Farouk *et al*, 2005; Tangteerawatana *et al*, 2008). As the parasites possess complex life cycles, distinct stage structures and high antigenic variations, human immune responses, both innate and acquire immunity, against the various specific parasite antigens have not been completely elucidated. Research in immunity and malarial vaccines, *eg* against pre-erythrocytic parasite and pathogenic asexual stage or to limit parasitemia, have intensively been developed for more than 40 years (Tongren *et al*, 2006; Greenwood *et al*, 2008). However, there have been very few molecular biological studies focusing on investigating mechanisms of classes/subclasses of Ig production, particularly Ig class switching in falciparum malaria.

In general, class switch recombination (CSR) at the Ig heavy-chain (IgH) locus occurs in antigen (Ag) stimulated naive B cells, which firstly synthesize IgM and IgD. Subsequently, the activated B cells often switch their H-chain isotype to express IgG, IgA, and IgE without changing their specificity for Ag (Stavnezer, 2000). The process of H-chain class switching proceeds in three distinct stages: 1) germline gene transcription (GLT), 2) DNA recombination (CSR), and 3) B cell differentiation into Ig-secreting plasma cells or memory B cells (Gould *et al*, 2003). In addition, activation-induced cytidine deaminase (AID), which is a B cell-specific and CD40-inducible RNA-editing enzyme, obligatorily initiates CSR. AID promotes deletion of intervening IgH DNA between Sμ and the targeted switch (S) region (Manis *et al*, 2002). GLT synthesized by this transcription and consequent splicing from an I exon to its downstreams C_H exons is necessary in order to procede to CSR (Iwasato *et al*, 1990). During active CSR, the intervening DNA, between the recombined S regions, is looped out to form extrachromosomal circular DNA, called the switch circle (SC), which contains the I exon promoter upstream of the targeted S region, the DNA segment between Sμ and the targeted S region, and Cμ (Xu *et al*, 1993). SC further is transcribed to produce circle transcript (CT) or chimeric I-Cμ product under the influence of the I_{μ} promoter. CSR generates VDJ-C_H transcripts or mature transcripts (MT), which are then translated into IgH proteins (Storb and Stavnezer, 2002).

Isotype class switching in activated B cells is regulated by Th cells through physical contact with B cells, *eg*, through CD40 and such cytokines as IL-4, IL-10, IL-13 or transforming growth factor (TGF)-β (Storb and Stavnezer, 2002). These signals initiate GLT, which increases the accessibility of the targeted S region to the CSR machinery (Muramatsu *et al*, 2000).

Here, by transcription analysis of AID mRNA, CT and MT, we investigated Ig CSR in human purified B cell culture stimulated with crude *P. falciparum* antigen (cPfAg) plus anti-CD40 in comparison with culture in medium alone plus anti-CD40 with or without IL-4. Pf-specific IgG and IgE assays using ELISA indicated complete ongoing CSR by the antigen.

MATERIALS AND METHODS

Approval for this study was obtained from the Ethics Committee, Faculty of Tropical Medicine, Mahidol University and Thai Red Cross Society, Bangkok, Thailand. All participants involved were informed of the objectives of study and signed consent forms.

Isolation of human peripheral B lymphocytes

Buffy coats of O^+ blood from healthy donors were purchased from The Thai Red Cross Society, Bangkok, Thailand. Peripheral blood mononuclear cells (PBMCs) were separated from buffy coat by gradient centrifugation on

Lymphoprep (Axis-Shield, Oslo, Norway) according to the previous study (Boyum, 1968). B lymphocytes were purified by means of positive selection with anti-CD19 monoclonal Ab (mAb) coated immunomagnetic beads (Detachabead Dynal Biotech, Oslo, Norway) as described by Romero-Rojas *et al* (2001). Purity of B cells was determined using a FACSCalibur Flow Cytometer and CellQuest software (BD Biosciences, CA) by immunostaining with anti-CD20 mAb conjugated with FITC (SouthernBiotech, Birmingham, USA) and anti-CD27 mAb conjugated with PE (Dako, Glostrup, Denmark). Purified B cells consisted of more than 97% $CD20^+$, with less than 0.8% detectable $CD27^+$ (Fig 1).

Parasite culture and crude parasite preparation

Parasites from a laboratory isolate of *P. falciparum*, TM 267, was cultured using the conventional *in vitro* method (Trager and Jensen, 1976). Late stage parasitized red blood cells (PRBC), enriched by percoll gradient method (Troye-Blomberg *et al*, 1999), were lysed by sonication (Ultrasonic Processor XL; Heat System, NY) and then filtered through 0.2 µm membrane filter. The infected-red cell lysate was used as crude Ag of late stage *P. falciparum* (cPfAg) (Troye-Blomberg *et al*, 1999). The concentration of protein in soluble crude Ag was determined using Coomassie Plus Protein Assay Reagent Kit (Pierce, IL).

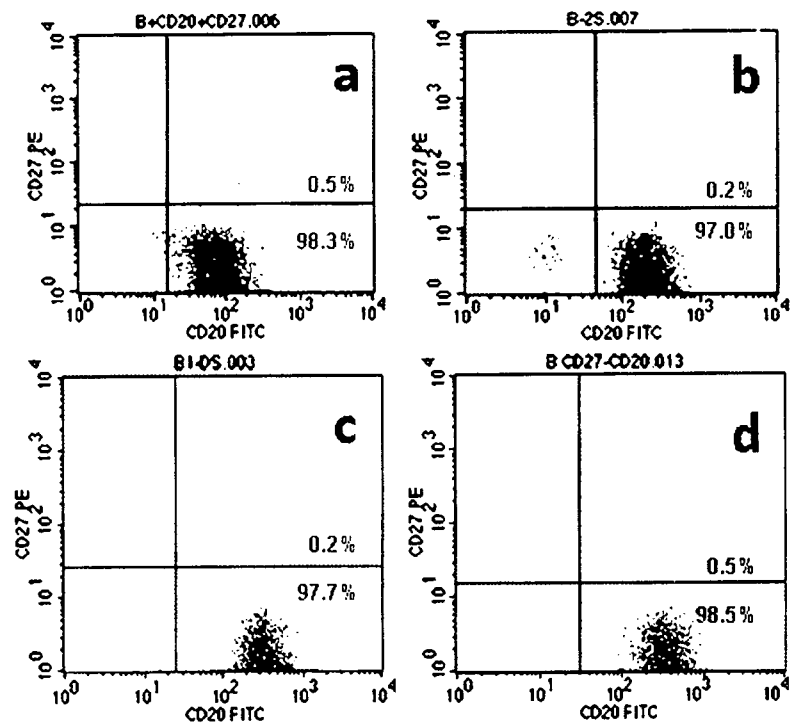


Fig 1–FACS analysis of purified B cells obtained from 4 donors. B cells were separated by anti-CD19 Dynal bead and Detachabead. Purified B cells were immunostained with FITC-conjugated mAb to CD20 and PE-conjugated mAb to CD27 and analyzed by flow cytometry. The results indicate percentage of naïve B cells ($CD20^+CD27^-$) obtained from donors 1-4 (panel a-d) was more than 97% while memory B cells were less than 1%.

Cultures and reagents

Purified B cells were cultured in complete RPMI 1640 culture medium supplemented with 10% fetal bovine serum (FBS). Anti-human CD40 mAb (Mabtech, Nacka, Sweden), recombinant human IL-4 (R&D Systems, MN) and cPfAg was used at the concentration of 10 µg/ml, 300 U/ml, and 10 µg/ml, respectively. After 4 days of culture, stimulated B cells were harvested for total RNA extraction. The culture supernatant was individually collected on Days 7 and 14 and assayed for Ig levels ELISA.

Detection of Ig CSR markers and AID expression by reverse transcriptase-polymerase chain reaction (RT-PCR) and quantitative real-time PCR (QRT-PCR)

Total RNA was extracted from $2x10^6$ of stimulated and control B cells using RNeasy total RNA kit (Qiagen, Hilden, Germany). Approximately 5 µg of total RNA were reverse transcripted to cDNA as described previously (Takhar *et al*, 2005). AID mRNA, γ-GLT, ε-GLT, γ-mRNA, and ε-mRNA transcripts were amplified by RT-PCR for 35 cycles. CTs (Iγ-Cµ, Iε-Cµ and Iε-Cγ) were amplified using seminested PCR strategy (Takhar *et al*, 2007). β-actin mRNA, a housekeeping gene, was amplified from all samples to check for integrity of cDNA and control of cDNA loading (Cerutti *et al*, 1998). The specific primer pairs and expected PCR product sizes are shown in Table 1. The amplified PCR products were confirmed by DNA sequencing using HiYield Gel/ PCR DNA Extraction kit (Real Biotech, Taipei, Taiwan), Bigdye Terminators Cycle Sequencing Ready Reaction kit and an automated sequencer (ABI PRISM, MA). A Blast search was performed on each sequence, providing alignments with IgH chain and AID sequences in GenBank database.

For QRT-PCR analysis, AID mRNA expression were performed in triplicate on Rotor-Gene 3000 and software version 6.0 (Corbett Research, Sydney, Australia) with Quantimix Easy SYG kit (BIOTOOL, Madrid, Spain). The amount of AID mRNA was normalized relative to the amount of β-actin mRNA. Generation of amplification products of the correct size was confirmed by dissociation curve and agarose gel-electrophoresis. Relative quantification of AID mRNA gene expression data was analyzed using the $2^{-\Delta\Delta C}{}_T$ method (Xu *et al*, 2007). The fold change in AID gene expression was normalized to the endogenous reference gene, β-actin, and relative to the unstimulated control (calibrator). The following primers pairs were used: AID, 5′ AGA GGC GTG ACA GTG CTA CA 3′ (sense) and 5′ TGT AGC GGA GGA AGA GCA AT 3′ (antisense); β-actin, 5′ GGA TGC AGA AGG AGA TCA CT 3′ (sense) and 5′ CGA TCC ACA CGG AGT ACT TG 3′ (antisense).

Detection of Ig production by ELISA

Total IgG and IgE produced by B cell cultures were determined by ELISA as reported previously (Cerutti *et al*, 2002). Levels of *P. falciparum* specific IgG and IgE were measured as described (Perlmann *et al*, 1994) using plates coated with cPfAg at the concentration of 10 µg/ml. Optical density (OD) was measured using an ELISA reader at 405 nm.

RESULTS

Expression of CSR molecular markers in cultured B cells

Amplified PCR products corresponding to expression of CSR markers in the stimulated human B cells, including expression of AID mRNA, CT and mature transcripts from 4 independent experiments, are summarized in Table 2. Analysis patterns of the amplified products for these markers and GLT in 2% ethidium bromide-stained agarose gel, obtained from one of the four experiments,

Table 1
Sequences of oligonucleotide primers used to amplify AID mRNA, germline transcripts (GLT or I_H-C_H), circle transcripts (CT), mRNA encoding the heavy chain of IgE (ε-mRNA), IgG (γ-mRNA) and β-actin.

Specific gene	Expected product size (bp)	Primer name	Sequence	GenBank accession number
AID	382	AID S	5′-TGCTCTTCCTCCGCTACATCTC-3′	NM_020661
		AID AS	5′-AACCTCATACAGGGGCAAAAGG-3′	NM_020661
ε-GLT	379	Iε S	5′-GGCCACACATCCACAGGC-3′	X56797
(Iε-Cε)		Cε AS	5′-GGGGTGAAGTCCCTGGAGC-3′	X95746
γ-GLT	500	Iγ S	5′-GATGCCAGGATGGGCACGAC-3′	S79588
(Iγ-Cγ)		Cγ AS	5′-CCAACTCTCTTGTCCACCTTGG-3′	X04646
ε-CT	320	Iε S1[a]	5′-GGGAGCTGTCCAGGAACCCGACAGAGC-3′	X56797
(Iε-Cμ)		Iε S2[b]	5′-GGCCACACATCCACAGGC-3′	X56797
		Cμ AS	5′-GTTGCCGTTGGGGTGCTGG-3′	X17115
ε-CT	339	Iε S1[a]	5′-GGGAGCTGTCCAGGAACCCGACAGAGC-3′	X56797
(Iε-Cγ)		Iε S2[b]	5′-GGCCACACATCCACAGGC-3′	X56797
		Cγ AS	5′-CCAACTCTCTTGTCCACCTTGG-3′	X04646
γ-CT	500	Iγ S1	5′-CATGACTGGATGCGGCAGAG-3′	DQ083944
(Iγ-Cμ)		Iγ S2	5′-GATGCCAGGATGGGCACGAC-3′	DQ083944
		Cμ AS	5′-GTTGCCGTTGGGGTGCTGG-3′	X17115
ε-mRNA	800	VDJ S	5′-TCGACTTCTGGGGCCAAGGG-3′	L06224
		Cε AS	5′-GCCAGGTCCACCACCAGACAGGTGA-3′	AK130825
γ-mRNA	400	VDJ S	5′-TCGACTTCTGGGGCCAAGGG-3′	L06224
		Cγ AS	5′-ATGGTCCCCCCAGGAACTCAGGT-3′	AJ294733
β-actin	593	β-actin S	5′-GTACCACTGGCATCGTGATGGACT-3′	NM_001101
		β-actin AS	5′-ATCCACACGGAGTACTTGCGCTCA-3′	NM_001101

Note: S1[a] and S2[b] refer to sense primers for first round PCR and second round PCR, respectively.

are shown in Fig 2. For determination of the initiating step of CSR, amplified PCR products representing γ-GLT (500 bp) and ε-GLT expression (379 bp) were detected in all conditions of cultured B cells, including in medium alone, with anti-CD40 alone, and with anti-CD40 plus IL-4 or cPfAg. Whereas amplified PCR products (356 bp) corresponding to AID mRNA expression was observed in all stimulated conditions of B cell cultures, but not in unstimulated B cells cultured with medium alone. QRT-PCR for AID mRNA expression showed marked differences in amounts among all conditions of cultures. B cells cultured with IL-4 and anti-CD40 were stimulated to express the highest levels of AID mRNA (Fig 3). Anti-CD40 in combination with cPfAg stimulated AID mRNA expression more than anti-CD40 alone.

The marker used to indicate active CSR going on to switch from IgM to IgG, IgM to IgE, and IgG to IgE is Iγ-Cμ, Iε-Cμ, and Iε-Cγ CTs, respectively. Iε-Cμ CT was observed in B cells cultured with IL-4 plus anti-CD40 in all 4 experiments and only in those cultured with cPfAg in experiment 4 (Table 2). Iγ-Cμ CT was observed only in B cells stimulated with anti-CD40 plus IL-4 in experi-

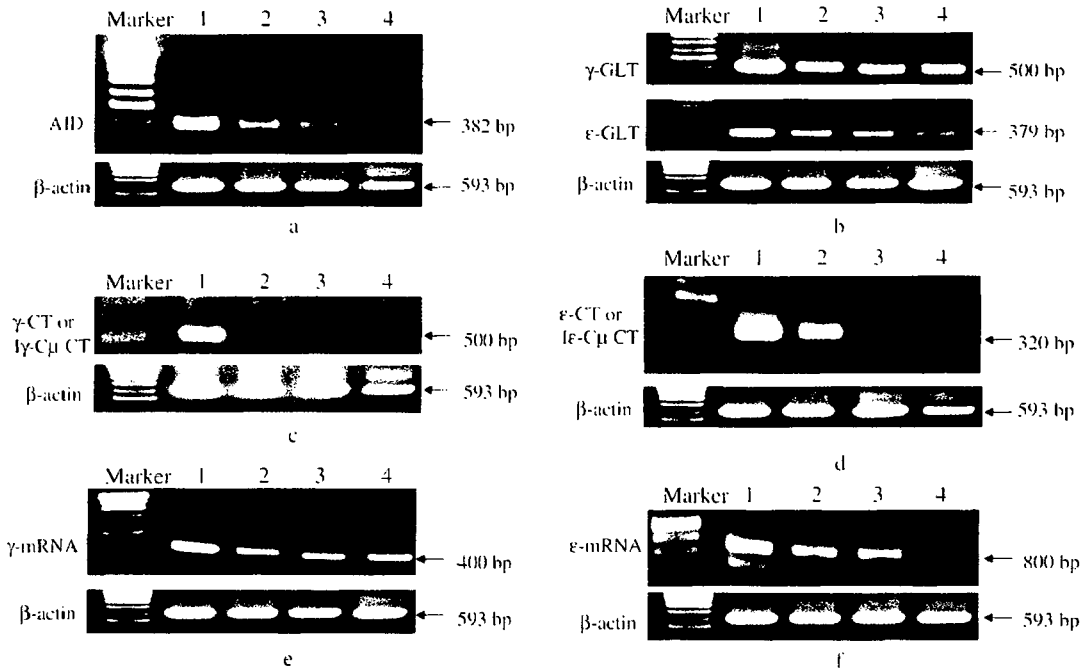


Fig 2–Expression of AID mRNA, germ line transcripts (γ-GLT and ε-GLT), circle transcripts (I γ-Cμ CT and I ε-Cμ CT) and mature transcripts (γ-mRNA and ε-mRNA). PCR products were amplified from $2x10^6$ purified B cells cultured for 4 days in medium with anti-CD40 (10 μg/ml) plus IL-4 (300 U/ml), lane 1; crude *P. falciparum* Ag (10 μg/ml), lane 2; anti-CD40 alone; lane 3; and medium alone, lane 4. The data illustrated here were obtained from the experiment 4.

ments 3 and 4, and those stimulated with cPfAg in experiments 1 and 3, whereas no Iε-Cγ CT was observed in all B cell cultures (data not shown).

Presence of the mature, γ-mRNA and ε mRNA transcript encoding for IgG and IgE respectively, was investigated to indicate the final step of the CSR. Amplified PCR product corresponding to expression of γ-mRNA (400 bp) was observed in all unstimulated and stimulated B cell cultures. ε-mRNA (800 bp) expression was found in all B cells cultured with anti-CD40 plus IL-4 but not in unstimulated B cells in medium alone. B cells stimulated with cPfAg from 2 of 4 experiments and all stimulated B cell cultures in experiment 4 showed ε-mRNA expression (Table 2).

Production of immunoglobulin classes in supernatant of cultured B cells

Increase of total IgG in culture supernatant of all conditions was observed on both Day 7 and Day 14 of culture. Levels of total and *P. falciparum* specific Ig classes produced by B cells cultured with cPfAg plus anti-CD40 from the 4 experiments are summarized in Tables 3 and 4. Increased levels of anti- *P. falciparum* IgG were observed on both Day 7 and Day 14 while increased specific IgE levels were noticeable in culture supernatants collected on Day 14.

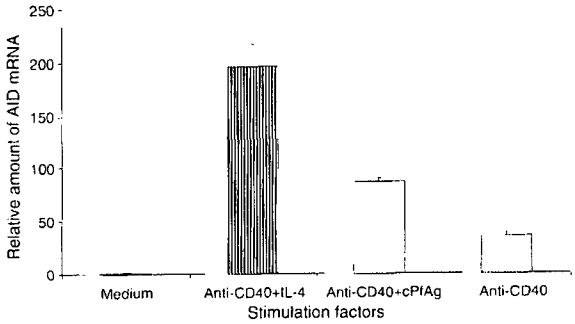


Fig 3–Relative amount of AID mRNA expression in human B cells determined by QRT-PCR. Purified B cells ($2x10^6$) were cultured for 4 days in medium, medium with anti-CD40 (10 μg/ml) plus IL-4 (300 U/ml), crude *P. falciparum* Ag (10 μg/ml), and anti-CD40 alone. The Y-axis represents amount of AID mRNA, normalized to an endogenous reference gene, β-actin, relative to unstimulated human B cells. Each data column and error bar represents mean value and standard deviation of triplicate from three independent QRT-PCR experiments. The data illustrated here were obtained from one of four independent experiments, which yielded similar results.

DISCUSSION

Evidence from both clinical and experimental studies have shown elevation of Ig profiles including IgM, IgE, IgG and IgG subclasses specific to various components of asexual stage *P. falciparum* parasites (Greenwood *et al*, 2008). Here, we demonstrated the occurrence of Ig class switching and its production by cPfAg-activated human B cells. This study, using naïve B cells, was designed to observe the consequences of the activated B cells, particularly Ig production, after exposure to a crude lysate of *P. falciparum* infected RBC. Most of the previous studies revealed that B cell activation by this parasite is T cell dependent (Troye-Blomberg *et al*, 1999; Heddini, 2002; Miller *et al*, 2002). Several studies commonly used anti-CD40 or CD40L instead of T cell cocultures (Zhou *et al*, 2003; Dedeoglu *et al*, 2004; Fear *et al*, 2004). Here, to limit the other factors and to focus the study on activated B cells ongoing Ig class switching, we cultured approximately 97% of purified mature B cells with anti-CD40 instead of PBMC culture. Our investigations demonstrated 2 phases. Firstly, ongoing Ig CSR was observed by determination of CSR molecular markers (Cerutti *et al*, 2002; Takhar *et al*, 2007). Secondly, complete process of Ig class switching was shown by Ig production in the culture supernatant (Perlmann *et al*, 1994; Cerutti *et al*, 2002). In agreement with the previous study (Tongren *et al*, 2006), variations in profiles of CSR markers and levels of specific Ig production were dependent upon individual samples and immune response to malaria infection.

Both γ-GLT and ε-GLT were found in all conditions of B cell cultures and unstimulated B cells from the 4 experiments (data not shown). This finding is consistent with earlier studies suggesting that these germline genes are constitutively transcribed in the majority of naïve human B cells in a population (Klien *et al*, 1998; Fear *et al*, 2004). As both GLT markers could not indicate whether CSR actually occured after stimulation, therefore determination of GLTs does not provide useful markers for investigation of active CSR.

In the present study, there was no expression of AID mRNA observed in B cells cultured in unstimulated B cells indicating that our purified B cells were inactive before stimulation. This finding is consistent with a previous study showing that naïve B cells express AID mRNA only upon stimulation (Nagumo *et al*, 2002). Further, in agreement with previous studies (Muramatsu *et al*, 2000; Takhar *et al*, 2007), our findings demonstrated that AID mRNA expression is a

Table 2
Expression of Ig class switching markers in human B cells.

Stimulated factor	PCR amplification of gene expression																			
	AID mRNA				γ-CT (Iγ-Cμ)				ε-CT (Iε-Cμ)				γ-mRNA				ε-mRNA			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Medium	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-
Anti-CD40+IL-4	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Anti-CD40+cPfAg	+	+	+	+	+	-	+	-	-	-	-	+	+	+	+	+	-	+	-	+
Anti-CD40	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	+

2x10^6 purified B cells were cultured for 4 days in medium, medium with anti-CD40 (10 μg/ml) plus IL-4 (300 U/ml) or cPfAg (10 μg/ml), and anti-CD40 alone. Results are expressed as presence (+) and undetectable (-) amplified PCR products. Expression of AID mRNA and mature transcripts (γ-mRNA and ε-mRNA) was amplified by RT-PCR. Expression of circle transcripts (Iγ-Cμ, Iε-Cμ and Iε-Cγ) were amplified by RT-PCR and second round seminested PCR. Four separate experiments (1, 2, 3 and 4) were performed in duplicate.

Table 3
Level of total IgG in supernatant from purified B cell culture.

Stimulating factor	Mean of total IgG concentration (ng/ml)							
	Day 7				Day 14			
	Exp1	Exp2	Exp3	Exp4	Exp1	Exp2	Exp3	Exp4
Medium	130.7	47.5	43.4	170.9	120.2	106.9	216.3	151.4
Anti-CD40+IL-4	667.6	277.7	730.9	940.8	661.0	524.6	774.9	832.6
Anti-CD40+cPfAg	307.6	270.8	440.7	439.0	424.7	442.3	552.2	461.4
Anti-CD40	369.3	134.7	333.9	416.3	600.5	300.7	796.6	472.0

Purified B cells (1x10^6 cells/1 ml) were cultured in medium, medium with anti-CD40 (10 μg/ml) plus IL-4 (300 U/ml) or cPfAg (10 μg/ml), and anti-CD40 alone. Supernatants were collected on Days 7 and 14 for the assay of total IgG. Data are expressed as mean for duplicate measurement. Four experiments (Exp 1-4) were conducted separately.

Table 4
Production of Ig classes by purified B cells stimulated with cPfAg.

Experiment no.	Level of Ig			
	Total IgG (ng/ml)	Total IgE (pg/ml)	Specific IgG (pg/ml)	Specific IgE (pg/ml)
1	424.7	324.7	7,213.1	21.3
2	442.3	1,366.5	6,310.2	152.6
3	552.2	730.3	7,870.6	290.4
4	461.4	4,892.6	12,797.8	153.5

Purified B cells ($1x10^6$ cells/1 ml) were cultured in medium in the presence of anti-CD40 (10 µg/ml) plus cPfAg (10 µg/ml). Supernatants were collected on Day 14 for the assay of total and *P. falciparum*-specific IgG and IgE. Data are expressed as mean for duplicate measurement. Four experiments were conducted separately.

good indicator for the early stage of active Ig CSR. In addition, IL-4 plus anti-CD40 was a positive inducer of AID expression and CSR study, in agreement with results from previous studies (Cerutti *et al*, 1998; Zhou *et al*, 2003; Fear *et al*, 2004; Takhar *et al*, 2005).

Quantitative determination of AID mRNA expression revealed a different potency of each stimulator to induce CSR. Our study demonstrated cPfAg in combination with anti-CD40 could induce higher levels of AID mRNA expression compared with anti-CD40 alone. Thus, we consider that initiation of active CSR for Pf specific Ig was successful. We consistently found variations in AID expression among B cells stimulated with cPfAg prepared from different parasite isolates (data not shown). This finding may indicate that AID mRNA expression is a hallmark to show efficiency to initiate Ig CSR among different stimuli. However, an earlier study suggested that efficient occurrence of CSR might require a threshold level of AID and that increased AID mRNA expression, which correlates with an increased amount of AID protein level, could confirm complete CSR induced by the stimuli (Rush *et al*, 2005). In the present study, the findings of increased level of AID mRNA expression and the presence of Pf-specific Igs may indicate complete CSR by stimulation of cPfAg. Similar to previous findings (Nagumo *et al*, 2002; Fear *et al*, 2004), we suggest that expression of AID mRNA may indicate an ongoing CSR due to current stimuli. However, determination amount of AID expression, by means of QRT-PCR and Western blot analysis, should be performed to confirm successful CSR in this study.

Amplified PCR product of γ-mRNA observed in all conditions of B cell cultures from the 4 separate experiments confirmed that CSR to IgG synthesis could undergo completely. These findings were supported by the increased levels of total and specific IgG. Total IgG in our study was not produced by contaminating memory B cells because we selected only resting and unstimulated B cells, which had no AID expression when cultured in medium alone. Therefore, not only the target Ag, cPfAg, but other factors including anti-CD40 and media may also have activated CSR and finally produced different levels of total IgG. Earlier studies (Jabara *et al*, 1990; Zhang *et al*, 1991; Yoshimoto *et al*, 1997) also found some

baseline Ig level in B cell culture with medium alone. A previous study demonstrated in rat glioma cell culture that 10% FBS in culture medium could enhance cell proliferation (Liu *et al*, 1995). This result was indicated by expression of proliferating cell nuclear antigen (PCNA) gene after stimulation of its promoter. In addition, the authors showed similarity of PCNA promoter of human and rodents (Liu *et al*, 1995). This finding supports the present study that Igs may increase from proliferation of B cell in response to FBS in the culture medium. Similar to our finding, human purified resting B cells cultured with anti-CD40 mAb alone could produce IgM and IgG (Jabara *et al*, 1990; Zhang *et al*, 1991).

This study indicates that assays for the determination of specific Ig levels by ELISA are necessary in order to confirm the successful occurrence of active IgG CSR by Ag of interest (Cerutti *et al*, 1998; Garraud *et al*, 2002; Tangye *et al*, 2002; Fear *et al*, 2004). Quantitative instead of qualitative determination of γ-mRNA expression should be more advantageous to determine the correlation of IgG product with concurrent expression of γ-mRNA.

ε-mRNA expression was not observed in unstimulated B cells. This finding is in agreement with previous studies showing IgE switching needs stimulation and cannot occur simultaneously (Wood *et al*, 2004; Takhar *et al*, 2005). Only B cells from experiment 4 showed ε-mRNA expression consistent with the presence of increased IgE level in supernatant on Day 7 (data not shown). However, ε-mRNA expression in B cells stimulated with IL-4 and anti-CD40 for 4 days (Cerutti *et al*, 2002) confirmed that our PCR protocol regarding the measurement of ε-mRNA levels was reliable. The levels of total and specific IgE (Day 14) indicated that CSR for IgE synthesis by activated B cells were ongoing. The absence of ε-mRNA on Day 4 from the experiments 1-3 might be explained by a) limitation of the threshold of our RT-PCR method, and/or low capacity and individual response of B cell cultures to undergo CSR for IgE synthesis after stimulation using our conditions. Some earlier studies suggested the harvesting time to investigate ε-mRNA expression is from Day 3 to 5 (Cerutti *et al*, 1998; Takeuchi *et al*, 2000; Nagumo *et al*, 2002; Kajiwara *et al*, 2004). Comparing to IgG production, previous studies suggested stimulation of resting B cell to produce IgE by IL-4 plus anti-CD40 or some specific agents usually needs a longer period of time, of 14 up to 21 days (Zhang *et al*, 1991; Nagumo *et al*, 2002).

CT (Iγ-Cμ, Iε-Cμ or Iε-Cγ) can explain the occurrence of CSR from IgM to IgG, IgM to IgE, or IgG to IgE, respectively. As positive control, Iε-Cμ CT was observed in B cells stimulated with anti-CD40 plus IL-4 in all 4 experiments while Iγ-Cμ CT was observed only in the experiments 3 and 4 (Table 2). Previous studies indicated that anti-CD40 or CD40L plus IL-4 is a good positive control for IgE switching (Zhang *et al*, 1991; Cerutti *et al*, 1998; Tangye *et al*, 2002). To study IgG switching, stimulation B cells with anti-CD40 or CD40L/IL-4/IL-10 showed better response than those stimulated with only anti-CD40 or CD40L/IL-4 (Tangye *et al*, 2002; Fear *et al*, 2004). In the present study, expression of CT (Iγ-Cμ and Iε-Cμ) indicating active CSR marker of active CSR to IgG and IgE showed discrepancies and was not consistent with Ig production. Previous studies indicated the importance of CT as one of the most reliable markers to indicate active CSR because it decays rapidly after removal of stimulation (Cerutti *et al*, 1998; Kinoshita *et al*, 2001; Cameron *et al*, 2003). One of the limitations in determining CT is its weak expression (Kinoshita *et al*, 2001). Therefore, alternative techniques, *eg* nested

PCR (Cameron *et al*, 2003), semi-nested PCR (Takhar *et al*, 2005) or Southern blot analysis (Cerutti *et al*, 1998; Kinoshita *et al*, 2001), were applied to enhance expression signal. In this study we amplified CT-PCR product by semi-nested PCR modified from the previous study (Takhar *et al*, 2005). The discrepancies of CT expression in the 4 separate experiments might possibley be caused by B cells stimulated with different factors individually undergoing CSR and expressing CT at different times. Using a fixed time (Day 4) of investigation may lead to inaccurate interpretation. We realize the importance of CT analysis improvement and different time course analysis (during Days 3-5) in furture studies.

Taken together, after stimulation with lysate PfAg, we could determine specific antibody, both IgG and IgE, against crude PfAg. This means that class switching recombination has already occurred although we were not successful to demonstrate complete biomarkers that we had designed for indicating initiation step, intermediate and final steps. However, the obligatory expression of AID expression and mature transcript indicates the beginning and final step respectively, and in combination with the evidence of increased specific Igs it can be assumed that CSR by cPfAg stimulation has undergone completely. Without specific Ig assay, only these biomarkers are not sufficient to indicate occurrence of CSR induced by any specific stimuli.

In summary, this study showed some biomarkers and established procedures for transcript analysis to inspect the occurrence of Pf-specific Ig CSR. Further studies, including time course analysis, quantitative examination of biomarkers and using specific molecules instead of cPfAg, are needed in order to find more reliable procedures.The improved strategy may be one of practicable means to evaluate properties and efficiency of any target molecules in initiating specific protective Ig classes/subclasses against the malaria parasites.

ACKNOWLEDGEMENTS

This study was supported by grants from Swedish International Development Cooperation Agency (SIDA); Faculty of Tropical Medicine, Mahidol University; Mahidol-Oxford Tropical Medicine Research Unit (MORU); and The Thailand Research Fund (to Pachuen Potup from The Royal Golden Jubilee PhD Program; 5MMU45R1); The Commission on Higher Education through The Co-operative Research Network (CRN), Thailand; and The Ellison Medical Foundation Fellowship in Nutrition and Infection (2006) (for Dr Yaowapa Maneerat). We thank Dr Andrea Cerutti for guidance in Ig CSR study and reagents for ELISA work. We are grateful to Dr Paron Dekumyoy for evaluating our antigen quality and Dr Yuvadee Mahakunkijcharoen for guidance. We also acknowledge RC Andrew Thompson and Hannes Wickert for suggestions and proof reading.

REFERENCES

Adams S, Brown H, Turner GDH. Breaking down the blood-brain barrier: signaling a path to cerebral malaria? *Trends Parasitol* 2002; 18: 360-6.

Bereczky S, Montgomery SM, Troye-Blomberg M, Rooth I, Shaw MA, Farnert A. Elevated antimalarial IgE in asymptomatic individuals is associated with reduced risk for subsequent clinical malaria. *Int J Parasitol* 2004; 34: 935-42.

Bolad A, Farouk SE, Israelsson E, *et al*. Distinct interethnic differences in immunoglobulin G class/subclass and immunoglobulin M antibody responses to malaria antigens but

not in immunoglobulin G responses to nonmalarial antigens in sympatric tribes living in West Africa. *Scand J Immunol* 2005; 61: 380-6.

Boyum A. Isolation of mononuclear cells and granulocytes from human blood. *Scand J Clin Lab Invest* 1968; Suppl 97: 77-89.

Calissano C, Modiano D, Sirima BS, *et al.* IgE antibodies to *Plasmodium falciparum* and severity of malaria in children of one ethnic group living in Burkina Faso. *Am J Trop Med Hyg* 2003; 69: 31-5.

Cameron L, Gounni AS, Frenkiel S, Lavigne F, Vercelli D, Hamid Q. SεSμ and SεSγ switch circles in human nasal mucosa following *ex vivo* allergen challenge: evidence for direct as well sequential class switch recombination. *J Immunol* 2003; 171: 3816-22.

Cerutti A, Zan H, Schaffer A, *et al.* CD ligand and appropriate cytokines induce switching to IgG, IgA, and IgE and coordinated germinal center and plasmacytoid phenotypic differentiation in a human monoclonal IgM^+ IgD^+ B cell line. *J Immunol* 1998; 160: 2145-57.

Cerutti A, Zan H, Kim EC, *et al.* Ongoing *in vivo* immunoglobulin class switch DNA recombination in chronic lymphocytic leukemia B cells. *J Immunol* 2002; 169: 6594-603.

Dedeoglu F, Horwitz B, Chaudhuri J, Alt FW, Geha RS. Induction of activation-induced cytidine deaminase gene expression by IL-4 and CD40 ligation is dependent on STAT6 and NFkB. *Int Immunol* 2004; 16: 395-404.

Farouk SE, Dolo A, Bereczky S, *et al.* Different antibody- and cytokine-mediated responses to *Plasmodium falciparum* parasite in two sympatric ethnic tribes living in Mali. *Microbes Infect* 2005; 7: 110-7.

Fear DJ, McCloskey N, O' Connor B, Felsenfeld G, Gould HJ. Transcription of Ig germline genes in single human B cells and the role of cytokines in isotype determination. *J Immunol* 2004; 173: 4529-38.

Garraud O, Perraut R, Diouf A, *et al.* Regulation of antigen-specific immunoglobulin G subclasses in response to conserved and polymorphic *Plasmodium falciparum* antigens in an *in vitro* model. *Infect Immun* 2002; 70: 2820-7.

Gould HJ, Sutton BJ, Beavil AJ, *et al.* The biology of IgE and the basis of allergic disease. *Annu Rev Immunol* 2003; 21: 579-628.

Greenwood BM, Fidock DA, Kyle DE, *et al.* Malaria: progress, perils, and prospects for eradication. *J Clin Invest* 2008; 118: 1266-76.

Heddini A. Malaria pathogenesis: a jigsaw with an increasing number of pieces. *Int J Parasitol* 2002; 32: 1587-98.

Iwasato T, Shimizu A, Honjo T, Yamagishi H. Circular DNA is excised by immunoglobulin class switch recombination. *Cell* 1990; 13: 143-9.

Jabara HH, Fu SM, Geha RS, Verceli D. CD40 and IgE: Synergism between anti-CD40 monoclonal antibody and interleukin 4 in the induction of IgE synthesis by highly purified human B cells. *J Exp Med* 1990; 172: 1861-4.

Kajiwara K, Shinazawa M, Morishima H, Yanagihara Y. Differential effect of IL-4 and IL-13 on the expression of recombination activating genes in mature B cells from human peripheral blood. *Cell Immunol* 2004; 227: 121-8.

Kinoshita K, Harigai M, Fagarasan S, Muramatsu M, Honjo T. A hallmark of active class switch recombination: transcripts directed by I promoters on looped-out circular DNAs. *Proc Natl Acad Sci USA* 2001; 98: 12620-33.

Klien U, Rajewsky K, Kuppers R. Human immunoglobulin IgM^+ IgD^+ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells. *J Exp Med* 1998; 188: 1679-89.

Liu YC, Liu WL, Ding ST, Chen HM, Chen JT. Serum responsiveness of the rat PCNA promoter. *Exp Cell Res* 1995; 218: 87-95.

Manis JP, Tian M, Alt FM. Mechanism and control of class-switch recombination. *Trends Immunol* 2002; 23: 31-9.

Miller LH, Baruch DI, Marsh K, Doumbo OK. The pathogenesis basis of malaria. *Nature* 2002; 415: 673-9.

Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* 2000; 102: 553-63.

Nagumo H, Agematsu K, Kobayashi N, Shinozaki K, Hokibara S, Nagase H. The different process of class switching and somatic hypermutation; a novel analysis by CD27 (-) naïve B cells. *Blood* 2002; 99: 567-75.

Perlmann H, Helmby H, Hagstedt M. IgE elevation and IgE anti-malarial antibodies in *Plasmodium falciparum* malaria: association of high IgE levels with cerebral malaria. *Clin Exp Immunol* 1994; 97: 284-92.

Perlmann P, Perlmann H, Looareesuwan S, *et al.* Contrasting functions of IgG and IgE antimalarial antibodies in uncomplicated and severe *Plasmodium falciparum* malaria. *Am J Trop Med Hyg* 2000; 62: 373-7.

Romero-Rojas A, Ponce-Hernandez C, Ciprian A, Estrada-Parra S, Hadden JW. Immunomodulatory properties of Mycoplasma pulmonis. I. Characterization of the immunomodulatory activity. *Int Immunpharmacol* 2001; 1: 1679-88.

Rush JS, Liu M, Odegard VH, Unniraman S, Schatz DG. Expression of activation-induced cytidine deaminase is regulated by cell division, providing a mechanistic basis for division-linked class switch recombination. *PNAS* 2005; 102: 13242-7.

Seka-Seka J, Brouh Y, Yapo-Crezoit AC, Atseye NH. The role of serum immunoglobulin E in the pathogenesis of *Plasmodium falciparum* malaria in Ivorian children. *Scand J Immunol* 2004; 59: 228-30.

Stavnezer J. Molecular processes that regulate class switching. *Curr Trop Microbiol Immunol* 2000; 245: 127-68.

Storb U, Stavnezer J. Immunoglobulin genes: generating diversity with AID and UNG. *Curr Biol* 2002; 12: R725-7.

Takeuchi H, Kayano H, Hirose T. Differences in IgE isotype switching induced by anti-CD40 monoclonal antibody and cytokines among subtypes of chronic B lymphoid leukemias. *Exp Hematol* 2000; 28: 543-50.

Takhar P, Corrigan CJ, Smurthwaite L, *et al.* Class switch recombination to IgE in the bronchial mucosa of atopic and nonatopic patients with asthma. *J Allergy Clin Immunol* 2007; 119: 213-8.

Takhar P, Smurthwaite L, Coker HA, *et al.* Allergen drives class switching to IgE in the nasal mucosa in allergic rhinitis. *J Immunol* 2005; 174: 5024-32.

Tangteerawatana P, Montgomery SM, Perlmann H, Looareesuwan S, Troye-Blomberg M, Khusmith S. Differential regulation of IgG subclasses and IgE antimalarial antibody responses in complicated and uncomplicated *Plasmodium falciparum* malaria. *Parasite Immunol* 2008; 29: 475-83.

Tangye SG, Ferguson A, Avery DT, Ma CS, Hodgkin PD. Isotype switching by human B cells is division-associated and regulated by cytokines. *J Immunol* 2002; 169: 4298-306.

Tongren JE, Drakeley CJ, McDonald SLR, *et al.* Target antigens, age, and duration of antigen exposure independently regulate immunoglobulin G subclass switching in malaria. *Infect Immun* 2006; 74: 257-64.

Trager W, Jensen JB. Human malaria parasite in continuous cultures. *Science* 1976; 193: 673-5.

Troye-Blomberg M, Perlmann P, Mincheva Nilsson L, Perlmann H. Immune regulation of protection and pathogenesis in *Plasmodium falciparum* malaria. *Parasitologia* 1999; 41: 131-8.

Wood N, Bourque K, Donaldson DD, *et al.* IL-21 effects on human IgE production in response to IL-4 or IL-13. *Cell Immunol* 2004; 231: 133-45.

Xu L, Gorham B, Li SC, Bottaro A, Alt FW, Rothman P. Replacement of germ-line epsilon promoter by gene targeting alters con-

trol of immunoglobulin heavy chain class switching. *Proc Natl Acad Sci USA* 1993; 90: 3705-9.

Xu W, He B, Chiu A, *et al.* Epithelial cells trigger frontline immunoglobulin class switching through a pathway regulated by the inhibitor SLPI. *Nat Immunol* 2007; 8: 294-303.

Yoshimoto T, Okamura H, Tagawa YI, Iwakura Y, Nakanishi K. Interleukin 18 together with interleukin 12 inhibits IgE production by induction of interferon-γ production from activated B cells. *Proc Natl Acad Sci USA* 1997; 94: 3948-53.

Zhang K, Clark EA, Saxon A. CD40 stimulation provides an IFN-γ- independent and IL-4-dependent differentiation signal directly to human B cells for IgE production. *J Immunol* 1991; 146: 1836-42.

Zhou C, Saxon A, Zhang K. Human activation-induced cytidine deaminase is induced by IL-4 and negatively regulated by CD45: implication of CD45 as a Janus kinase phosphatase in antibody diversification. *J Immunol* 2003; 170: 1887-93.

Evidence for the Transmission of *Plasmodium vivax* in the Republic of the Congo, West Central Africa

Richard Culleton,[1,3] Mathieu Ndounga,[5] Fadile Yildiz Zeyrek,[6] Cevayir Coban,[2] Prisca Nadine Casimiro,[5] Satoru Takeo,[4] Takafumi Tsuboi,[4] Anjali Yadava,[8] Richard Carter,[7] and Kazuyuki Tanabe[1]

[1]Laboratory of Malariology, International Research Centre of Infectious Diseases, Research Institute of Microbial Diseases, and [2]Laboratory of Host Defense, World Premier International Immunology Frontier Research Center, Osaka University, Osaka, [3]Department of Protozoology, Institute of Tropical Medicine (NEKKEN) and the Global Centers of Excellence Program, Nagasaki University, Nagasaki, and [4]Cell-Free Science and Technology Research Centre, Ehime University, Ehime, Japan; [5]Centre d'Etudes sur les Resources Vegetales, Brazzaville, Republic of the Congo; [6]Department of Microbiology, Medical Faculty, Harran University, Sanliurfa, Turkey; [7]Institute of Immunology and Infection Research, University of Edinburgh, Edinburgh, United Kingdom; [8]Division of Malaria Vaccine Development, Walter Reed Army Institute of Research, Silver Spring, Maryland

***Plasmodium vivax* is not thought to be transmitted in western and central Africa, because of the very high prevalence of the red blood cell Duffy-negative phenotype in local populations, a condition which is thought to confer complete resistance against blood infection with *P. vivax*. There are, however, persistent reports of travelers returning from this region with *P. vivax* infections. To investigate whether transmission occurs in this region, the presence of antibodies specific to *P. vivax* preerythrocytic-stage antigens was assessed in individuals from the Republic of the Congo. A total of 55 (13%) of 409 samples tested by enzyme-linked immunosorbent assay had antibodies to *P. vivax*–specific antigens.**

Transmission of *Plasmodium vivax* is not generally thought to occur in western or central continental Africa, where 95%–99% of the human population is refractory to *P. vivax* blood infection because of the protective effect of the red blood cell (RBC) Duffy-negative condition [1, 2]. Despite this, reports of Duffy-positive nonimmune travelers returning from these areas with infections diagnosed as being due to *P. vivax* are common and have persisted over many years of surveillance [3]. Furthermore, a recent report has implied that *P. vivax* transmission may occur in a population consisting of very high percentages of Duffy-negative individuals, with the presence of *P. vivax*–specific proteins reported in 0.65% of mosquitoes from an area of western Kenya [4]. An additional study reported evidence of *P. vivax* infections in 2 Duffy-negative individuals in Brazil [5]. Some investigators have interpreted such findings as implying that the parasite may be in the process of evolving the ability to infect Duffy-negative individuals [6]. However, we have argued elsewhere [3] that *P. vivax* transmission can be expected in populations with high levels of RBC Duffy negativity and in which malaria transmission intensities are sufficiently high, as is the case in many areas of western and central Africa. Notwithstanding this expectation, a recent polymerase chain reaction (PCR)–based parasite species–typing survey of 2588 blood samples obtained from patients in 9 western and central African countries failed to find any *P. vivax* parasites, except on the island of Sao Tome, where *P. vivax* transmission is known to occur [3].

In the present study, we used serological testing to search for evidence of *P. vivax* transmission in Pointe-Noire, a city on the west coast of the Republic of the Congo, where >95% of the population is expected to be RBC Duffy negative and, thus, refractory to *P. vivax* blood infection. In September 2007, we collected blood samples from 415 Pointe-Noire residents and searched for the presence of antibodies to the *P. vivax*–specific antigens *P. vivax* circumsporozoite protein (PvCSP) and *P. vivax* merozoite surface protein 1 (PvMSP1). Both antigens are expressed in liver-stage parasites and induce antibodies even in the absence of *P. vivax* blood infection [7]. Detection of antibodies to these *P. vivax*–specific antigens in a largely Duffy-negative human population could be evidence of its transmission there.

Materials and methods. By means of passive case detection, 415 samples were collected from the Mbota health center in Pointe-Noire, located on the west coast of the Republic of

Received 16 March 2009; accepted 10 June 2009; electronically published 2 October 2009.

Reprints or correspondence: Dr. Richard Culleton, Dept. of Protozoology, Institute of Tropical Medicine, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852–8523, Japan (richard@nagasaki-u.ac.jp); or Dr. Kazuyuki Tanabe, Laboratory of Malariology, International Research Centre of Infectious Diseases, Research Institute of Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita, Osaka, Japan (kztanabe@biken.osaka-u.ac.jp).

The Journal of Infectious Diseases 2009;200:1465–9

0022-1899/2009/20009-0015$15.00
DOI: 10.1086/644510

Potential conflicts of interest: none reported.

Presented in part: 17th International Conference for Tropical Medicine and Malaria, Jeju Island, South Korea, 1 October 2008 (abstract LB-P15); British Society for Parasitology Malaria Meeting, Edinburgh, United Kingdom, 6–8 April 2009 (abstract M8).

Financial support: Japanese Ministry of Education, Culture, Sports, Science, and Technology (grant-in-aid 18073013 for scientific research on priority areas); Japan Society for the Promotion of Science (grants-in-aid 17-05495 and 20390120 for scientific research).

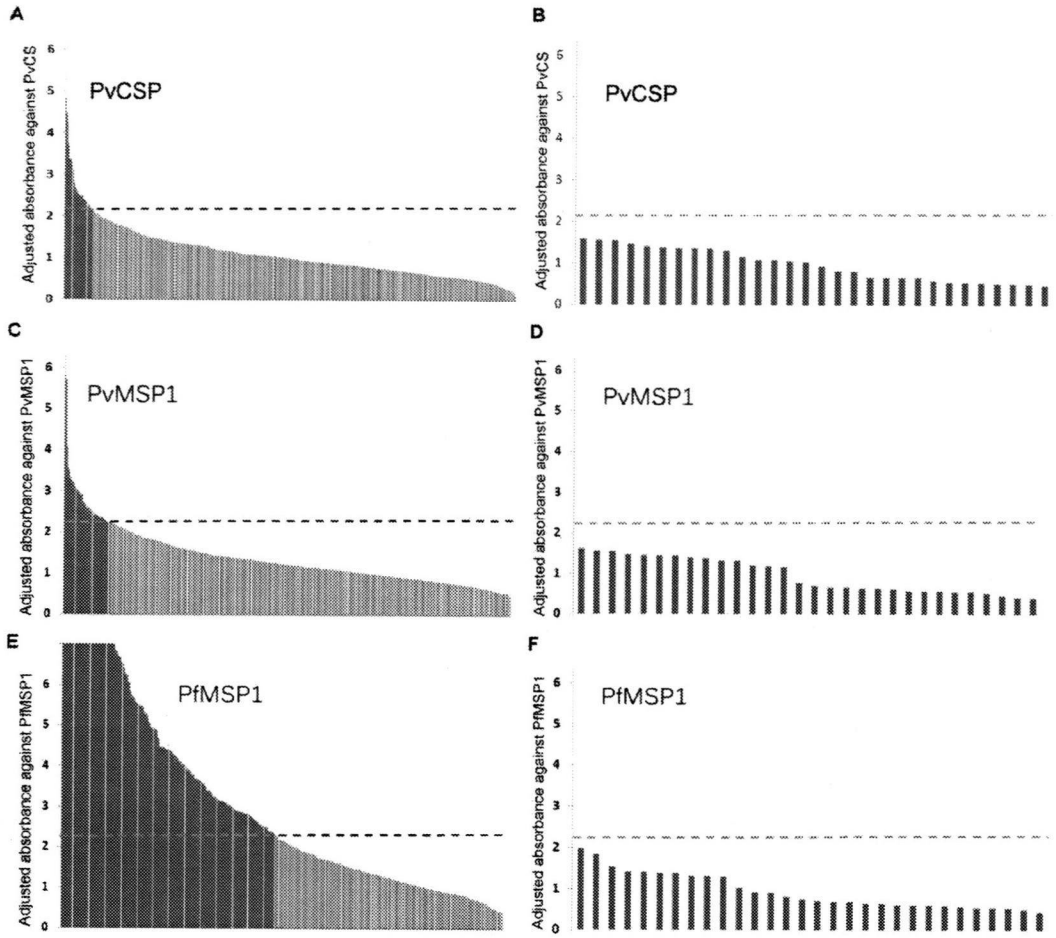


Figure 1. Adjusted absorbances against *Plasmodium vivax*–specific antigens *P. vivax* circumsporozoite protein (PvCSP) *(A), P. vivax* merozoite surface protein 1 (PvMSP1) *(C),* and the *Plasmodium falciparum* antigen *P. falciparum* merozoite surface protein 1 (PfMSP1) *(E)* for 409 individuals from Pointe-Noire, Republic of the Congo. *B, D,* and *F,* Corresponding adjusted absorbances for nonexposed individuals (from Japan and the United Kingdom). Cutoff values are denoted by horizontal dashed lines, and positive individuals are denoted by the areas shaded dark gray. The cutoff value was calculated as the mean value (+3 standard deviations) of the adjusted absorbances of 30 nonexposed individuals. All absorbances were measured at 405 nm.

the Congo, during September 2007. No age restrictions were applied to individuals from whom samples were obtained. The samples were collected on Whatman 31ETCHR filter paper. Travel histories were obtained from individuals before sample collection, and those who had traveled outside of the Republic of the Congo were excluded from the study ($n = 6$). Approval of the sample collection was obtained from the ethics committee at the Research Institute of Microbial Diseases, Osaka University (Osaka, Japan), and sampling was authorized by the administrative authority of the Ministry for Research and the Ministry for Health in the Republic of the Congo. Written informed consent was obtained from individual patients, and antimalarial treatment was provided when appropriate. An additional 10 blood samples were collected from *P. vivax*–infected patients from Siverek-Sanliurfa in the southeast of Turkey, for use as positive controls, and from 30 individuals from Japan and the United Kingdom with no previous exposure to *P. vivax* (ie, nonexposed individuals), for use as negative control samples (for collection details, see the description of supplementary methods in the Appendix, which appears only in the electronic version of the *Journal*).

All samples were screened by enzyme-linked immunosorbent assay (ELISA) for the detection of immunoglobulin G antibodies to 3 *Plasmodium*-specific proteins. The first of these proteins was PvCSP recombinant protein. This *Escherichia coli*–expressed recombinant protein encompasses the N-terminal and C-terminal regions of PvCSP flanking a chimeric repeat region [8]. The second protein, PvMSP1 recombinant protein, was expressed using a wheat germ cell-free protein translation system [9] that encompasses N-terminal blocks 1 and 2 of

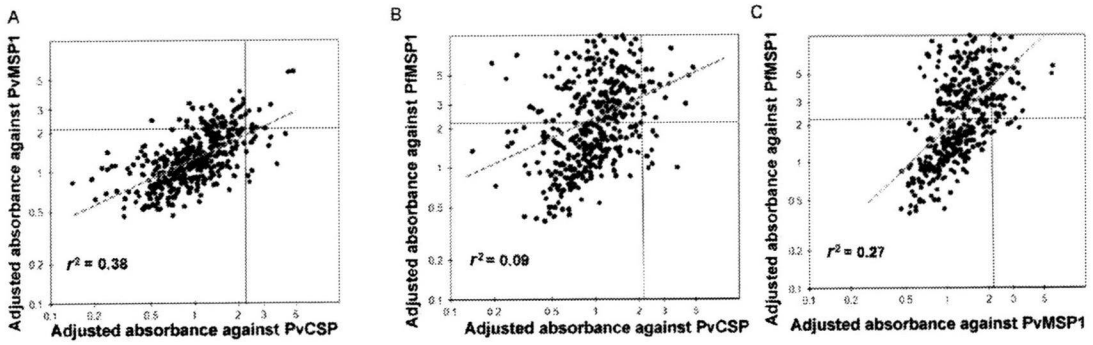


Figure 2. Linear regression analysis of the association between the adjusted absorbances against the *Plasmodium vivax* antigens *P. vivax* merozoite surface protein 1 (PvMSP1) and *P. vivax* circumsporozoite protein (PvCSP) *(A)*, the *Plasmodium falciparum* antigen *P. falciparum* merozoite surface protein 1 (PfMSP1) and PvCSP *(B)*, and PfMSP1 and PvMSP1 *(C)* for 409 individuals from Pointe-Noire, Republic of the Congo. Coefficient of determination values (r^2) for each antigen pair are shown on the graph, and linear regression lines are denoted by dashed gray lines. The solid horizontal and vertical lines denote the positive cutoff values for each antigen.

PvMSP1 (Ser_{72} to His_{432} [based on the *SalI* sequence {GenBank accession number PVX_099980}]). The third protein, *Plasmodium falciparum* merozoite surface protein 1 (PfMSP1) recombinant protein, was an *E. coli*–expressed recombinant protein of N-terminal blocks 1–6 (M1/6) of the *P. falciparum* MAD20 sequence [10]. A detailed description of the ELISA protocol is provided in the Appendix.

To adjust for interplate variations, adjusted absorbances were obtained by dividing the mean optical density (OD) value of 2 repeats for each individual sample by the mean OD value for the same 4 nonexposed individuals assayed on the same 96-well plate (under identical conditions). Samples with adjusted OD values (+3 standard deviations) that were greater than the mean value for 30 nonexposed serum samples were considered to be positive for antibodies to the antigen tested. In the case of a positive result for PvCSP or PvMSP1, blood samples from the same patients were used for extraction of host and parasite DNA for parasite species typing and determination of host Duffy status.

Results. Figure 1 shows the results of ELISAs performed on the 409 samples collected from patients presenting to Mbota health center in Pointe-Noire, Republic of the Congo. For 25 (6%) of these samples, adjusted anti-PvCSP absorbance readings were greater than the mean value (+3 standard deviations) for 30 serum samples obtained from nonexposed individuals and were therefore considered to be positive for antibodies to this protein. For 39 (10%) of the samples, adjusted absorbance readings were greater than the cutoff value noted for PvMSP1. A total of 197 individuals (48%) were found to be positive for antibodies to PfMSP1, a *P. falciparum* antigen. All *P. vivax*–positive samples were independently tested twice more in duplicate, and the same positive results were obtained.

Of the 25 samples that were positive for PvCSP antibodies, 9 (36%) were also positive for antibodies to PvMSP1, and 16 (64%) were positive for antibodies to PfMSP1. Of the 39 samples that were positive for PvMSP1, 31 (79%) were also positive for PfMSP1. To investigate the possibility that there was cross-reactivity between antibodies to *P. falciparum* and *P. vivax* antigens, correlation and linear regression analyses were performed for the antigen pairs PvCSP/PvMSP1, PvMSP1/PfMSP1, and PvCSP/PfMSP1. Adjusted absorbance values were log transformed to meet the normality and homoscedasticity assumptions of the analysis, and coefficient of determination (r^2) values and linear regression lines were generated (Figure 2). There was a highly significant medium-strength positive correlation between antibody responses against PvCSP and PvMSP1 ($r^2 = 0.38$; 409 *df*; $P < .001$) but a much weaker, although still significant, low correlation between PvCSP and PfMSP1 ($r^2 = 0.09$; 409 *df*; $P < .001$). There was a stronger correlation between PvMSP1 and PfMSP1 ($r^2 = 0.27$; 409 *df*; $P < .001$), but this was also much weaker than the correlation between the 2 *P. vivax* antigens. Furthermore, serum antibody absorbance ELISA experiments performed with known positive serum samples incubated separately with *P. vivax* and *P. falciparum* MSP1 antigens showed no evidence of cross-reactivity between the respective antibodies (figure 3, which appears only in the electronic version of the *Journal*).

We assessed whether various factors (patient age or sex; pres-

This figure is available in its entirety in the online version of the *Journal of Infectious Diseases*.

Figure 3. Enzyme-linked immunosorbent assay results for known *Plasmodium falciparum* antibody– and *Plasmodium vivax* antibody–positive serum samples incubated with *P. vivax* merozoite surface protein 1 (PvMSP1) and *P. falciparum* merozoite protein surface protein 1 (PfMSP1) antigens and tested for antibody responses to PvMSP1 and PfMSP1 antigens.

Table 1. Descriptive Statistics for Adjusted Absorbencies against 3 Antigens for 409 Individuals from Pointe-Noire, Republic of the Congo

This table is available in its entirety in the online edition of *The Journal of Infectious Diseases.*

ence or absence of parasites; parasite species, if infected; and district of residence) were associated with an increased probability of seropositivity against *P. vivax* antigens. There was no association between the district of residence and the presence of antibodies to *P. vivax* or *P. falciparum* (Table 1, which appears only in the electronic version of the *Journal*). Similarly, there was no strong correlation between age and the presence of antibodies to PvCSP ($r^2 = 0.09$, 409 *df*; $P < .01$), PvMSP1 ($r^2 = 0.13$; 409 *df*; $P < .01$), and PfMSP1 ($r^2 = 0.16$; 409 *df*; $P < .01$), as determined by Spearman rank correlation tests. The presence or absence of parasites in blood, as detected by microscopy, as well as whether those parasites were *P. falciparum, Plasmodium malariae,* or *Plasmodium ovale,* was not correlated with the presence of antibodies to either *P. vivax* or *P. falciparum;* however, the numbers of *P. malariae*– and *P. ovale*–infected individuals were low ($n = 5$ and $n = 7$, respectively), precluding statistical analysis (Table 1). Interestingly, females were significantly more likely than males to be seropositive for *P. vivax* antibodies, with 45 (17%) of 269 females positive for antibodies to PvCSP or PvMSP1, or both, compared with 10 (7%) of 145 males (6.51, by χ^2 test; 1 *df*; $P = .01$). There was, however, no difference in seropositivity for *P. falciparum* antibodies between the sexes, with 137 (51%) of 269 females and 60 (41%) of 145 males having positive responses against PfMSP1 (1.81, by χ^2 test; 1 *df*; $P = .18$).

DNA was extracted from the 55 samples for which positive antibody responses against either of the 2 *P. vivax*–specific antigens were demonstrated by ELISA. *Plasmodium* species identification was performed by polymerase chain reaction (PCR), and *P. vivax* DNA was not detected in any samples. The Duffy genotype status of the 55 individuals was determined by PCR [11], and all these individuals were found to be homozygous carriers of the FY^*B^{null} allele and, thus, of the RBC Duffy-negative phenotype.

Discussion. We have shown that the serum samples from 55 (13%) of 409 individuals from Pointe Noire in the Republic of the Congo contained antibodies to the *P. vivax*–specific antigens PvCSP (25 samples [6%]), PvMSP1 (39 samples [9.5%]), or both (9 samples [2.2%]). These results suggest that *P. vivax* is transmitted in an area of west central Africa where the frequency of the Duffy-negative genotype is 95%–99% [1]. This finding goes against the current orthodoxy that *P. vivax* is not transmitted in western Africa and offers an explanation for the many cases of *P. vivax* contracted by Duffy-positive travelers in this region.

It has been established elsewhere [7] that Duffy-negative individuals who are refractory to the blood stages of *P. vivax* may develop antibodies to such antigens as CSP and MSP1, which are expressed in the preerythrocytic stages of this parasite in areas of endemicity. This finding is supported by evidence of the establishment of preerythrocytic immunity in individuals undergoing anti–blood-stage chemoprophylaxis for *P. falciparum* [12] and in mice with *Plasmodium yoelii* [13].

Although initial experiments indicated that there was no cross-reactivity between antibodies to the PvMSP1 and PfMSP1 antigens used in the present study, we did find a weak correlation between the antibody responses to the 2 species-specific versions of this antigen. There was also a very weak correlation between antibody responses to the PvCSP and PfMSP1 antigens. We do not consider, however, that these correlations are, in themselves, evidence for antigenic cross-reactivity between *P. vivax* and *P. falciparum* antigens. Indeed, if 2 species of malaria parasites are coendemic, this result is predicted from the fact that exposure to infection by one species of malaria parasite will be highly correlated with the risk of exposure to infection by other species.

Our data indicate that, in the region of study in western and central Africa, there is an endemic entity present that is inducing antibodies specific to the preerythrocytic stages of *P. vivax* in the RBC Duffy-negative human populations of the region. We suggest that this entity is most likely sporozoites of *P. vivax* itself, delivered by the local malaria vector mosquitoes. In conjunction with the frequent reports of travelers returning from western and central Africa with diagnosed *P. vivax* infections, these findings make a strong argument for the presence and continued transmission of *P. vivax* in this region. Given the very high malaria transmission intensity in this area, it is possible that the transmission of *P. vivax* is maintained within the local population by the ~1%–5% of Duffy-positive individuals who are presumed to be present in the local population.

Acknowledgments

We thank all the participants in this study who provided blood samples.

References

1. Cvalli-Sforza LL. Africa: the history and geography of human genes. Princeton, NJ: Princeton University Press, **1994**:158–94.
2. Miller LH, Mason SJ, Clyde DF, McGinniss MH. The resistance factor to *Plasmodium vivax* in blacks: the Duffy-blood-group genotype, FyFy. N Engl J Med **1976**; 295:302–4.
3. Culleton RL, Mita T, Ndounga M, et al. Failure to detect *Plasmodium vivax* in West and Central Africa by PCR species typing. Malar J **2008**;7: 174.
4. Cavasini CE, Mattos LC, Couto AA, et al. *Plasmodium vivax* infection

among Duffy antigen–negative individuals from the Brazilian Amazon region: an exception? Trans R Soc Trop Med Hyg **2007**; 101:1042–4.
5. Ryan JR, Stoute JA, Amon J, et al. Evidence for transmission of *Plasmodium vivax* among a Duffy antigen negative population in Western Kenya. Am J Trop Med Hyg **2006**; 75:575–81.
6. Rosenberg R. *Plasmodium vivax* in Africa: hidden in plain sight? Trends Parasitol **2007**; 23:193–6.
7. Herrera S, Gómez A, Vera O, et al. Antibody response to *Plasmodium vivax* antigens in Fy-negative individuals from the Colombian Pacific coast. Am J Trop Med Hyg **2005**; 73:44–9.
8. Yadava A, Sattabongkot J, Washington MA, et al. A novel chimeric *Plasmodium vivax* circumsporozoite protein induces biologically functional antibodies that recognize both VK210 and VK247 sporozoites. Infect Immun **2007**; 75:1177–85.
9. Tsuboi T, Takeo S, Iriko H, et al. Wheat germ cell-free system-based production of malaria proteins for discovery of novel vaccine candidates. Infect Immun **2008**; 76:1702–8.
10. Tolle R, Fruh K, Doumbo O, et al. A prospective study of the association between the human humoral immune response to *Plasmodium falciparum* blood stage antigen gp190 and control of malarial infections. Infect Immun **1993**; 61:40–7.
11. Olsson ML, Hansson C, Avent ND, Akesson IE, Green CA, Daniels GL. A clinically applicable method for determining the three major alleles at the Duffy (FY) blood group locus using polymerase chain reaction with allele-specific primers. Transfusion **1998**; 38:168–73.
12. Guerin-Marchand C, Druilhe P, Galey B, et al. A liver-stage-specific antigen of *Plasmodium falciparum* characterized by gene cloning. Nature **1987**; 329:164–7.
13. Belnoue E, Costa FT, Frankenberg T, et al. Protective T cell immunity against malaria liver stage after vaccination with live sporozoites under chloroquine treatment. J Immunol **2004**; 172:2487–95.

Parasitology International 58 (2009) 243–248

Contents lists available at ScienceDirect

Parasitology International

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

Enzymatic characterization of the *Plasmodium vivax* chitinase, a potential malaria transmission-blocking target

Satoru Takeo [a], Daisuke Hisamori [a], Shusaku Matsuda [a], Joseph Vinetz [b], Jetsumon Sattabongkot [c], Takafumi Tsuboi [a,d,*]

[a] Cell-Free Science and Technology Research Center, Ehime University, 3 Bunkyo-cho, Matsuyama, Ehime 790-8577, Japan
[b] Division of Infectious Diseases, University of California San Diego School of Medicine 9500 Gilman Drive, 0741 Palade Laboratories, La Jolla, CA 92093-0741, USA
[c] Department of Entomology, Armed Forces Research Institute of Medical Sciences, Bangkok 10400, Thailand
[d] Venture Business Laboratory, Ehime University, 3 Bunkyo-cho, Matsuyama, Ehime 790-8577, Japan

ARTICLE INFO

Article history:
Received 18 November 2008
Received in revised form 29 April 2009
Accepted 2 May 2009
Available online 8 May 2009

Keywords:
Chitinase
Transmission-blocking
Plasmodium vivax
Allosamidin

ABSTRACT

The chitinase (EC 3.2.1.14) of the human malaria parasite *Plasmodium falciparum*, PfCHT1, has been validated as a malaria transmission-blocking vaccine (TBV). The present study aimed to delineate functional characteristics of the *P. vivax* chitinase PvCHT1, whose primary structure differs from that of PfCHT1 by having proenzyme and chitin-binding domains. The recombinant protein rPvCHT1 expressed with a wheat germ cell-free system hydrolyzed 4-methylumbelliferone (4MU) derivatives of chitin oligosaccharides (β-1,4-poly-*N*-acetyl glucosamine (GlcNAc)). An anti-rPvCHT1 polyclonal antiserum reacted with *in vitro*-obtained *P. vivax* ookinetes in anterior cytoplasm, showing uneven patchy distribution. Enzymatic activity of rPvCHT1 shared the exclusive endochitinase property with parallelly expressed rPfCHT1 as demonstrated by a marked substrate preference for 4MU-$GlcNAc_3$ compared to shorter GlcNAc substrates. While rPvCHT1 was found to be sensitive to the general family-18 chitinase inhibitor, allosamidin, its pH (maximal in neutral environment) and temperature (max. at ~25 °C) activity profiles and sensitivity to allosamidin (IC_{50} = 6 μM) were different from rPfCHT1. The results in this first report of functional rPvCHT1 synthesis indicate that the *P. vivax* chitinase is enzymatically close to long form *Plasmodium* chitinases represented by *P. gallinaceum* PgCHT1.

1. Introduction

To complete transmission between a vertebrate host and an anopheline mosquito, *Plasmodium* male and female gametes merge in the mosquito midgut to form zygotes that elongate into the invasive motile form, the ookinete. The ookinete must traverse the chitin-containing peritrophic matrix (PM) *en route* to invading the midgut epithelium to become a sporozoite-forming oocyst. The ookinete secretes a chitinase [1] that facilitates this process, as has been shown in gene knockout studies [2,3], membrane feeding assays with a chitinase inhibitor allosamidin [4], and with chitinase-specific antibodies [5,6]. Therefore, the chitinase is a potential target for transmission blocking of malaria with chemical or immunological strategies.

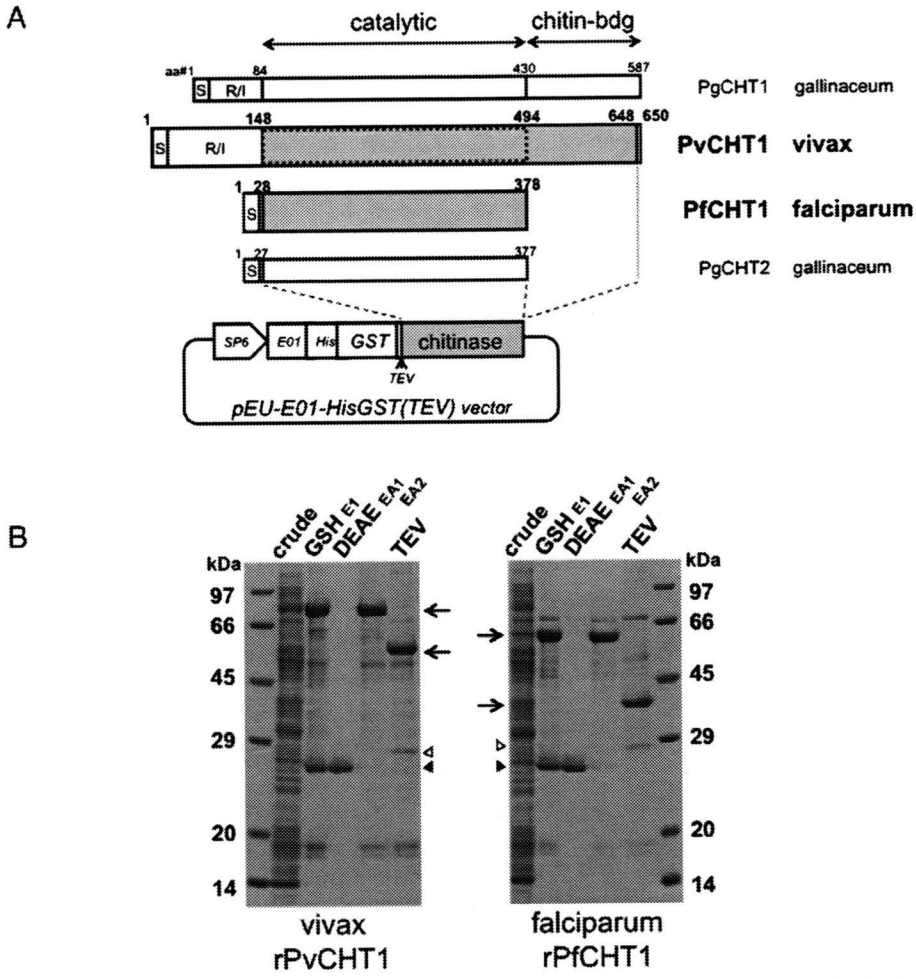
Genes encoding chitinases have been identified from several *Plasmodium* species, but functional analysis including studies on enzymatic activity has only been done with the chitinases PfCHT1 of *Plasmodium falciparum* [7] and PgCHT1 of *P. gallinaceum* [8]. *P. gallinaceum* is the only malaria parasite species in which more than one chitinase genes have been identified. Although both PgCHT1 [4,8] and PgCHT2 [5,8–10] are members of the family 18 chitinases, these enzymes differ significantly in their enzymatic properties including pH optima and quantitative sensitivity to allosamidin [8]. The short form PgCHT2 lacks two structure characteristics present in the long form PgCHT1; 1) a "repeat/insert" region to form a proenzyme domain, between N-terminal signal peptide and a catalytic domain, and 2) a putative chitin-binding domain at the C-terminus. The *P. falciparum* PfCHT1 and the chitinase of the chimpanzee malaria parasite *P. reichenowi* PrCHT1 are short forms; the enzymatic characteristics of PfCHT1 has been experimentally demonstrated to be more similar to the orthologous PgCHT2 than to PgCHT1 [7].

Detailed functional analysis including enzymatic characterization of PvCHT1, the chitinase of the other major human malaria parasite *P. vivax* is fundamental to assessing the potential of *Plasmodium* chitinase as a candidate transmission-blocking target. The deduced amino acid sequence from cloned *pvcht1* [11] indicates that this enzyme is orthologous to the long form of *Plasmodium* chitinase represented by PgCHT1. An unrooted phylogenetic tree of the conserved catalytic domain supports the clustering of PvCHT1 and PgCHT1 (TBLASTN amino acid identity values = 81%/positives = 92%) distant from another

* Corresponding author. Cell-Free Science and Technology Research Center, Ehime University, 3 Bunkyo-cho, Matsuyama, Ehime 790-8577, Japan. Tel.: +81 89 927 8277; fax: +81 89 927 9941.
E-mail address: tsuboi@ccr.ehime-u.ac.jp (T. Tsuboi).

1383-5769/$ – see front matter © 2009 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.parint.2009.05.002

clustering of PgCHT2 (I38%/P61%), PfCHT1 (I37%/P62%) and PrCHT1 (I37%/P61%) [10]. No other chitinase appears to be present in the *P. vivax* genome [12]. Indirect immunofluorescence assay (IFA) using antiserum raised against the synthetic peptide corresponding to the catalytic active site of PgCHT1 detected the protein in *P. vivax* ookinetes [11]. Since it is difficult to obtain sufficient native PvCHT1 protein for functional analysis, analysis of recombinant PvCHT1 (rPvCHT1) *in vitro* is necessary. Given the difficulties of expressing rPvCHT1 in *Escherichia coli* (J. Vinetz, unpublished data), we have utilized a cell-free expression system using wheat germ extracts [13,14] which has been demonstrated to be useful for making recombinant *Plasmodium* proteins [15,16] for obtaining functional rPvCHT1. Use of this system included production not only of the common catalytic domain but also of the chitin-binding domain specific to PgCHT1 and PvCHT1 (long form). The system was



	Protein (μg)		Total activity (*FI / min)		Yield (%)		Specific activity (*FI / min / mg protein)		Fold	
step	vivax	falciparum	vivax	falciparum	vivax	falciparum	vivax	falciparum	vivax	falciparum
GSH E1	636	357	915	153 x 10^3	100	100	1,438	429 x 10^3	1.0	1.0
DEAE EA2	80	29	315	17.8 x 10^3	34	12	3,955	608 x 10^3	2.8	1.4
TEV	33	7	240	14.7 x 10^3	26	10	7,313	2,070 x 10^3	5.1	4.8

*FI : arbitrary relative fluorescence units

Fig. 1. Synthesis and purification of recombinant *P. vivax* chitinase (PvCHT1) and *P. falciparum* PfCHT1 proteins. A. Schematic representation and translated sequence of PvCHT1 and PfCHT1 constructs in a cell-free expression vector pEU-E01-HisGST(TEV). The diagram of two *P. gallinaceum* chitinases PgCHT1 and PgCHT2 as well as the positions of signal peptide (S), repeat/insert region (R/I), and catalytic or chitin-binding domains are placed for comparison. The shaded regions are cloned to obtain recombinant (r) proteins. The second rPvCHT1 mimicking such a short form species as PfCHT1 is bordered by dotted line (see Section 2.1). The locations of SP6 promoter (SP6), translational enhancer (E01), hexa-histidine (His), glutathione S-transferase (GST), and rPvCHT1 or rPfCHT1 (chitinase) on the vector are shown. B. A representative result after sequential purification steps (see Sections 2.3 and 2.4). 2.4 μl of total cell-free synthesis products (crude) or 10 μl of the samples at various purification steps were analyzed by SDS-PAGE under reducing condition. The protein products at each step are shown by arrows. 1) A contaminant glutathione (GSH)-binding protein in the eluates E1 after GSH/GST affinity chromatography, or in EA1 after DEAE anion exchange chromatography, and 2) the remnants of the N-terminal His-GST portion and possibly AcTEV protease itself (28–29 kDa) after AcTEV treatment and dialysis, are respectively shown by closed and open triangles. The chitinase specific activities of rPvCHT1 and rPfCHT1 samples are shown in the table format below; in the eluates E1, EA2, and in the final products after AcTEV treatment and dialysis.