Possible relationship between Plasmodium falciparum *ring-infected erythrocyte surface antigen (RESA) and host cell resistance to destruction by chemicals*

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Possible relationship between *Plasmodium falciparum* ring-infected erythrocyte surface antigen (RESA) and host cell resistance to destruction by chemicals

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Abstract Repeated incubation of *Plasmodium falciparum* culture in 0.015 % saponin solution for a total of 35 min destroys most of the uninfected cells, leaving only the ring-infected erythrocytes (RIEs). Parasites concentrated by this method can subsequently complete the asexual cycle and infect other erythrocytes. It is possible that resistance to saponin is mediated by one or more of the numerous parasite proteins present in the host erythrocyte membrane. We have found that schizonts are as susceptible as uninfected erythrocytes to saponin, indicating that the protective protein is parasite stage specific. Studies with cultured parasites have shown that ring-infected erythrocyte surface antigen (RESA) strengthens host erythrocyte membrane and protects against destruction. Therefore, we hypothesize that RESA could be involved in resistance to saponin. Here, we have carried out PCR test on RESA gene, using three different primers. One of them showed that *P. falciparum* isolates collected directly from infected humans and cultured only for a few days, or not at all, have amplicon sizes ranging from 372 to 510 bp. However, the amplicon size changed to 873 bp when in vitro growth was continued for one or more weeks. This genetic transformation precedes acquisition of the ability to confer saponin resistance to RIEs.

Introduction

Plasmodium species are obligate intracellular protozoan parasites that depend on intact erythrocytes while digesting the hemoglobin for nutrients. It is, therefore, not surprising that they produce and incorporate different types of proteins into the host erythrocyte membranes to enable them perform the functions that would protect from destruction (Deitsch and Wellems 1996; Cooke et al. 2004; Haldar and Mohandas 2007). It has been found that immediately a *Plasmodium falciparum* merozoite enters an erythrocyte, it produces a protein called Pf155 or ring-infected erythrocyte surface antigen (RESA), which it incorporates into the cytoplasmic side of the erythrocyte membrane by interaction with spectrin (Aikawa et al. 1990; Deloron et al. 1987). This antigen induces production of specific antibodies that are easily detectable in people infected with *P. falciparum*. Indeed, RESA is one of the proteins currently being studied as potential candidate for malaria vaccine (Genton et al. 2003; Kabilan et al. 1994; Moorthy et al. 2004).

Through genetic manipulation of *P. falciparum* cultures and normal erythrocytes, it has been found that RESA strengthens erythrocyte membrane (Diez-Silva et al. 2005; Mills et al. 2007; Pei et al. 2007). Several reports have suggested that the stabilization plays a role in the flow of ring-infected erythrocytes through capillaries and venules, protects the parasites from febrile temperature of up to 41° C, and protects an already infected erythrocyte from invasion by additional merozoites (Diez-Silva et al. 2005; Mills et al. 2007; Pei et al. 2007). The question is whether RESA protein in vivo is identical to that produced in vitro, knowing that in vitro cultivation induces structural changes in RESA sequence (Hernandez-Rivas et al. 1996). Whatever the case, the finding that RESA strengthens host erythrocyte membrane provides an opportunity to speculate on what could account for the relative resistance of in vitro produced ring-infected erythrocytes (RIEs) to saponin. It was first reported in 1994 that *P. falciparum* RIEs could be concentrated by carefully controlled rounds of exposure to low dose of saponin (Orjih 1994). Later, it was found that this concentration method was not effective with RIEs collected directly from patients and

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never subjected to in vitro cultivation (Orjih 2008). It is known that saponin initiates hemolysis by forming complexes with erythrocyte membrane cholesterol (Bangham and Horne 1962; Bottger et al. 2012). However, infected erythrocyte membrane contains many parasite proteins which could affect the action of saponin (Maier et al. 2009; Goldberg and Cowman 2010). Because only RIEs are resistant to saponin, we suggest that RESA could be the mediator.

Materials and methods

P. falciparum isolates

Blood samples from imported falciparum malaria cases were obtained from hospital laboratories in Kuwait, a non-endemic area. The infections originated from India and Africa. The blood samples were primarily collected for routine malaria diagnosis by microscopy, after which DNA extraction and PCR analysis were done to confirm the species. The laboratory strains included in the study are HB3 and PB112 which have been described earlier (Orjih 2008). A total of 35 isolates tested by PCR were never subjected to in vitro cultivation and are referred to here as wild or uncultured. In addition, seven wild isolates were tested by PCR before and after being cultured for the first time.

P. falciparum cultivation

The standard method for in vitro cultivation of *P. falciparum* was followed as has been described (Orjih 2008) The RPMI 1640 growth medium was supplemented with HEPES, D-glucose, hypoxanthine, gentamicin, and 10 % human serum. The cultures were incubated at 37° C in a 5-L modular incubator chamber (Billups-Rotthenberg, Del Mar, CA, USA), which was filled with 3 % oxygen, 4 % carbon dioxide, and 93 % nitrogen. Spent medium was replaced daily, and fresh erythrocytes were supplied every three to four asexual cycles, unless stated otherwise.

Wild *P. falciparum* isolates tested for PCR before and after in vitro cultivation

The isolates were code-named as BRA, HE17, M328, SSH, PVR, ML21, and S165. The blood samples from which they were isolated were received within 24 h after collection from the patients. About 0.2 ml of each blood sample was utilized directly for DNA extraction. The remaining fraction of the sample, usually 0.3 to 0.5 ml, was centrifuged for 10 min at 1,614 g and the plasma was discarded. The pellet was washed three times by suspending the cells in 5 ml of the RPMI 1640 growth medium and centrifuging each time for 10 min at 1,614 g. The final pellet was suspended in 6 ml of growth medium and transferred into a 50-ml sterile plastic culture flask. The flask was loosely covered with the cap before placing it in the modular incubator chamber. Air in the chamber was displaced completely with a gas mixture containing 3 % oxygen, 4 % carbon dioxide, and 93 % nitrogen (Kuwait Oxygen & Acetylene Co., Kuwait), and the gas out and inlets were sealed airtight. The culture was incubated continuously for up to 14 days at 37° C, replacing spent supernatant daily with equal volume of fresh medium. Thin smears were prepared daily and stained with Giemsa stain for morphological evaluation and parasitemia level, using standard microscopy procedures (Orjih 2012). DNA was extracted every 2 to 4 days.

Modified Krebs/Ringer phosphate buffer

This buffer was used to prepare saponin solution. The followings salts (quantity in grams) were dissolved in 1 L of double distilled water: NaCl, 3.974; Na_2HPO_4, 7.1; KCl, 0.358; and $MgSO_4{\cdot}7H_2O$, 0.296. The pH was adjusted to 7.4 with HCl. The buffer was sterilized with a 0.2-μm membrane filter.

Saponin solution

Saponin powder from Quillaja bark was obtained from Sigma, St. Louis, MO, USA. It contained 11.1 % sapogenin, 15.1 % sulfate ash, and 43.9 % carbon. A 0.015 % working solution of it was prepared in modified Krebs/Ringer phosphate (MKRP) buffer and sterilized by membrane filtration and was used within 24 h.

Test for saponin resistance

The method has been described previously (Orjih 1994, 2008). For example, a synchronized *P. falciparum* culture containing 5×10^{10} erythrocytes and 3.7 % parasitemia was suspended in 28 ml of cold MKRP buffer containing 0.015 % saponin. Four aliquots, each containing 7 ml of the suspension, were prepared in 15-ml screw cap plastic centrifuge tubes. The aliquots were first incubated on ice for 30 min, during which the suspension was mixed by hand at intervals of approximately 5 min. The tubes were then centrifuged for 5 min at 1,614 g, after which the supernatant was discarded. The four pellets were pooled in 7 ml of saponin-free MKRP. This suspension was centrifuged, and the supernatant was discarded. The pellet was again suspended in 7 ml of fresh 0.015 % saponin solution and incubated on ice with constant mixing by hand until it appeared almost completely hemolyzed, usually within 3–5 min. The suspension was centrifuged for 5 min at 1,614 g, after which the supernatant was discarded and 7 ml of RPMI 1640 medium, supplemented

with 10 % human serum, was added to the tube to stop saponin action. The tube was kept on ice for 10 min before repeating centrifugation. The supernatant was discarded, and the pellet was washed twice, using 7 ml of growth medium each time. Where required, the concentrated rings were cultured further to complete the asexual life cycle as previously described (Orjih 2008).

Rapid test for saponin resistance

Typically, about 0.1 ml of MKRP washed unsynchronized culture or infected blood was suspended in 1 ml of cold 0.015 % saponin solution and incubated on ice for 4–6 min with constant mixing. Six milliliters of complete medium, containing 10 % serum, was added to the suspension to terminate saponin action. It was centrifuged, and the pellet was washed twice with 7 ml of complete medium containing 10 % serum. Thin smears of the pellet were stained with Giemsa stain and examined by microscopy to evaluate the effect on uninfected erythrocytes, as well as those containing ring forms, trophozoites, and schizonts.

Production of *P. falciparum* ring forms in fresh erythrocytes

P. falciparum culture was synchronized with sorbitol, and the RIEs were concentrated to 98 % parasitemia by saponin hemolysis (Orjih 2008). The parasites were allowed to grow in culture for approximately 40 h, during which most of the parasites have matured to schizonts. Five milliliters of O-positive blood was collected from a male adult volunteer in a tube containing the anticoagulant EDTA. The blood sample was centrifuged, and the buffy coat containing the white cells was discarded with the plasma. The erythrocyte pellet was washed thrice with cold RPMI 1640 medium. The final pellet was suspended in 13 ml of complete growth medium containing 10 % O-positive serum. The schizont-rich parasite culture was centrifuged, and the pellet was mixed with the fresh erythrocyte suspension, and two aliquots, 6 ml each, were transferred into culture flasks. The parasites were cultured for 24 h under the growth conditions described above. Giemsa-stained thin smears of the cultures showed that the schizonts had produced ring forms in the fresh erythrocytes. The cultures were then synchronized with sorbitol and subjected to saponin hemolysis as described above.

DNA isolation

DNA was isolated directly from 0.2 ml of *P. falciparum* infected blood or cultured cells, using a NucleoSpin® kit from Macherey-Nagel GmbH (Dueren, Germany) and the procedure described by the manufacturer. The DNA was stored at −20° C until required for PCR. As a negative control, DNA was also extracted from human leukocytes prepared from the blood of uninfected donors.

Polymerase chain reaction amplification

The sequences of the oligonucleotide primers used in this study were published by Wooden et al. (1993) as shown in Table 1. For amplification, 3 μl of DNA extract was mixed with 12.5 μl of 2×AmpliTaq Gold® PCR Master Mix (ABI) containing 3 mM $MgCl_2$ and 4 μM of each of the forward and reverse primers, and made up to a final volume of 25 μl with RNase/DNase-free water (Sigma, USA). Using a Gene Amp PCR System 9700 thermocycler, amplification was performed by heating to 94° C for 5 min followed by 40 cycles of denaturation at 94° C for 45 s, annealing at 50 C for 45 s and extension at 72° C for 45 s, with a final extension step at 72 C for 7 min. Amplified products (3–5 μl) were mixed with loading dye and electrophoresed through a 1.5 % agarose gel for about 50 min. The amplicon bands were visualized with ethidium bromide and photographed using a gel documentation system (UVItech, Cambridge, UK); sizes were estimated by its software package using a 100-bp ladder marker as standard.

Sequencing of RESA PCR amplicons

This was performed as described by Alfadhli et al. (2004). Briefly, PCR product was ethanol precipitated and finally dissolved in 15 μl of water. From this, 4 μl were mixed with 3 μl (1 pmol) of either the forward or the reverse primer, and 8 μl of the dye terminator ready sequence reaction mix (PrismTM Ready Reaction Big DyeTMTerminator Cycle Sequencing Kit from ABI). The sequencing reaction was performed in a 9700 ABI thermocycler programmed to 25 cycles of 96 °C for 10 min, 50 °C for 5 s, and 60 °C for 4 min. The DNA was then ethanol precipitated and suspended in 25 μl of HiDiformamide, heated for 2 min at 95 °C, and run on an ABI3100 DNA sequencer.

Table 1 List of oligonucleotide primers used in this study. They were originally published by Wooden et al. (1993)

Gene		Sequence 5′-3′
RESA	Forward	gatcaaggaggagagaacc
	Reverse	cagcattaacaccaacacc
CSP	Forward	atagtagatcacttggaga
	Reverse	gcatattgtgaccttgtcca
MSP1	Forward	gaagatgcagtattgacagg
	Reverse	gagttctttaatagtgaacaag
MSP2	Forward	gagtataaggagaagtatgg
	Reverse	cctgtacctttattctctgg

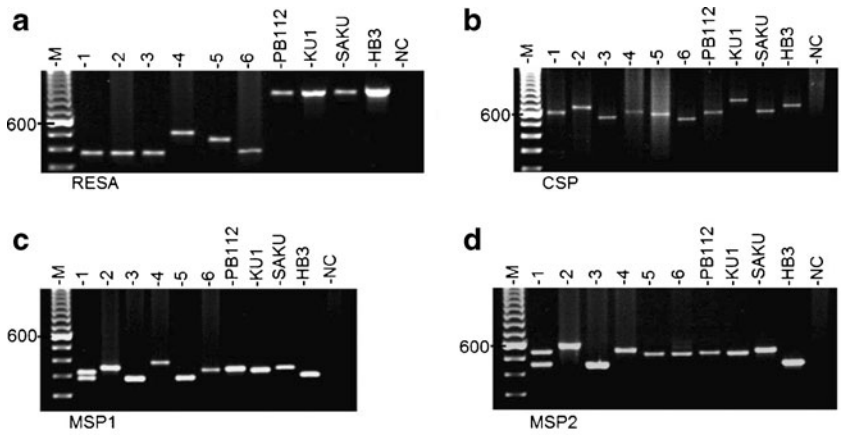

Fig. 1 PCR amplification of *P. falciparum* genes. DNA was extracted directly from uncultured *P. falciparum* infected blood of six persons (*lanes 1–6*) and four cultured strains (*PB112*, *KU1*, *SAKU*, and *HB3*). PCR amplification was performed using the primers for RESA (**a**), CSP (**b**), MSP1(**c**), and MSP2 (**d**) genes. With RESA, the amplicon sizes of uncultured parasite DNA varied between 387 and 514 bp, whereas those of cultured parasites were all circa 890 bp. CSP amplicons (**b**) show that all the isolates, including those in culture, were genetically different from one another. Additional differentiation, including mixed infections (*lane 1*) were provided by MSP1 and MSP2. The amplicon sizes (bp) range from 553–800 for CSP, 200–385 for MSP1, and 455–654 for MSP2

Results

The infected blood samples used in this study contained only *P. falciparum*. Most of them contained the ring forms with or without some gametocytes. The cultured parasite strains contained all the asexual stages as well as gametocytes, except HB3 which was not producing gametocytes. The parasitemia levels in the uncultured isolates ranged between 0.1 and 3.8 %. The established cultures were usually used for the study when the parasitemia levels were between 3 and 7 %.

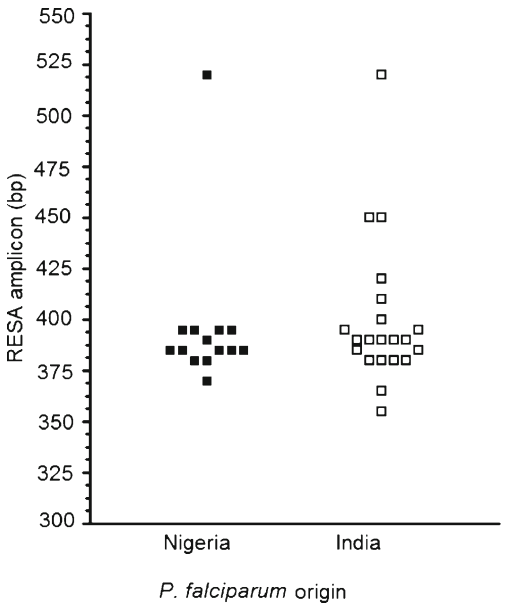

Fig. 2 DNA amplicons for RESA detected in uncultured *P. falciparum* isolates. Infected blood samples were collected from 14 Nigerians (*closed box*) and 21 Indians (*open box*). The sizes ranged between 375 and 520 bp. Samples 1–6 of Fig. 1 are also included in this figure

DNA amplification products of uncultured and cultured *P. falciparum* isolates

Figure 1 shows an example of PCR results obtained in this study, using primers for the genes encoding different cell surface proteins. The amplicon sizes given in here were estimated on gel by computer, unless stated otherwise. We have determined that they are usually longer that the sizes obtained by nucleotide sequencing. The RESA primer pair used for Fig. 1a has the following sequence: 5′-gatcaaggaggagagaacc-3′ and 5′-cagcattaacaccaacacc-3′. It shows that uncultured isolates (lanes 1 to 6) have amplicon sizes ranging from 387 to 514 bp, whereas those of cultured isolates were around 888 bp. Other RESA primers, such as those described by

Table 2 PCR amplification of DNA from *P. falciparum* isolates before and after growth in vitro

	RESA		MSP1	MSP2	CSP
Isolate	Uncultured	Cultured	(No difference between uncultured and cultured)		
BRA	504[a]	873[a] (7)[b]	335	510	615
HE17	510	510 (10)	342	513	654
M328	405	890 (7)	246	624	759
SSH	400	890 (8)	369	612	747
PVR	410	890 (7)	305	528	768
ML21	375	890 (10)	245	477	753
S165	400	890 (10)	350	495	Not done

[a] Exact sizes determined by DNA sequencing of PCR product. Other sizes were estimated on gel

[b] Day first detected in culture. The day the parasites were introduced into culture for the first time is day 0

Moyano et al. (2001) and Vazeux et al. (1993) did not differentiate between uncultured and cultured parasites (data not shown). Also included in the figure are the amplicons obtained from the same isolates shown in (a), using primers for circumsporozoite protein (CSP) (b), merozoite surface protein (MSP1) (c), and MSP2 (d). These three primers amplify genes unrelated to RESA. They did not differentiate between uncultured and cultured parasites. Unless stated otherwise, references to the RESA PCR in this study are about data obtained with the primer pair 5′-gatcaaggaggagagaacc-3′ and 5′-cagcattaacaccaacacc-3′.

The results for RESA gene obtained from a total of 35 different isolates, including those shown in Fig. 1, are summarized in Fig. 2. These were falciparum malaria infections acquired in Nigeria and India. No geographical difference in the amplicon size distribution was detected. Primers for CSP, MSP1, and MSP2 genes also did not detect any specific geographical difference (data not shown).

To demonstrate that the increase in RESA amplicon size happens in vitro, we utilized the DNA that was extracted from seven wild isolates before and after cultivation. The PCR results are summarized in Table 2. In six cases, the expanded PCR product was of a similar size to that obtained for the established laboratory-maintained cultures, including HB3. PCR amplification was also performed for MSP1, MSP2, and CSP genes and they confirmed isolate diversity but did not differentiate between cultured and uncultured parasites.

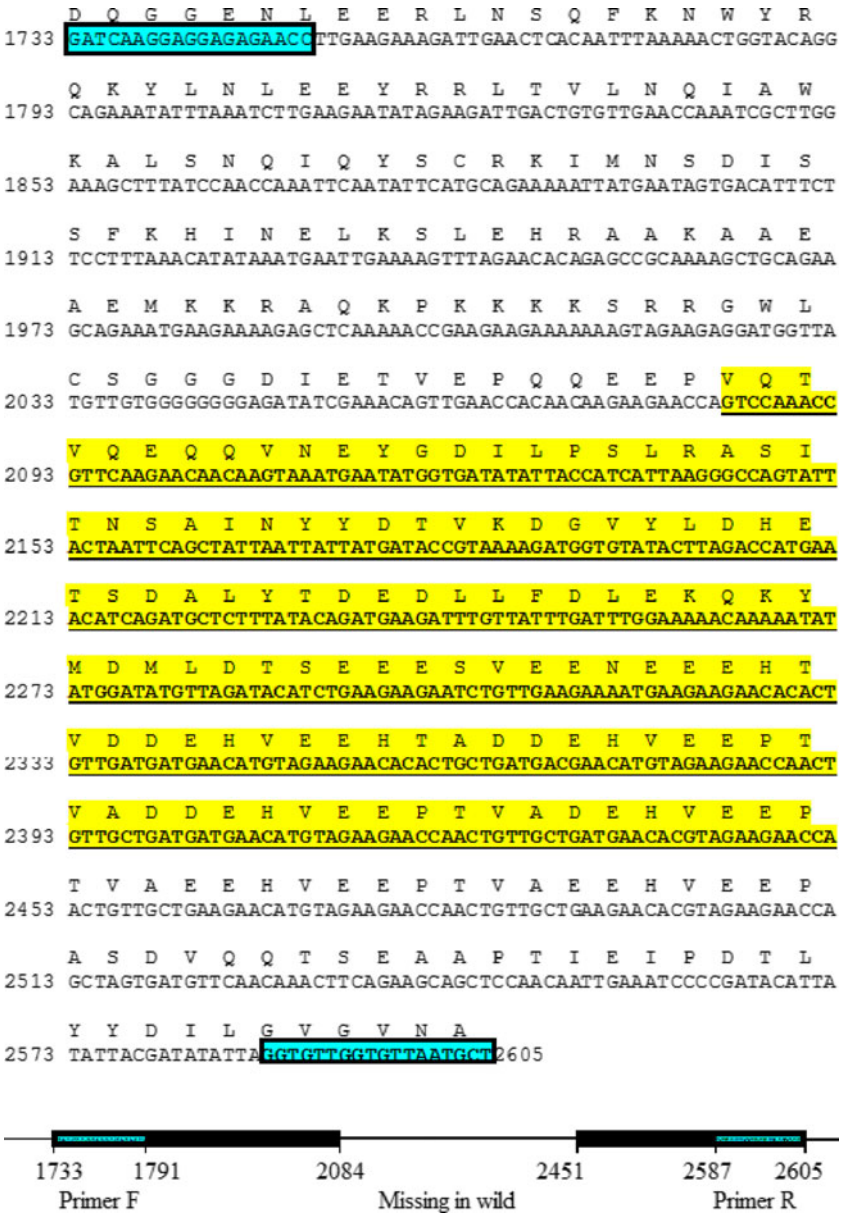

Fig. 3 Nucleotide and predicted amino acid sequence of the RESA amplification product from DNA of *P. falciparum* BRA (**a**). The entire 873 nucleotide sequence bracketed by the primers (shown boxed in blue) is homologous to nucleotides 1732-2605 of the FC27 sequence by Favaloro et al. (1986). The highlighted underlined region in the sequence (369 nucleotides) is only present in the DNA amplicon from in vitro culture but absent from DNA extracted directly from blood without culturing. The sequence is further illustrated in the sketch at the bottom of the figure

Nucleotide and amino acid sequences of RESA amplicon

The RESA amplicons from the DNA of BRA isolate before cultivation and after 12 days in culture were sequenced. The results are shown in Fig. 3. The DNA sequence from the cultured parasites was found to be completely homologous with that of the *P. falciparum* FC27 EcoRI chromosomal fragment sequence containing the RESA gene sequence published by Favaloro et al. (1986) and has been numbered here in the same way for ease of comparison. The entire 873 nucleotide sequence of cultured BRA, bounded by the sequences of the forward and reverse primers, is homologous to nucleotides 1732-2605 of the published FC27 sequence. The in-frame highlighted underlined region (369 nucleotides) is present in the DNA amplicons from the in vitro culture but absent from DNA extracted directly from the wild type without prior cultivation. We also sequenced the RESA amplicons of two other uncultured isolates shown in Fig. 4a, b. In both cases, the 5′ part includes 351 nucleotides of the FC27 sequence 1733-2083. For AO1 (Fig. 4a), this is joined to nucleotides 2453-2605, giving a total size of 504 nucleotides identical to that seen for uncultured BRA, and for AO2, to

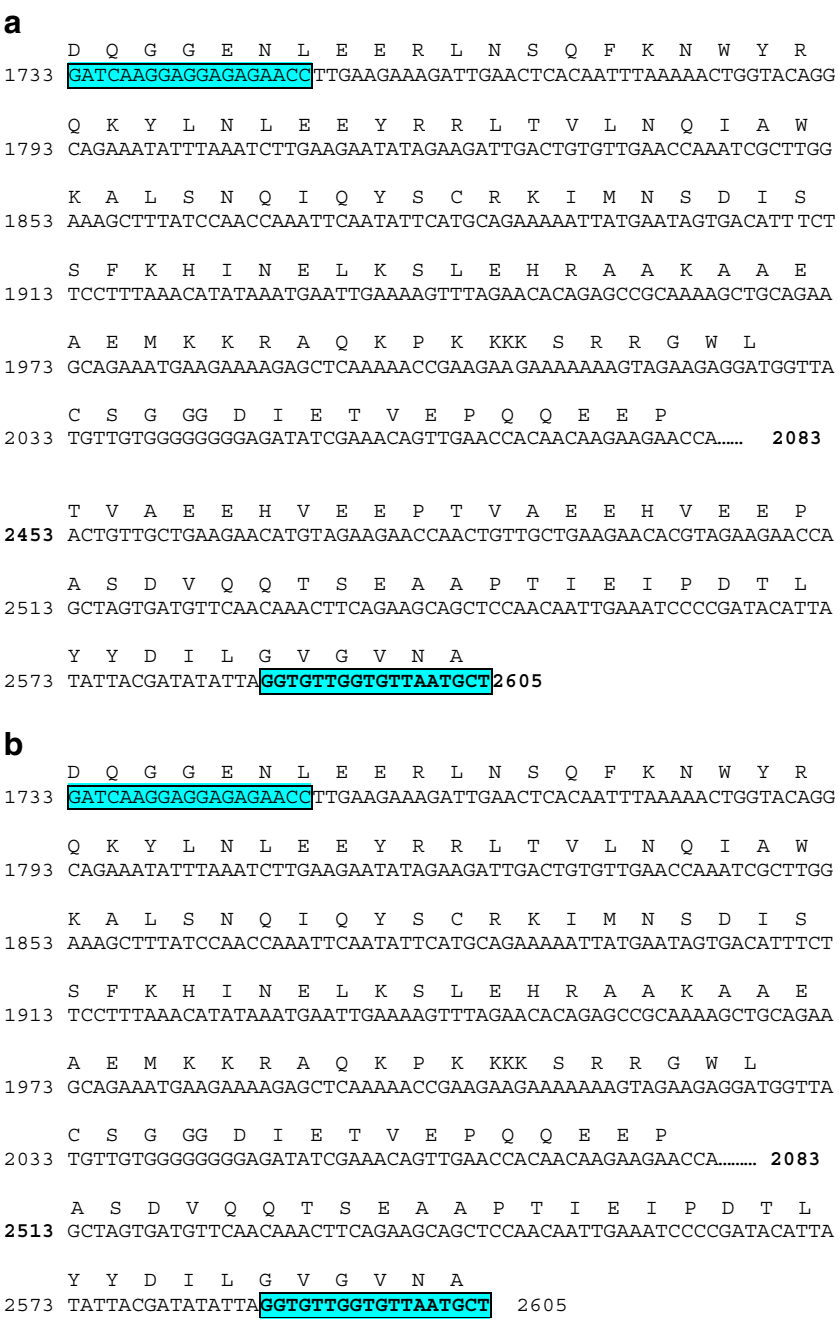

Fig. 4 Nucleotide and predicted amino acid sequence of RESA amplification product from DNA of uncultured *P. falciparum* isolates. The isolates were code-named AO1 (**a**) and AO2 (**b**). The 3′ part of AO1 (**a**) is identical with that of BRA. The 3′ part of AO2 (**b**) consists of a shorter sequence of nucleotides 2033-2065. In both isolates, a large part of the FC27 sequence published by Favaloro et al. (1986) is missing

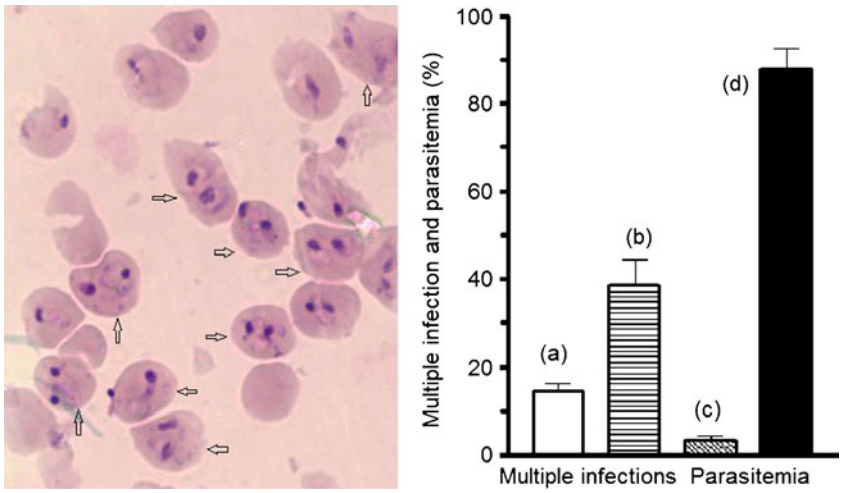

Fig. 5 Saponin treatment increases the proportion of MIEs in *P. falciparum* culture. The microphotograph shows a field (100X oil immersion) in a Giemsa-stained thin film of saponin concentrated HB3 culture. *Arrows* indicate MIEs. *Bars* represent mean ± SEM of three experiments; *a*, *b* represent proportions of MIEs before and after saponin treatment, while *c*, *d* represent the total parasitemia levels before and after saponin

nucleotides 2513-2605 giving a total fragment size of 444 nucleotides (Fig. 4b).

Effect of saponin on different stages of *P. falciparum*

In the rapid test for saponin resistance described in "Materials and methods", the culture contained the following mixture (%) of erythrocytes: 94 uninfected, 5 RIEs, 0.8 trophozoites, and 0.2 schizonts. After about 5 min exposure to saponin, the proportions (%) of surviving erythrocytes changed to 45.2 uninfected, 54.2 RIEs, 0.5 trophozoite, and 0.1schizont. The result is consistent with RIE resistance to saponin, relative to the other erythrocytes in the culture. Figure 5 illustrates the result obtained when sorbitol synchronized RIEs were treated with saponin; the parasitemia increased to between 68 and 99.5 %, mean 87.8 %. It was also found that the proportion of erythrocytes containing multiple rings increased further after the treatment with saponin. The saponin-resistant RIEs appeared intact morphologically in Giemsa-stained thin smears of the culture. When cultured further, the parasites remained viable and were able to complete the asexual life cycle (data not shown; see Orjih, 2008). Figure 6 shows infected blood sample collected directly from an adult male and cultured for 42 h before exposure to saponin. All the erythrocytes in the culture, including uninfected and those containing trophozoites and schizonts, were destroyed. The RIEs were also destroyed, possibly because the parasite had not yet acquired the ability to confer resistance to host cells.

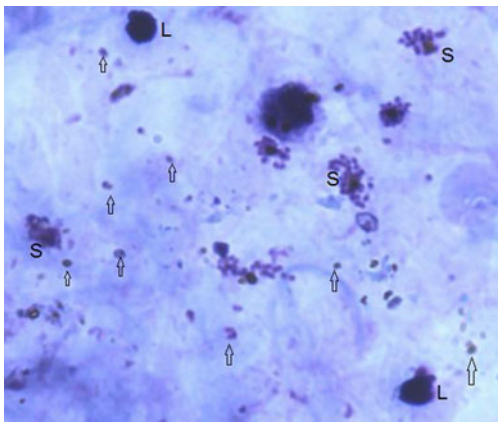

Fig. 6 Brief in vitro growth of wild *P. falciparum* isolate does not confer resistance to saponin. Infected blood was collected from an adult male and cultured for 42 h before exposure to saponin. All the erythrocytes in the culture, including the uninfected and those containing parasites, were destroyed. *Arrows* point at free-ring forms. Examples of hemoglobin-free schizonts (S) and resistant leukocytes (L) are also identified. The thin smear of the culture was stained with Giemsa stain

Discussion

In this study, we have shown that the RESA primer pair, 5′-gatcaaggaggagagaacc-3′ and 5′-cagcattaacaccaacacc-3′, can differentiate between uncultured (wild) and cultured *P. falciparum* parasites. Isolates collected directly from infected humans had relatively short amplicons (387–514 bp), even when cultured briefly. However, when cultivation was continued for seven or more days, the RESA size increased to approximately 890 bp. It is unclear whether the increase in size is induced in vitro or whether wild isolates naturally contain both the short and the long fragments, one masking the other, depending on growth environment. Several reports on changes in RESA during in vitro cultivation have been published, but they have mostly indicated deletions and duplications in the subtelomic region (Scherf and Mattei 1992; Biggs et al, 1989; Ribacke et al. 2007; Bottius et al, 1998). However, it has never been reported that such changes consistently differentiated between uncultured and cultured parasites.

On the potential association of RESA to saponin resistance, published experiments have shown that unlike cultured

parasites, *P. falciparum* RIEs collected directly from infected humans are not resistant to saponin (Orjih 2008). Perhaps, uninfected erythrocytes obtained directly from humans respond differently to saponin treatment than stored blood. This possibility was tested here in two ways. First, it was found that there was no reproducible difference in percent hemolysis of erythrocyte suspension prepared with blood from blood bank (stored for 15 or more days) and those collected directly from humans. Second, erythrocytes collected from human were immediately made available to highly concentrated *P. falciparum* schizonts in culture for 24 to 40 h, during which ring forms were produced in the fresh erythrocytes. The culture was then synchronized and treated with saponin as described in materials and methods. The experiment was done three times and the average parasitemia after saponin was 92 %, all at the ring stage of development. Furthermore, during the first few weeks following the introduction of the strain PB112 to growth in vitro for the first time, attempts to concentrate the RIEs were unsuccessful. It was not until after about 2 months of continuous maintenance in culture that the strain acquired resistance to saponin. This strain has subsequently been concentrated by saponin in erythrocytes from different sources, both fresh and stored.

Model experiments with erythrocyte membrane ghosts and *P. falciparum* culture have shown that RESA stabilizes erythrocyte membrane, making it resistant to mechanical damage, among other things (Diez-Silva et al. 2005; Mills et al. 2007; Pei et al. 2007). It is, therefore, reasonable to hypothesize that RESA produced in culture is the mediator of resistance to saponin. *P. falciparum* acquires the ability to induce saponin resistance in host erythrocyte membrane during adaptation to continuous growth in vitro. Experience with HB3 and PB112 strains suggest that once the ability is acquired, the parasite can confer saponin resistance to fresh, as well as stored, erythrocytes.

The finding that erythrocytes containing multiple rings of *P. falciparum* are more resistant to saponin than those containing only one parasite raises the question whether the protective effect of RESA against erythrocyte membrane damage is dependent on the quantity of RESA protein incorporated into the membrane. Since every merozoite that invades erythrocyte incorporates its RESA into the host cell membrane, it is reasonable to assume that multiple infections would increase the amount of RESA protein in the membrane of their shared host cell. Other investigators have demonstrated that the stabilization effect increases with quantity of RESA present in the membrane (Pei et al. 2007).

The observation that ring-infected erythrocytes are more resistant than uninfected erythrocytes was first made almost 20 years ago (Orjih 1994). It has been repeatedly used to remove uninfected erythrocytes from freshly synchronized culture of *P. falciparum* for research purposes. From the present study, it appears that the methodology could also contribute to better understanding of RESA development and function. It is known that RESA strengthens the membrane of host erythrocyte during the first 24–30 h of the intraerythrocytic life, after which the effect is masked or taken over by mature parasite proteins. Our hypothesis here is that the membrane stabilization effect of RESA can also protect against chemical damage. Experiments not presented here showed that when cultured *P. falciparum* parasites were killed at the ring stage by artemisinin or chloroquine, the host erythrocytes remained more resistant to saponin than uninfected erythrocytes (Orjih 1996; observation with chloroquine not published). In contrast, the infected erythrocytes were not protected against saponin when the parasites were killed at trophozoites and schizonts stages by the antimalarial drugs. It has been reported by other investigators that RIEs still remained RESA positive in serological tests even when the parasites were no longer in the erythrocytes (Angus et al. 1997). These observations strongly suggest possible involvement of RESA in stage-specific protection of *P. falciparum* RIEs against destruction by some chemicals, including antimalarial drugs.

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