

BLOOD STAGE *PLASMODIUM FALCIPARUM* ANTIGENS INDUCE IMMUNOGLOBULIN CLASS SWITCHING IN HUMAN ENRICHED B CELL CULTURE

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Abstract. This study aimed to demonstrate class switch recombination (CSR) in heavy chain expressing immunoglobulin G (IgG) and IgE in human B cells after exposure to *Plasmodium falciparum* schizont lysate. Human B cells (CD20⁺CD27⁻) were cultured with crude *P. falciparum* antigen (cPfAg) and anti-CD40. On Day 4 post-exposure, total RNA from B cells was prepared and the occurrence of CSR from IgM to IgG and/or IgE was investigated by reverse transcription-polymerase chain reaction. Molecular markers to detect active CSR included enzyme activation-induced cytidine deaminase mRNA, γ and ϵ -germline transcripts (γ , ϵ -GLT), circle transcript (CT) and mature transcript (γ and ϵ -mRNA) expression. On Day 7 and Day 14 after exposure, levels of Igs in the culture supernatant were determined by enzyme-linked immunosorbent assay. Our findings showed that we could demonstrate cPfAg-stimulated B cells undergoing CSR by use of the expressed CSR markers and the increase in specific IgG and IgE indicating the potential of this approach in the study of CSR in *P. falciparum*-stimulated B cells.

INTRODUCTION

Human malaria still causes approximately 1 million deaths from 500 million malaria cases each year. *Plasmodium*

falciparum is the major cause of severe and fatal malaria with complications, eg, coma from cerebral malaria, renal failure and severe anemia (Greenwood *et al*, 2008). Antibodies and T cells are among the immune factors thought to play a role in mediating protection and also pathology (Adams *et al*, 2002; Heddi, 2002). In *P. falciparum* infection serum levels of immunoglobulin M (IgM), IgG and IgE increase in individuals living in endemic areas (Perlmann *et al*, 2000; Calissano *et al*, 2003; Bereczky *et al*, 2004; Seka-Seka *et al*, 2004; Bolad *et al*, 2005;

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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/sub-classes 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 S_{μ} 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 proceed 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 up-

stream of the targeted S region, the DNA segment between S_{μ} and the targeted S region, and C_{μ} (Xu *et al*, 1993). SC further is transcribed to produce circle transcript (CT) or chimeric I- C_{μ} product under the influence of the I_H 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 μ m 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).

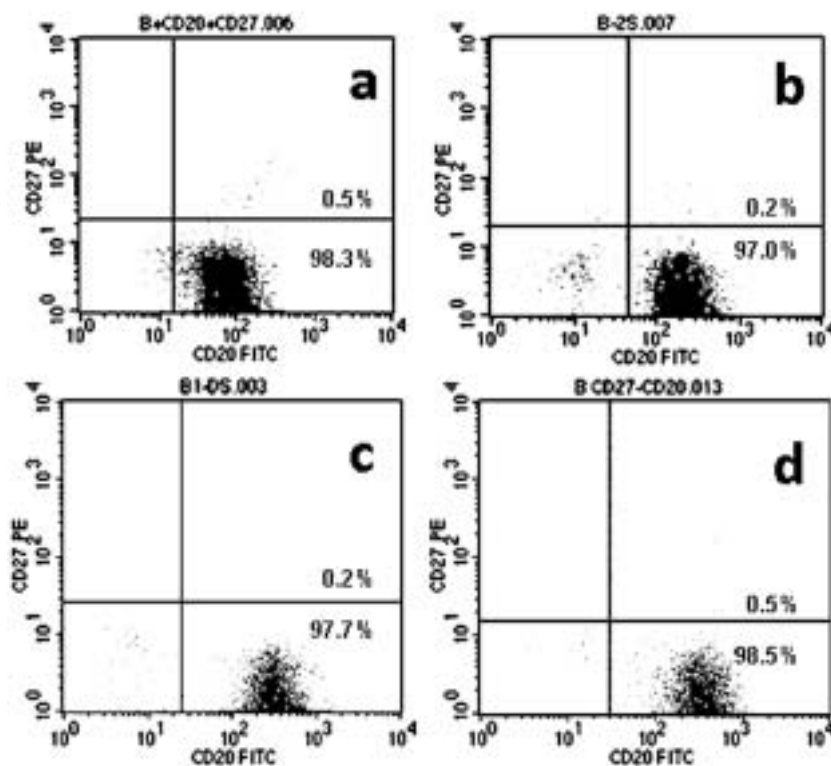


Fig 1—FACS analysis of purified B cells obtained from 4 donors. B cells were separated by anti-CD19 Dynal bead and Detachabead. Purified B cells were immunostained with FITC-conjugated mAb to CD20 and PE-conjugated mAb to CD27 and analyzed by flow cytometry. The results indicate percentage of naïve B 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 2×10^6 of stimulated and control B cells using RNeasy total RNA kit (Qiagen, Hilden, Germany). Approximately 5 µg of total RNA were reverse transcribed 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 (I γ -C μ , I ϵ -C μ and I ϵ -C γ) were amplified using seminested PCR strategy (Takhar *et al*, 2007). β -actin mRNA, a house-keeping 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' GGATGC AGAAGG AGATCA 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'-AACCTCATACAGGGCAAAAGG-3'	NM_020661
ε-GLT (Iε-Cε)	379	Iε S	5'-GGCCACACATCCACAGGC-3'	X56797
		Cε AS	5'-GGGGTGAAGTCCCTGGAGC-3'	X95746
γ-GLT (Iγ-Cγ)	500	Iγ S	5'-GATGCCAGGATGGGCACGAC-3'	S79588
		Cγ AS	5'-CCAACCTCTTTGTCCACCTTGG-3'	X04646
ε-CT (Iε-Cμ)	320	Iε S1 ^a	5'-GGGAGCTGTCCAGGAACCCGACAGAGC-3'	X56797
		Iε S2 ^b	5'-GGCCACACATCCACAGGC-3'	X56797
		Cμ AS	5'-GTTGCCGTTGGGGTGCTGG-3'	X17115
ε-CT (Iε-Cγ)	339	Iε S1 ^a	5'-GGGAGCTGTCCAGGAACCCGACAGAGC-3'	X56797
		Iε S2 ^b	5'-GGCCACACATCCACAGGC-3'	X56797
		Cγ AS	5'-CCAACCTCTTTGTCCACCTTGG-3'	X04646
γ-CT (Iγ-Cμ)	500	Iγ S1	5'-CATGACTGGATGCGGCAGAG-3'	DQ083944
		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'-ATGGTCCCCCAGGAACTCAGGT-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-

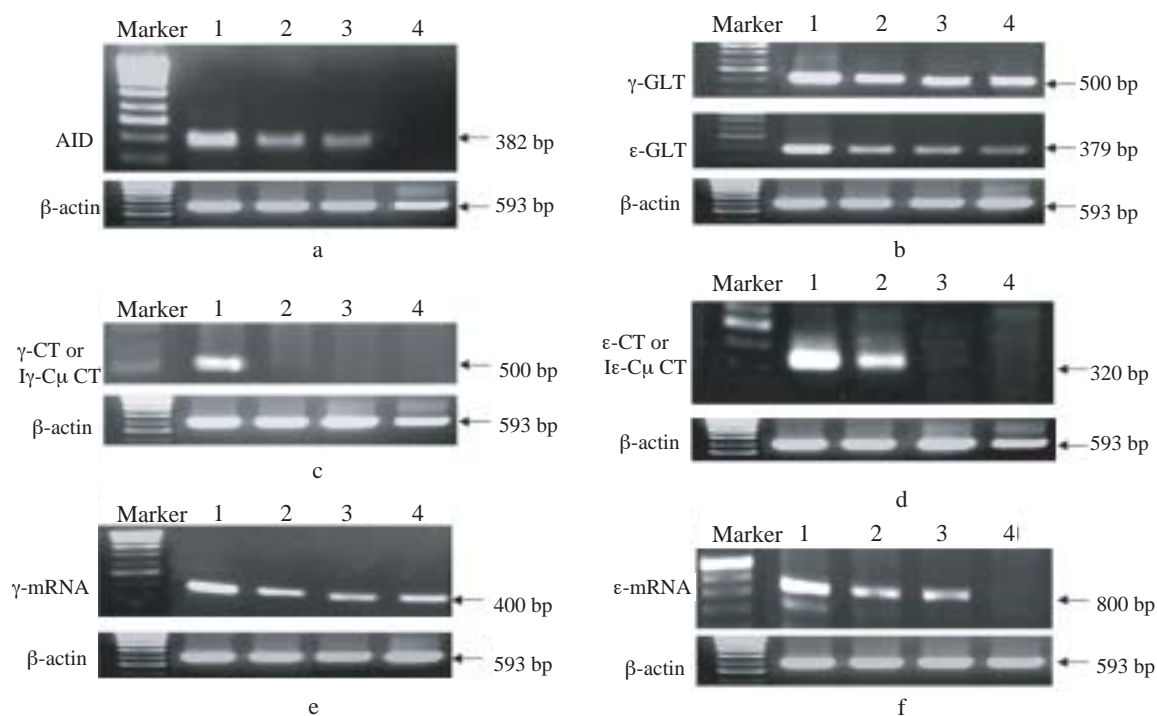


Fig 2—Expression of AID mRNA, germ line transcripts (γ -GLT and ϵ -GLT), circle transcripts (I γ -C μ CT and I ϵ -C μ CT) and mature transcripts (γ -mRNA and ϵ -mRNA). PCR products were amplified from 2×10^6 purified B cells cultured for 4 days in medium with anti-CD40 (10 μ g/ml) plus IL-4 (300 U/ml), lane 1; crude *P. falciparum* Ag (10 μ g/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 I ϵ -C γ 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.

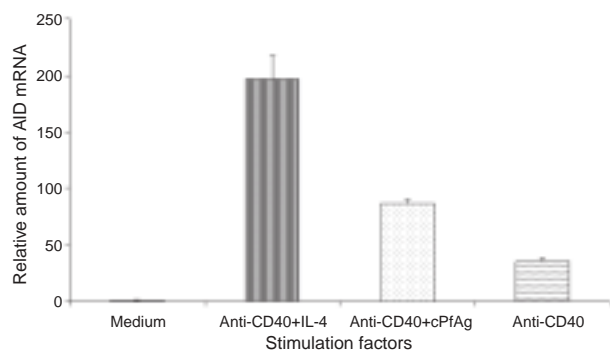


Fig 3—Relative amount of AID mRNA expression in human B cells determined by QRT-PCR. Purified B cells (2×10^6) were cultured for 4 days in medium, medium with anti-CD40 (10 μ g/ml) plus IL-4 (300 U/ml), crude *P. falciparum* Ag (10 μ g/ml), and anti-CD40 alone. The Y-axis represents amount of AID mRNA, 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 occurred 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	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-

2x10⁶ 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 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	Exp1	Exp2	Exp3	Exp4				
Medium	130.7	47.5	43.4	170.9	120.2	106.9	216.3	151.4	667.6	277.7	730.9	940.8	661.0	524.6	774.9	832.6
Anti-CD40+IL-4	667.6	277.7	730.9	940.8	661.0	524.6	774.9	832.6	307.6	270.8	440.7	439.0	424.7	442.3	552.2	461.4
Anti-CD40+cPFAg	307.6	270.8	440.7	439.0	424.7	442.3	552.2	461.4	369.3	134.7	333.9	416.3	600.5	300.7	796.6	472.0
Anti-CD40	369.3	134.7	333.9	416.3	600.5	300.7	796.6	472.0								

Purified B cells (1x10⁶ 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 (1×10^6 cells/1 ml) were cultured in medium in the presence of anti-CD40 (10 μ g/ml) plus cPfAg (10 μ g/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 ($I\gamma$ -C μ , $I\epsilon$ -C μ or $I\epsilon$ -C γ) can explain the occurrence of CSR from IgM to IgG, IgM to IgE, or IgG to IgE, respectively. As positive control, $I\epsilon$ -C μ CT was observed in B cells stimulated with anti-CD40 plus IL-4 in all 4 experiments while $I\gamma$ -C μ 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 ($I\gamma$ -C μ and $I\epsilon$ -C μ) 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 possibly 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 future 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.

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