figure 1a

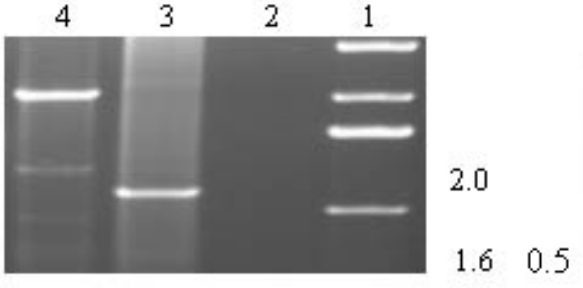

Figure 1b

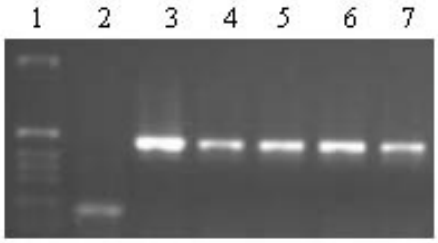

Figure 1c

Figure 1: DNA constructs and molecular analysis of transfected parasites. (1a) DNA constructs used to genetically modify *P. knowlesi* parasite to express baboon IFN-γ cytokine. (1b) Demonstrates presence of plasmids in the transfected P. knowlesi parasites. (1c) PCR of interferon gamma gene in the Transfected P.knowlesi parasites.

minneapolis, minn) and according to manufacturer's instructions. Parasite DNA was analysed through plasmid rescue by electroporation into *Escherichia coli*, and PCR according to standard procedures (Ozwara *et al*., 2003b). The PCR was done for analysis of selectable marker and IFN-γ genes. The pyrimethamine resistant mutant form of the *T. gondii dhfr-ts* gene (Donald and Roos, 1993) was amplified by PCR using primers 5'-cgtgatcaatgcataaaaccggtgtgtc-3' and 5'-cgtgatcaaagcttctgtatctccgc-3'. The primers A (5'-ggcttttcagctctgcattg-3') and B (5'-ccgctcgaggctgggatgctcttcgacc-3') were used to detect IFN-γ gene (Fig.-1).

In vitro analysis of monkey IFN-γ expression

Transfection and control parasite cultures were expanded *in vitro* as previously described (Kocken *et al*., 2002) for up to three cycles. Parasite growth *in vitro* rapidly declines after a few cycles in culture (Kocken *et al*., 2002). Culture supernatants were harvested from the parasite cultures and frozen at –70°C until use. The culture supernatants were analysed by Enzyme-Linked Immunosorbent Assay (ELISA) for the presence of monkey IFN-γ using monkey IFN-γ ELISA kit (U-cytech, Netherlands) and according to manufacturer's instructions.

Analysis of baboon antibody responses to transfected parasites

The blood for serum preparation was collected at 14-day post inoculation for the transfected parasites and the controls. Before the collection of the blood, the baboons were anaesthetized with Ketamine. The blood was collected aseptically in serum tubes and spun at 2000 rpm for 10 minutes. The serum was then sucked off into sterile serum tubes and stored at -20°C until needed for antibody ELISA. The serum was prepared from the donor monkey PAN 2574, PAN 2593 infected with passaged transfected *P. knowlesi* and from PAN 2838 and PAN 2851

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both infected with transfected parasites for selection.

Polystylene Micro ELISA plates (Dynatech laboratories, Sussex, UK) were coated overnight with crude whole parasite antigen at a concentration of $1x10^8$ parasites/ml diluted in bicarbonate buffer, pH 9.6. Excess coating buffer was flicked off and non-specific binding sites were blocked with 3% BSA in PBS for 1 hr at 37ºC. Unbound BSA was washed off six times with 0.05% Tween 20 in PBS. One hundred micro litres of diluted serum (1/25 in BSA in PBS) samples dispensed into the wells and incubated for 1hour at 37ºC. Unbound serum was washed off six times as above and 50µl of 1/2000 alkaline phosphatase conjugated rabbit anti-monkey IgG (Cappel, Organon Teknika, OR, USA) was added and followed by incubation for 1 hour at 37°C. Unbound conjugate was washed off as above before adding 50µl of p-nitrophenyl phosphate substrate (pNPP, sigma, UK, final concentration 1mg/ml in 10% diethanolamine buffer. The plates were incubated at 37°C for 30 minutes in the dark. Optical density was read at 405nm in a microplate reader (Dynatech laboratories). In assaying for IgM antibodies in serum, 100µl of 1/2000 Horse radish peroxidase conjugated rabbit anti monkey IgM (Cappel Organon Teknika, OR USA) was added and incubated for 1 hour at 37°C. One hundred microlitres Orthophenyldiamine (OPD) (Sigma, UK, final concentration of 0.4µg/ml) in phosphate citrate buffer was used as substrate. Optical density was read at 450nm after 30 minutes of inocubation.

RESULTS

Transfection of *P. knowlesi* in the baboon

In order to generate adequate parasites for transfection donor monkey PAN 2574 was infected with wild-type *P. knowlesi* parasites. The development of wild-type parasites in the donor monkey was followed by parasitaemia determination on daily basis. The infected baboon with wild type *P. knowlesi* parasites developed acute infection with peak parasitaemia with about 800 parasites/10,000 erythrocytes by day 8 post-inoculation (Fig. 2, PAN 2574). To avoid disappearance of wild-type parasites from the system of donor monkey, the baboon was anaesthetized.and bled. The blood was spun at 1800 rpm and the top brown layer harvested, contained 6.5 x 10^8 schizonts blood stage parasites, required for transfection.

The *P. knowlesi* parasites were transfected by electroporation with transfection construct $pD_B.D_{TM}.D_B/AB.\gamma_{MM}.D_B$ (Fig.1). Electroporation condition and time constants are shown in Table 1. The electroporated parasites were pooled and equal volumes injected into two baboons (PAN 2838 and PAN 2851).The development and the selection of transformants was closely monitored by parasitaemia determination and pyrimethamine treatment. There were no parasites observed during the first two days post-inoculation. However, day 3 post-inoculation newly invaded parasites were readily detectable with very low parasitaemia and upon treatment with pyrimethamine drug, parasitaemia rapidly dropped to levels undetectable by thick film analysis from day 4 up to day 10 for PAN 2851 and up to day 10 for PAN 2838 (Fig. 2, PAN 2838 and PAN 2851). The results further shows that parasitaemia rose to detectable levels from day 10 post-inoculation for PAN 2851 and for both baboon from day 11 post-inoculation. After day 12 post-inoculation, the parasitaemia of pyrimethamine resistant parasites in both baboons stabilised with fluctuation in parasitaemia ranging between 0.2% and 0.4% (Fig. 2).

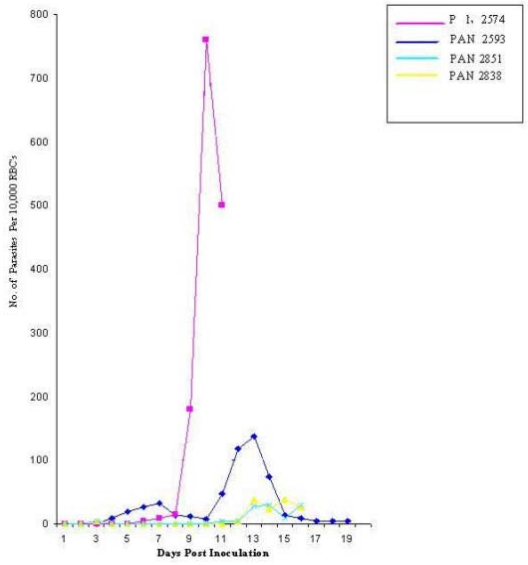

Figure 2: parasitaemia profile of parasites in the donor monkey PAN 2574 Transfected parasites in PAN 2838, 2858 and 2593

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Table 2: IFN-γ cytokine released from transfected parasites and controls.

Culture	Parasitaemia %	Day post inoculation	Transfection Construct	IFN-γ Release in Pg
Wild-type parasites				
PAN 2669	0.09	8	N/A	>>
PAN 2698	0.19	9	N/A	>>
Erythrocytes	-	-	N/A	>>
Transfected parasites				
PAN 2838	0.09	14	pD_B. D_{TM}. D_B/A_B. γM_m. D_B	31.143
PAN 2851	0.14	14	pD_B. D_{TM}. D_B/A_B. γM_m. D_B	37.584
PAN 2593	0.16	14	pD_B. D_{TM}. D_B/A_B. γM_m. D_B	40.182

KEY: >> Below detectable levels , N/A Transfection did not take place.

At day 14 post-inoculation, when the parasitaemia of the selected transfected *P. knowlesi* was stable, the two baboons were anaesthetised and 2.5 ml of blood was separately collected from each baboon. The blood was used to prepare separately the stock of transfected parasites. The stock of transfected parasites were prepared by spinning the blood at 1200 rpm for 10 minutes after which the supernatant was sucked off. The pellets were suspended and mixed together, 1PCV of cryoprotectant was added and the mixture was allowed to equilibrate for 5 to 10 minutes at room temperature. The mixture was then aliquoted in 400µl /vial and snap frozen in liquid Nitrogen at -150°C (J B Jensen, 1983 modification of Rowe *et al*., **5**: 119). Some of the cryopreserved transfected *P. knowlesi*, parasites was later retrieved cultured overnight and used to infect PAN 2593 to determine the development of passaged transfected parasites under pyrimethamine pressure. The level of parasites of passaged transfected parasites was below detectable level by thin blood smear, at day three post-inoculation. However, from day 4 post-inoculation parasitaemia stabilised with low parasitaemia slightly higher than that in PAN 2838 and PAN 2851 (Fig. 2 PAN 2593). The baboon (PAN 2593) never developed acute infection like the donor monkey which was infected with wild-type parasites since the parasitaemia remained low and the baboon looked normal with no malaria symptoms for about 20 days (Fig. 2).

Molecular Characterization of transfected *P. knowlesi*.

Protocol described by Ozwara *et al*.(2000b) was followed in isolation and analyses of parasite DNA. The PCR was done for analyses of the transfected *P. knowlesi* plasmids carrying selectable marker gene (*Tgdhf-ts)* and IFN-γ genes. This is because the PCR results of the DNA sample displayed conspicuous bands in well 3 and 4 for PAN 2838 and PAN 2851 respectively, while the negative control well 4, of the wild-type *P. knowlesi* had no band (Fig. 1b), showing that the transfected parasites had taken up the plasmids carrying the selectable marker gene, hence transfection of *P. knowlesi* parasites had occurred.

The PCR analysis, which amplified the IFN-γ Open Reading Frame (ORF) of the isolated DNA sample from transfected parasites showed the presence of plasmid carrying gene for IFN-γ cytokine (Fig. 1c). The positive results indicated that the gene for IFN-γ cytokine was intact and stable in the parasites.

Characterisation of IFN- γ expression in baboon derived *P. knowlesi*

To determine host IFN-γ production in transfected *P. knowlesi* parasites, cultures were expanded, and their supernatants harvested, and analysed for the presence of host IFN-γ by ELISA with a macaque IFN-γ ELISA kit. Host IFN-γ was detected in culture supernatants from parasites episomally transfected with plasmid

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pDB.DTM.DB/AB.γMM.DB but not from control cultures of wild-type parasites (Table 2). The absence of detectable IFN-γ in the controls confirmed that the IFN-γ gene that expressed IFN-γ cytokine in the transfected parasites was intact, stable and viable in the plasmid. The IFN-γ cytokine detected was from IFN-γ gene of transfected parasites and was bioactive, since the ELISA kit used detects only bioactive cytokine. The results further show that, the higher the parasitaemia of the transfected parasites the greater was the quantity of the IFN-γ released (Table 2).

Table 3: Antibody production against transfected and passaged transfected *P. knowlesi* parasites and controls in baboon

Day	Baboon (PAN)	Mean O.D 1gM	Mean O.D 1gG	Parasitaemia (%)
0	(2838) (transfected)	0.0	0.0	0
9	"	0.2	0.0	0.30
14	"	0.2	0.0	0.34
0	(2851) (transfected)	0.0	0.0	0
9	"	0.1	0.0	0.08
14	"	0.1	0.0	0.09
0	2593 (passaged)	0.0	0.0	0
9	"	0.1		0.12
14	"	0.4	0.0	0.33
			0.0	
0	2574 (wild-type)	0.0	0.1	0
9	"	0.4	0.6	6.9

Antibody responses to transfected *P. knowlesi* in the baboon

The serum used for ELISA for antibody response to transfected parasites and the controls was prepared as previously described. Two isotypes IgG and IgM were assayed for, using crude whole parasites antigens. The Polystyrene Micro ELISA plates (Dynatech laboratories, Sussex, UK) were used and appropriate steps of coating the plate and washing was followed as per protocol. To assay for IgG isotype, alkaline phosphatase conjugated rabbit anti-monkey (Cappel, Organon Teknika, OR USA), while to assay for IgM, Horse Radish Peroxidase conjugated rabbit anti-monkey IgM (Cappel, Organon Teknika, OR USA) was used. The ELISA for antibody response against transfected parasites showed that antibody response was positive for IgM isotype but not for IgG isotype in baboons infected with transfected and passaged transfected parasites. However, antibody response toward wild-type parasites (PAN 2574) which induced acute infection in the donor monkey was strong with rise in both IgM and IgG isotype and the higher the parasitaemia, the stronger was the antibody response (Table 3)

DISCUSSION

The result of this study indicated that baboons are fully susceptible to experimental *P. knowlesi* as previously established (Ozwara *et al.*, 2003a). This is because parasitaemia profile observed in PAN 2574 (Fig. 2) was comparable to those in rhesus monkey following infection with the same parasite (Kocken *et al.*, 2002), indicating that virulence of this strain is similar in both monkeys. At peak infection PAN 2574 was bled, processed and adequate parasites required for transfection by electroporation were obtained. About 5.0 x 10^8 schizonts/ml were used for every electroporation. In this study transfection by electroporation succeeded using electroporation conditions of 2.5kv, 25μF and 200Ω and 0.5x10^9 to 1x10^9 parasites with 50μg DNA in cytomix (van der Hoff *et al.*, 1992) in a 0.4 cm curette (Biorad).

The transfection procedure used in this study is only effective for mature schizonts stage of *P. knowlesi* parasites when merozoites are mature and about to leave the cell, hence damage of the erythrocytes by electroporation would not affect the survival of the transfected parasites. Transfection by electroporation succeeded in this study as it was confirmed by *in vivo* selection of transfected parasites by use of oral dosing of pyrimethamine**.** It is appreciated that the best selection system could have been *in vitro* in terms of cost and time. However, due to limitation of *P. knowlesi* culture survival in our laboratory the selection had to be done in a baboon system. The *in vivo* development and selection of transfected *P. knowlesi* parasites, confirmed in this study that pyrimethamine administered orally had killed *P. knowlesi* parasites that had not taken the plasmids. The non-transfectants were selectively eliminated

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from the baboon system by pyrimethamine administered orally as observed in other studies (Anna van der Wel *et al.,* 1997) when rhesus monkeys were used.

The PCR analysis of DNA-isolated from pyrimethamine resistant was positive for plasmid carrying the selectable marker, *T. gondii dhfr-ts*. This result is the first to confirm that *T. gondii dhfr-ts* was actually present in the transfected parasites and conferred pyrimethamine resistance of *P. knowlesi* in baboons as previously established in rhesus monkey (van der Well *et al.,* 1997). Further PCR analysis of isolated DNA of transfected parasites confirmed presence of plasmids carrying IFN-gene that could express host interferon gamma. The detection of IFN-gamma cytokine in the culture supernatant of transfected parasites showed for the first time that episomally transfected parasites with plasmids pD_B. D_{TM}. D_B/A_B. γM_m. D_B (Fig. 1) could express host IFN-γ in baboon as previously expressed using rhesus monkey (Ozwara *et al.,* 2003b). This is the first example of a host molecules being produced by *P. Knowlesi* malaria in baboon. The development of the passaged transfected parasites in PAN 2593 was not significantly different from those in PAN 2838 and PAN 2851, as they all developed low parasitaemia without an indication of malaria symptoms. The passaged transfected parasites also expressed host IFN-γ cytokine just like in PAN 2838 and PAN 2851. The poor development of passaged transfected *P. knowlesi* parasites that could express host IFN-γ cytokine, showed that the transfected parasites were well modulated in baboon system as previously established in rhesus monkey (Ozwara *et al.,* 2003b). The results further indicated that the transfected *P. knowlesi* parasites could infect baboon, but couldn't induce acute malaria infection like the donor monkey that was infected with wild-type parasites.

In this study ELISA assay for antibody response indicated 1gM isotype-antibody titre rose during infection with transfected *P. Knowlesi* parasites, which is the main antibody produced during primary immune response. This study shows that transfected *P. Knowlesi* parasite that express host IFN-γ induced mainly mild and transient infection since there was no significant rise in IgG and that explains possibly why both baboons infected with transfected parasites were able to control parasitaemia since it failed to rise above 0.4% and similar results was observed in the baboon infected with passaged transfected *P. knowlesi* parasites. Mild and transient infection observed in the baboon with transfected parasite is probably due to the fact that IFN-γ is required for protection against malaria parasites (Su and Stevenson 2000; Yoneto *et al*., 1999). Interestingly, antibody response toward wild-type parasites that induced acute infection in the donor monkey was strong with significant rise in IgG isotype production. This result could be exploited in future to develop attenuated malaria vaccine. Interferon- γ transfected parasites may pose a possible risk if there is over expression of the cytokine leading to inflammatory reactions, which could be disastrous. This may happen if the parasites multiply rapidly especially if the initial inoculum is extremely high. Transfected parasites must be characterised in animal models in order to establish their safety before such a strategy is experimented in human. Nevertheless, the knowledge acquired is useful in understanding immunobiology of malaria parasites in the host. This study reveals that malaria parasites may be used as vectors for gene delivery to develop malaria vaccine. Further, this strategy may have potential in formulation of attenuated malaria vaccine and in immunotherapy where interferon - γ may have therapeutic roles.

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