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Plasmodium falciparum 19 kDa of merozoite surface protein-1 ($MSP\text{-}1_{19}$) expressed in *Mycobacterium bovis* bacille Calmette Guerin (BCG) is reactive with an inhibitory but not a blocking monoclonal antibody

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Abstract. Proteins on the surface of *Plasmodium falciparum* merozoites are good targets for vaccine development against malaria because they are accessible to antibodies in the plasma. The 19kDa C-terminus of merozoite surface protein-1 ($MSP\text{-}1_{19}$) has been shown to induce both inhibitory as well as blocking antibodies, the latter blocking the protective effects of the former. Inhibitory antibodies bind to $MSP\text{-}1_{19}$ and inhibit merozoite invasion of red blood cells (RBC) but the binding of blocking antibodies can prevent binding of inhibitory antibodies thereby allowing the parasite to invade RBC. We constructed a synthetic version of the $MSP\text{-}1_{19}$ of the *P. falciparum* using mycobacterium codon usage by assembly PCR. The synthetic $MSP\text{-}1_{19}$ was mutated at various sites to promote the production of inhibitory but not blocking antibodies as previously reported. The native and mutated $MSP\text{-}1_{19}$ were cloned and expressed in *Mycobacterium bovis* bacille Calmette-Guerin (BCG) and the expressions of the recombinant proteins were detected by specific monoclonal antibodies (mAbs) namely, 12.10 and 1E1 against $MSP\text{-}1_{19}$ using Western blotting. The mutated $MSP\text{-}1_{19}$ protein reacted with the inhibitory mAb, 12.10, but not the blocking mAb, 1E1, paving the way for the construction of a potential recombinant BCG (rBCG) blood stage vaccine against malaria.

INTRODUCTION

Malaria remains an important global health problem, in terms of infection, morbidity and mortality due to the increased incidence of parasite resistant to anti-malarial drugs. Approximately 247 million people are affected by malaria with nearly 1 million deaths annually (WHO, 2008). Thus vaccine development is widely recognized as one of the most efficient and cost-effective ways to improve public health and to protect humans against infectious diseases.

Since the completion of the *Plasmodium falciparum* genome project in 2002, scientists have identified hundreds of parasite proteins that could potentially form the basis for vaccine development (Greenwood, 2005). One of the three important targets for a malaria vaccine is from the asexual blood stage antigens which are aimed at inducing inhibitory antibodies towards the circulating merozoites, thus preventing them from infecting RBCs. One such blood stage candidate is the merozoite surface protein-1 (MSP-1). MSP-1 is expressed as a large and complex precursor (~200 kDa) on the merozoite surface (Patino *et al.*, 1997). At the time of merozoite invasion, the 42 kDa of merozoite surface protein-1 ($MSP\text{-}1_{42}$) is proteolytically cleaved into a soluble fragment of 33 kDa ($MSP\text{-}1_{33}$) and a 19 kDa fragment ($MSP\text{-}1_{19}$) that remains on the merozoite surface (Morgan *et al.*, 1999) and is carried into newly invaded RBCs (Blackman *et al.*, 1990). Antibodies to $MSP\text{-}1_{19}$ are important in preventing RBC invasion. Some mAbs inhibit secondary

processing of MSP-1_{19} and also inhibit RBCs invasion (Patino *et al.*, 1997). However, some antibodies specific for MSP-1_{19} are blocking antibodies. These blocking antibodies interfere with the binding of inhibitory antibodies. By competing for binding to the merozoite surface, blocking antibodies exclude inhibitory antibodies, allowing secondary processing and invasion to proceed (Blackman *et al.*, 1990; Patino *et al.*, 1997).

An important study by Uthaipibull *et al.* (2001) identified selected locations in the MSP-1_{19} molecule that could be recognized by inhibitory and blocking mAbs using a combination of site-directed mutagenesis and epitope mapping (PEPSCAN) approaches to measure the reaction of the antibodies with octapeptides of MSP-1_{19}. By selected modifications of these residues, they have engineered mutant forms of recombinant MSP-1_{19} that selectively bind to a panel of inhibitory antibodies and no longer recognized by known blocking antibodies. The modifications retained the structural integrity required for recognition by the protective antibodies and, induced the production of inhibitory antibodies (Morgan *et al.*, 1999). These interesting findings paved the way for a rational design of a more effective MSP-1_{19}-based blood-stage vaccine than the wild-type molecule.

Development of a live attenuated vaccine has been suggested to be a cost-effective method for vaccine design (Stover *et al.*, 1993). *Mycobacterium bovis* bacille Calmette-Guerin (BCG) is widely used as a live attenuated vaccine to protect against tuberculosis. Although BCG's effectiveness against tuberculosis is controversial, it has several advantages including an excellent safety record, can be administered at or any time after birth, demonstrates excellent adjuvant properties and induces long-lasting immunity (Stover *et al.*, 1993; Sterne *et al.*, 1998; Franco-Paredes *et al.*, 2006). Therefore, this mycobacterium constitutes an ideal live delivery vector for the presentation of foreign antigens to the immune system.

In the present study, we cloned and expressed the mutated version of $MSP1_{19}$ which induced inhibitory but not blocking antibodies (Uthaipibull *et al.*, 2001) in BCG. The mutated $MSP1_{19}$ was also generated in favour of mycobacterium codon usage to facilitate expression of this heterologous gene in BCG. The expression of the mutated $MSP1_{19}$ was driven by the heat shock protein 65 (hsp65) promoter of *Mycobacterium tuberculosis* and the signal peptide from the MPT63 of *M. tuberculosis*. We showed that the mutated $MSP1_{19}$ protein expressed in BCG successfully bound to the 12.10 inhibitory mAb, but not the 1E1 blocking mAb.

MATERIALS AND METHODS

Bacteria and media

The commercially available *Escherichia coli* TOP10 strain (Invitrogen, USA) was used in all initial cloning procedures. The recombinant *E. coli* TOP10 was grown in LB media supplemented with 50 µg/ml kanamycin (Sigma, USA). The *M. bovis* BCG Japan and Pasteur vaccine strains were cultured in 7H11 or 7H9 media (Difco Laboratories, USA) and rBCG was supplemented with 15 µg/ml kanamycin.

Synthesis and cloning of native and mutated MSP-1_{19}

Native and mutated versions of MSP-1_{19} were designed based on the native sequence of MSP-1_{19} of the Malaysian *P. falciparum* CAMP strain. The MSP-1_{19} was synthetically generated in favour of mycobacterium codon usage by assembly PCR (Stemmer *et al.*, 1995; Norazmi *et al.*, 1999). Briefly, 40 overlapping, 24–46 bp oligonucleotides coding for the composite DNA fragment were mixed at 250 µM concentrations each. The reaction mixture containing 2.5 µM of oligonucleotides mixture, 1x PCR buffer with 1.5 mM $MgCl_2$, 10 mM of dNTPs and 1 unit of *Taq* polymerase was used to perform the first PCR for gene assembly. The gene assembly was performed using the following PCR conditions: denaturation at 95°C for 1 min, followed by 55 cycles at 95°C for 1 min, 55°C for 1 min, 72°C for 1 min and a final incubation cycle at 72°C for 10 min.

A second PCR was performed using the following primers: sense 5'-CGGC TAGCATGAACATCAGCCAGCACCAG-3' and antisense 5'-GCTAGCCC TAGGTCAGT TCGACGACGAGCAGAA-3' primers at a concentration of 2.5 mM each. The forward primer included the *Nhe*I restriction enzyme site to facilitate cloning. The amplification of the mutated and native MSP-1_{19} was performed using the following PCR conditions; 25 cycles of 95ºC for 60s, 60ºC for 60s and 72ºC for 60s. The 314 bp PCR product was cloned into the cloning vector pCR®2.1-TOPO® (Invitrogen, USA) and confirmed by DNA sequencing. The mutated and native MSP-1_{19} were designated pNMN014 and pNMN015 respectively.

Construction of MSP-1_{19} expression vector

A shuttle plasmid, pNMN013 was constructed as follows. The heat shock protein 65 (hsp65) promoter and the MPT63 signal peptide of *M. tuberculosis* was obtained from pNMN008 (previously constructed by our group). The mycobacterium origin of replication (*myco* ORI) fragment was then excised from pUS1780 at the *Kpn*I site and cloned into the corresponding site of pNMN008. The shuttle plasmid was designated pNMN013. This shuttle plasmid also contains a kanamycin resistance gene, its ATG initiation codon and a muticloning site, which places the heterologous gene in an upstream orientation.

For cloning the MSP-1_{19} into BCG, the native and mutated MSP-1_{19} were re-cloned into corresponding sites of the shuttle vector, pNMN013. The shuttle plasmids containing the mutated and native MSP-1_{19} designated pNMN016 (Fig. 1a) and pNMN026 (Fig. 1b) respectively were transformed into BCG Japan and Pasteur by electroporation (Pulse Controller II & Gene Pulser® II-Bio-Rad, USA) with the pulse condition of 2.5 kV, 25 µF and 600 Ω as previously described (Norazmi *et al.*, 1999). The recombinant BCG clones were designated as rBCG016 and rBCG026.

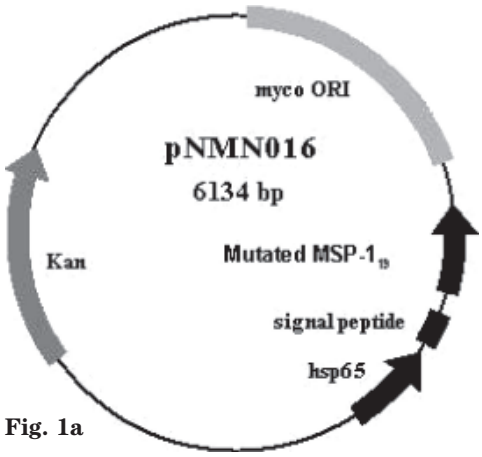

Fig. 1a

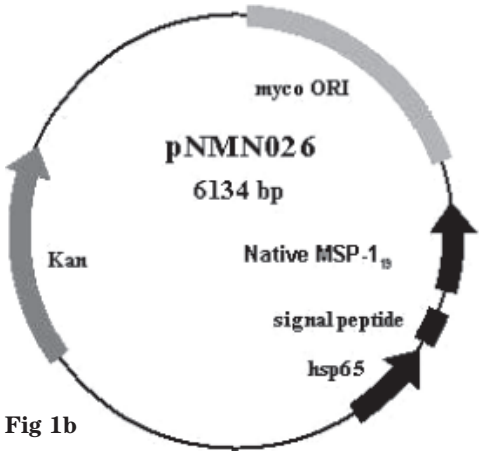

Fig 1b

Figure 1. Plasmid maps of the expression vectors used to express the MSP-1_{19} protein in *M. bovis* BCG. (a) pNMN016 expressing mutated MSP-1_{19} and (b) pNMN026 expressing native MSP-1_{19}.

Expression of MSP-1_{19} of *P. falciparum* in BCG

BCG strain Japan and Pasteur containing the recombinant plasmids, pNMN016 and pNMN026 were grown in 7H9 broth containing 10% oleic acid/albumin/dextrose/catalase (OADC) and 15 mg/ml kanamycin at 37ºC for 3 weeks. Following incubation, the rBCG cells were harvested by centrifugation at 4000 rpm for 10 minutes. For expression analysis, the rBCG cells were lysed on ice by sonication (Ultrasonic Processor XL Sonicator) for 5 minutes (15 s

on and 15 s off). The rBCG lysates were subjected to 12% polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane (Hybond C, Amersham Biosciences UK Ltd.) at a constant current of 13V for 1 hour. Western blotting was carried out by first blocking the membrane with 5% skimmed milk in 0.05% Tween 20 in PBS (PBS-T20) for 1 hour. The membrane was washed three times with PBS-T20 for 15 minutes each before probing with the specific primary antibodies against MSP-1_{19} – either the blocking 1E1 mAb or inhibitory 12.10 mAb (kind gifts from Dr. Anthony Holder, NIMR, UK) at a final concentration of 5 µg/ml. Horseradish peroxidase-conjugated IgG antibodies (Dako, Japan) was used as a secondary antibody at a dilution of 1:1000 and the reaction was detected using the chemiluminescence method according to the manufacturer's protocol (Amersham Biosciences, UK).

RESULTS AND DISCUSSION

Synthesis of mutated MSP-1_{19} by assembly PCR

The synthetic fragment of MSP-1_{19} gene was designed based on the native sequence of MSP-1_{19} of the Malaysian *P. falciparum* CAMP strain (Fig. 2). The mutated MSP-1_{19} gene encoding the 314 amino acids was generated by assembly PCR according to the method described by Norazmi *et al.* (1999). Some modifications were made to reduce the formation of "hairpin loops" and "primer dimers". To increase the expression of the mutated MSP-1_{19} in BCG, the sequence of mutated MSP-1_{19} was designed based on the frequently used codons in BCG (Dale & Patki, 1990) which is about 60% to 70% G+C compared to plasmodia genome which is about 82% A+T (Gardner *et al.*, 2002). Previous studies in cloning malarial epitopes into mycobacteria like BCG also showed variable results with regard to the expression

(1)	N	I	S	Q	H	Q	C	V	K	K	Q	C	P	E
(2)	AAC	ATC	AGC	CAG	CAC	CAG	TGC	GTC	AAG	AAG	CAG	TGC	CCG	GAG
(3)	AAC	ATC	AGC	CAG	CAC	CAG	TGC	GTC	AAG	AAG	CAG	TGC	CCG	GAG
(4)	N	I	S	Q	H	Q	C	V	K	K	Q	C	P	E
(1)	N	S	G	C	F	R	H	L	D	E	R	E	E	C
(2)	AAC	AGC	GGC	TGC	TTC	CGG	CAC	CTG	GAC	GAG	CGG	GAG	GAG	TGC
(3)	CGC	AGC	GGC	TGC	TTC	CGG	CAC	CTG	GAC	GAG	CGG	GAG	TAC	TGC
(4)	R	S	G	C	F	R	H	L	D	E	R	E	Y	C
(1)	K	C	L	L	N	Y	K	Q	E	G	D	K	C	V
(2)	AAG	TGC	CTG	TTG	AAC	TAC	AAG	CAG	GAG	GGC	GAT	AAG	TGC	GTC
(3)	AAG	TGC	CGC	TTG	AAC	TAC	AAG	CAG	GAG	GGC	GAT	AAG	TGC	GTC
(4)	K	C	R	L	N	Y	K	Q	E	G	D	K	C	V
(1)	E	N	P	N	P	T	C	N	E	N	N	G	G	C
(2)	GAG	AAC	CCG	AAC	CCG	ACC	TGC	AAC	GAG	AAC	AAT	GGT	GGC	TGC
(3)	CTG	AAC	CCG	AAC	CCG	ACC	TGC	AAC	GAG	AAC	AAT	GGT	GGC	TGC
(4)	L	N	P	N	P	T	C	N	E	N	N	G	G	C
(1)	D	A	D	A	K	C	T	E	E	D	S	G	S	N
(2)	GAC	GCC	GAC	GCC	AAG	TGC	ACC	GAG	GAG	GAC	TCG	GGC	TCG	AAC
(3)	GAC	GCC	GAC	GCC	AAG	TGC	ACC	GAG	GAG	GAC	TCG	GGC	TCG	AAC
(4)	D	A	D	A	K	C	T	E	E	D	S	G	S	N
(1)	G	K	K	I	T	C	E	C	T	K	P	D	S	Y
(2)	GGC	AAG	AAG	ATC	ACC	TGC	GAG	TGC	ACC	AAG	CCG	GAC	TCG	TAC
(3)	GGC	AAG	AAG	ATC	ACC	TGC	GAG	TGC	ACC	AAG	CCG	GAC	TCG	TAC
(4)	G	K	K	I	T	C	E	C	T	K	P	D	S	Y
(1)	P	L	F	D	G	I	F	C	S	S	S	N		
(2)	CCG	CTG	TTC	GAC	GGC	ATC	TTC	TGC	TCG	TCG	TCG	AAC		
(3)	CCG	CTG	TTC	GAC	GGC	ATC	TTC	TGC	TCG	TCG	TCG	AAC		
(4)	P	L	F	D	G	I	F	C	S	S	S	N		

Figure 2. The amino acid sequence of MSP-1_{19} designed in favour of mycobacteriums codon usage is indicated (1&2) and the locations of the residues and the modifications made within MSP-1_{19} is shown (3&4).

and immunogenicity of the recombinant mycobacterial clones (Matsumoto *et al.*, 1998; Norazmi *et al.*, 1999; Zheng *et al.*, 2003). Norazmi *et al.* (1999) showed that mycobacterium codon-optimized MSP-1_{19} of *P. falciparum* induced higher protein expression than the native gene when they were expressed in *M. smegmatis*. In the present study, approximately one in three nucleotides was changed to optimize the codon bias, and the G+C content was raised from approximately 33% to approximately 61% in the optimized sequences. Moreover, specific modifications were made to include specific mutations at various sites of MSP-1_{19} (Fig. 2) to induce the production of inhibitory but not the blocking antibodies as previously described by Uthaipibull *et al.* (2001). According to the study, 4 individual single amino acid substitutions in MSP-1_{19}, namely Asn15→Arg, Glu27→Tyr, Leu31→Arg and Glu43→Leu were made to abolish the binding of the blocking mAbs 7.5, 2.2, 1E1 and 111.4; but did not affect the binding of the inhibitory mAbs 12.8, 12.10 and 5B1 and neutral mAb 2F10. Hence, in the present study, these modifications were made to prevent the induction of blocking antibodies without affecting those of inhibitory antibodies.

Cloning and expression of MSP-1_{19} in BCG

The mutated and native MSP-1_{19} were cloned separately into the shuttle plasmid, pNMN013, to induce high expression of the malarial protein in BCG. To determine the expression of MSP-1_{19} in BCG, pNMN016 and pNMN026 were transformed into the BCG Japan and Pasteur strains. After 3 weeks, clones characterized by white and dry colonies were observed and the CFU was determined for each rBCG plate (Table 1). The BCG Japan strain produced a 10-fold higher CFU value compared to the BCG Pasteur strain. The difference in the growth and expression levels of various sub-strains of BCG has previously been demonstrated by Wiker *et al.* (1996). The genetic basis for these differences has been suggested to be due to a mutation in some BCG strains in the positive regulator of mpb83/mpb70 (Charlet *et al.*, 2005). Therefore for subsequent experiments, the BCG Japan strain was used.

In this study, the expression of MSP-1_{19} was driven hsp65 promoter and MPT63 signal peptide. The presence of MSP-1_{19} in BCG was first verified by PCR before Western blotting was performed. The rBCG016, rBCG026 and control groups (BCG and rBCG013) were then subjected to Western blotting, and probed with the inhibitory mAb 12.10 and the blocking mAb 1E1. Fig. 3 shows a protein band with a molecular weight of 19 kDa which indicated the presence of MSP-1_{19}. The expression of the mutated MSP-1_{19} by rBCG016 was successfully detected by the 12.10 inhibitory mAb but not the 1E1 blocking mAb. As expected rBCG026 reacted with both 12.10 and 1E1 mAbs. It has been previously suggested that the native conformation of the mutated MSP-1_{19} is crucial for the protective immune response (Ling *et al.*, 1994; Sachdeva *et al.*, 2004). In the present study, the conformational integrity of mutated MSP-1_{19} protein was established on the basis of its reactivities with inhibitory 12.10 mAb but not with the blocking 1E1 mAb. Thus the mutated MSP-1_{19} protein has resulted in reactivity with its specific mAb suggesting correct refolding of the recombinant protein to its native conformation.

Many studies have shown the expression of heterologous genes in BCG using various expression systems based on several promoters and signal sequences (Matsumoto *et al.*, 1998; da Cruz *et al.*, 2001; Wang *et al.*,

Table 1. CFU/mg DNA of rBCG on 7H11 media after 3 weeks of incubation period

rBCG	Strain	CFU/mg DNA
rBCG013	Pasteur	2.8×10^4
rBCG013	Japan	3.5×10^5
rBCG016	Pasteur	5.6×10^3
rBCG016	Japan	5.8×10^4
rBCG026	Pasteur	4.7×10^3
rBCG026	Japan	3.2×10^4

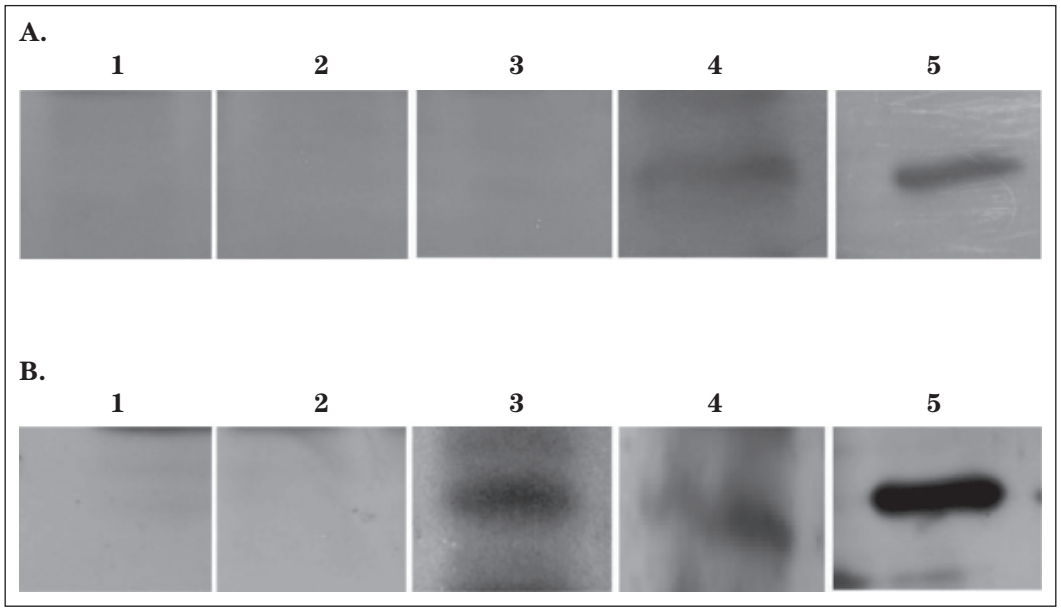

Figure 3. Western blot analysis of rBCG using 2 different antibodies, Panel A, 1E1 blocking mAb and Panel B, 12.10 inhibitory mAb. Lane 1, parent BCG (negative control); lane 2, shuttle vector (negative control); lane 3, rBCG016 expressing mutated MSP-1_{19}; lane 4, rBCG026 expressing native MSP-1_{19}; lane 5, purified MSP-1_{19} (positive control).

2007). The hsp65 promoter and MPT63 signal peptide of *M. tuberculosis* have been widely used in many research areas (Miller & Shinnick, 2000). However, limitations have been reported regarding the secretion and exportation of the recombinant proteins through the mycobacterial cell wall (Himmelrich *et al.*, 2000; Nascimento *et al.*, 2000, 2009). In this study, the MSP-1_{19} was expressed at detectable levels in the cell extract of rBCG but not detected in the culture supernatant; indicating that the antigen was localized in the cell cytoplasm. The result indicated that the use of hsp65 promoter and MPT63 signal peptide could induce expression of MSP-1_{19}, however translocation of the expressed protein may probably be blocked by the thick cell wall components of the BCG (Himmelrich *et al.*, 2000; Nascimento *et al.*, 2000). Even though previous studies had reported that the large protein size attributed to the failure of protein transportation, however it was also reported that other factors may also block the protein translocation through the mycobacterial cell wall (Nascimento *et al.*, 2000; Dennehy & Williamson, 2005).

In conclusion, these results demonstrated that specific modifications made to MSP-1_{19} protein produced strong reactivity with the 12.10 inhibitory mAb and abolished the binding of 1E1 blocking mAb. This suggests that rBCG016 expressing the mutated MSP-1_{19} protein possessed the correct conformation for the binding of inhibitory antibodies. Thus, a live candidate vaccine based on the mutated MSP-1_{19} protein expressed in BCG could be a potential developmental strategy for a malaria vaccine.

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