Molecular Cloning and Sequencing of the Circumsporozoite Protein Gene from \textit{Plasmodium falciparum} Strain FCC-1/HN and Expression of the Gene in Mycobacteria

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\textit{Mycobacterium bovis} bacillus Calmette-Guérin (BCG) has been used as a live bacterial vaccine to immunize more than 2 billion people against tuberculosis. In an attempt to use this vaccine strain as a vehicle for protective antigens, the \textit{Plasmodium falciparum} gene from strain FCC-1/HN encoding circumsporozoite protein (CSP) was amplified from the \textit{P. falciparum} genome, sequenced, and expressed in \textit{M. bovis} BCG under the control of an expression cassette carrying the promoter of heat shock protein 70 (HSP70) from \textit{Mycobacterium tuberculosis}. The recombinant shuttle plasmid pBCG/CSP was introduced into mycobacteria by electroporation, and the recombinant mycobacteria harboring pBCG/CSP could be induced by heating to express CSP; the molecular mass of recombinant CSP was about 42 kDa. This report of expression of the almost-full-length \textit{P. falciparum} CSP gene in BCG provides scientific evidence for the application of the HSP70 promoter in expressing a foreign gene in BCG and in development of BCG as a multivalent vectoral vaccine for malaria.

\textit{Mycobacterium bovis} bacillus Calmette-Guérin (BCG) has many advantages for the development of a recombinant polyvalent vaccine vector expressing antigens from a wide variety of pathogens, particularly those in which cell-mediated immunity is important for protection (9). \textit{M. bovis} BCG is an attenuated \textit{M. bovis} strain which has been used without major side effects to vaccinate more than 3 billion people; it is inexpensive to produce, can be given as a single dose at birth, and confers long-term immunity. \textit{M. bovis} BCG is a strong immunostimulant and has been used as an adjuvant in various protocols of immunization (23).

The recent development of genetic tools for mycobacteria has enabled the cloning of foreign genes in fast-growing mycobacteria such as \textit{Mycobacterium smegmatis} mc\textsuperscript{155} and in \textit{M. bovis} BCG. Various \textit{Escherichia coli}-mycobacterium shuttle plasmids which stably replicate in mycobacteria have been constructed, and foreign genes have been cloned in these vectors and shown to be expressed in \textit{M. smegmatis} mc\textsuperscript{155} and \textit{M. bovis} BCG. Cellular and humoral immune responses directed to some heterologous proteins were detected in mice immunized with different BCG recombinant strains expressing the corresponding genes (10, 17, 21).

Malaria, a disease caused by protozoan parasites of the genus \textit{Plasmodium}, is one of the most dangerous infectious diseases affecting human populations. Approximately 300 to 500 million people are infected annually, and 1.5 to 2.7 million lives are lost to malaria each year, with most deaths occurring among children in sub-Saharan Africa (24). Of the four species that cause malaria in humans, \textit{Plasmodium falciparum} is the greatest cause of morbidity and mortality. The resistance of the malaria parasite to drugs and insecticides has resulted in a resurgence of malaria in many parts of the world and a pressing need for a vaccine and new drugs.

Direct injections of radiation-attenuated sporozoites and immunization with the bites of irradiated infected mosquitoes have long been known to give both animals and humans excellent protection against subsequent viable challenge (4, 11, 15). This protection is mediated in part by antibodies, some of which are directed against the repeat region of the circumsporozoite protein (CSP), which covers the sporozoite surface (14). On the other hand, animal experiments have indicated that \textit{P. falciparum} sporozoites induce CSP-specific CD8\textsuperscript{+} cytotoxic T lymphocytes (CTL) (13) and that T cells alone are sufficient for sporozoite-induced immunity in mice (3). More recently, cloned CTL cell lines directed against the \textit{Plasmodium berghei} CSP have been shown to passively transfer protection against challenge (18). Also, various B- and T-cell epitopes have been mapped along the \textit{P. falciparum} CSP, and a CTL response against CSP has been detected in most seropositive donors (16). These results strongly suggest that the use of live recombinant vehicles might adequately present the antigen to the immune system.

In this study, the almost-full-length CSP coding gene, amplified from the \textit{P. falciparum} genome and sequenced, has been cloned into pBCG2100 under the control of the promoter of heat shock protein 70 (HSP70) from \textit{Mycobacterium tuberculosis}, yielding the recombinant shuttle plasmid pBCG/CSP, which enables an efficient expression of the \textit{P. falciparum} CSP gene in \textit{M. smegmatis} mc\textsuperscript{155} and \textit{M. bovis} BCG. We surmise that recombinant \textit{M. bovis} BCG expressing CSP might induce the destruction of infected cells which express CSP at an early stage of \textit{P. falciparum} infection, before they can release new sporozoites; this would be a desirable feature of a vaccine preparation.

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MATERIALS AND METHODS

Proliferation and preparation of parasite. The FCC-1/HN strain of *P. falciparum* was propagated and collected as previously described (22).

Bacterial strains and plasmids. The clone step was performed in *E. coli* DH5α (Pharmacia, Uppsala, Sweden). *M. smegmatis* mc2155 and the *Mycobacterium-E. coli* shuttle vector pBCG2100 were kindly provided by Huangfu Yongmu, and *M. bovis* BCG (Denmark strain) was purchased from the Beijing Institute of Biological Products (Beijing, People’s Republic of China).

Media and growth conditions. *E. coli* DH5α was cultivated in liquid under agitation or in solid Luria-Bertani medium at 37°C, and *M. smegmatis* mc2155 and *M. bovis* BCG were cultivated in liquid Middlebrook 7H10 medium (Difco Laboratories, Detroit, Mich.) supplemented with 10% Middlebrook 7H10 enrichment ADC (albumin-dextrose-catalase complex) (Difco Laboratories) and 0.05% Tween 80 (M-ADC-TW broth) with shaking at 37°C. For screening the recombinants, the transformed BCG was plated on an M-ADC-TW agar plate containing cycloheximide (100 μg ml⁻¹) (Sigma Chemical Co.) to prevent mold contamination during a long incubation period. When appropriate, media were supplemented with kanamycin (10 μg ml⁻¹).

Construction of expression vector. pBCG2100 is a 5.6-kb extrachromosomal plasmid designed for expression of foreign genes. The plasmid contains a mycobacterial plasmid origin of replication, the *E. coli* origin of replication, and the neo gene, conferring kanamycin resistance. The expression site is under the control of the *M. tuberculosis* HSP70 promoter and contains a multiple cloning site. The DNA manipulation was performed by using standard protocols as described by Sambrook et al. (19). Briefly, a pair of primers (sense primer, 5'-TCGAGGATCCATGCATACGGATCAGTGACCTGTA-3'; antisense primer, 5'-CGGGATCCATGATAGCTACTTACG-3') was designed according to the CSP-encoding sequence of strain 837 (Thailand strain) (12), with the *BamH* I site and start codon ATG inserted into the 5'-primer and the *KpnI* site and stop codon inserted into the 3'-primer; then, the CSP gene fragment was amplified by

FIG. 1. Nucleotide sequence of the CSP gene from FCC-1/HN. The nucleotides different from those in the CSP gene of strain 837 are boxed.
PCR from the genome of \textit{P. falciparum} strain FCC-1/HN (from Hainan Province of the People's Republic of China). It spanned conserved region I, the central immunodominant repeat region, the variable region behind the repeat region, and conserved region II. After purification, the resulting CSP gene fragment was digested with restriction enzymes \textit{Bam}HI and \textit{Kpn}I (Pharmacia) and ligated with pBCG2100, which was digested with the same enzymes, by T4 DNA ligase (Pharmacia), yielding the recombinant shuttle vector pBCG/CSP. The recombinant pBCG/CSP was transformed into \textit{E. coli} DH5α. Positive clones were screened by kanamycin resistance and identified by PCR and digestion with restriction enzymes.

Seqeuncing the CSP gene fragment. The recombinant pBCG/CSP identified by kanamycin resistance and enzyme digestion and purified by an extraction kit was sequenced in two directions on a Perkin-Elmer Applied Biosystems Division model 373A automated DNA sequencer using the primers listed above. The nucleotide sequence was determined by the dideoxynucleotide chain termination method.

**FIG. 2.** Comparison of the deduced amino acid sequences of CSPs of strains FCC-1/HN and 837. The different amino acids are boxed.

**FIG. 3.** SDS-PAGE of CSP expressed in BCG. Lane M, molecular mass markers (daltons); lane 1, BCG containing plasmid pBCG2100; lanes 2 to 4, recombinant BCG containing plasmid pBCG/CSP; lane 5, BCG control.

**FIG. 4.** Western blot of CSP expressed in BCG. Lanes 1 and 2, recombinant BCG containing plasmid pBCG/CSP; lane 3, BCG containing plasmid pBCG2100; lane 4, BCG control. The molecular mass marker is in daltons.
Electroporation of mycobacteria. Electroporation of mycobacterial cells (second-passage BCG) was carried out by using a Gene Pulser electroporator (Bio-Rad Laboratories, Richmond, Calif.), according to the instructions of the manufacturer. Briefly, cultures of M. smegmatis mc²155 and M. bovis BCG were grown in M-ADC-TW broth at 37°C in a shaking incubator. The cells were harvested when they reached an A₆₀₀ of 0.5 to 1.0, were washed in 10% glycerol three times, and then resuspended at 3 × 10⁶ CFU ml⁻¹ and allowed to stand on ice for 30 min. A 200-μl aliquot was then placed into an 0.2-cm-wide cuvette together with 1 μg of plasmid DNA and subjected to a 1,250-V pulse at 25 μF and 1,000 Ω of resistance. Cells were afterwards immediately diluted into 2 ml of liquid medium, preheated at 37°C, and incubated at the same temperature (3 h for M. smegmatis mc²155 and overnight for BCG) before being plated onto solid medium supplemented with kanamycin. Transformsants, selected for kanamycin resistance, appeared after 3 to 5 days for M. smegmatis mc²155 and after 3 to 4 weeks for BCG and were strained and screened for the presence of recombinant plasmid by PCR. Electroporations using 1 μg of plasmid DNA usually yielded about 2 × 10⁶ CFU for M. smegmatis mc²155 and 5 × 10⁵ CFU for BCG. The kanamycin-resistant transformsants were subcultured in M-ADC-TW broth containing kanamycin (10 μg ml⁻¹).

SDS-PAGE and Western blot analysis of recombinant proteins. In order to detect the synthesis of CSP, mycobacterial recombinants were grown in liquid medium with kanamycin. Ten milliliters of each culture was harvested at mid-log phase by centrifugation. The cells were resuspended in 1 ml of PBS (20 mM KPO₄, pH 7.5), 0.15 M NaCl supplemented with 0.1% Tween 80. Cells were sonicated for 15-s intervals (15 s on and 15 s off) for 5 min. Then, 1 ml of double-concentration sample buffer, containing 0.1 M Tris-Cl, 0.2 M dithiothreitol, 4% sodium dodecyl sulfate (SDS), 0.2% bromophenol blue, and 20% glycerol was added, and the extracts were boiled for 5 min, clearing the lysate by microcentrifugation. The cleared lysates were then subjected to polyacrylamide gel electrophoresis (PAGE) in the presence of SDS and with a 12% polyacrylamide gel as described by Sambrook et al. (19). After electrophoresis, the proteins were transferred onto nitrocellulose sheets (19). Immunodetection of CSP was achieved using polyclonal antibodies from malaria patient sera. The immunoblots were developed with goat anti-human horseradish peroxidase-conjugated antibodies (The Shanghai Institute of Biological Products, Shanghai, People’s Republic of China). Diaminobenzidine (Sigma) was used as a substrate and prepared according to the manufacturer's instructions. The substrate reaction was stopped with 20 mM EDTA.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper are available in the EMBL, GenBank, and DDBJ databases under accession no. AF315469.

RESULTS

Nucleotide and deduced amino acid sequences of the CSP gene. Both strands of the DNA containing the CSP gene were sequenced as described in Materials and Methods. The nucleotide sequence of the CSP gene is shown in Fig. 1. There are 47 nucleotides different from those of strain 837, and the sequence reveals 95.9% identity to strain 837. The deduced amino acid sequence is shown in Fig. 2 and compared with that of strain 837. The amino acid sequences for strains FCC-1/HN and 837 show 97.9% homology with eight differing amino acids. The theoretical molecular mass of the protein encoded by the cloned CSP gene was calculated to be 42.5 kDa.

Expression of CSP in M. smegmatis and M. bovis BCG. A gene encoding P. falciparum CSP was introduced into M. smegmatis and M. bovis BCG to examine their ability to express foreign DNA. The gene encoding the 42-kDa antigen P. falciparum CSP was chosen because it is a well-characterized target of the immune response in persons with malaria (7). The CSP DNA, which was amplified by PCR and identified by sequencing, was inserted into the unique BamHI and KpnI sites of Mycobacterium-E. coli shuttle vector pBCG2100 containing the HSP70 promoter to create pBCG/CSP. M. smegmatis and M. bovis BCG were transformed with plasmid pBCG/CSP, and cells were plated on medium containing 10 μg of kanamycin ml⁻¹.

Lysates of M. smegmatis and M. bovis BCG were prepared, subjected to SDS-PAGE, transferred to nitrocellulose, and probed with the polyclonal antibodies from malaria sera. The results (Fig. 3 to 6) showed that the CSP antigen could be expressed in both M. smegmatis and M. bovis BCG with a band of about 42 kDa corresponding to the expected molecular mass deduced from the gene encoding CSP.

DISCUSSION

In this study, we demonstrated that the cloned DNA sequence encoding CSP from P. falciparum FCC-1/HN shares 95.9% identity with the sequence encoding CSP from strain 837. Of 47 nucleotide differences, only 11 changes result in eight amino acid substitutions, while others are silent substitutions, so that the strains share 97.9% identity in amino acid sequence. Of the eight amino acid changes, there are six changes among nonrepeat regions, but the resulting amino acids are the same as those of the other strain (5). Although there are two changes in the repeat region, this changes the repeat unit from NNP to NVDP, the former having more repeat numbers than the latter. In short, the nucleotide and amino acid sequences of FCC-1/HN are highly homologous with those of strain 837, the two strains being adjacent geographically.

FIG. 6. Western blot of CSP expressed in M. smegmatis mc²155. Lane 1, recombinant M. smegmatis mc²155 containing plasmid pBCG/CSP; lane 2 and 3, M. smegmatis mc²155 containing plasmid pBCG2100. The molecular mass marker is in daltons.
To obtain a higher level of expression of CSP in mycobacteria, its coding sequence was cloned under the control of the stress response promoter from *M. tuberculosis*. As one of our aims was to induce an immune response to CSP after inoculation of mice with *M. bovis* BCG strains harboring the CSP gene, it was essential to clone CSP under the control of a promoter active when *M. bovis* BCG replicates in the macrophage. Buchmeier and Heffron reported that expression of a *Salmonella enterica* serovar Typhimurium gene under intracellular growth conditions showed that several HSPs are highly expressed upon infection of macrophages (2). The heat shock mycobacterial stress gene products have been demonstrated elsewhere to be dominant antigens (25). This suggests that transcription from the stress gene promoter might be efficient when *M. bovis* BCG grows intracellularly. Based on these observations, antigens were cloned under the control of the promoter of HSP70 from *M. tuberculosis*. The amplified almost full-length CSP was inserted into pBCG2100 downstream of the promoter. The resulting plasmid was electroporated into *M. smegmatis* and *M. bovis* BCG, and the results of SDS-PAGE and Western blotting show that the gene encoding the CSP antigen of *P. falciparum* could be expressed at detectable levels.

To date, CSP is the only malaria antigen that, when used as a subunit vaccine, has conferred protection against experimental sporozoite challenge in human volunteers (1, 6, 8), and CSP vaccine candidates have shown promise in clinical trials (20).

In conclusion, the data presented here show that the mycobacterial HSP70 promoter functions efficiently when cloned upstream from foreign DNA and that the two mycobacterial species *M. smegmatis* and *M. bovis* BCG constitute appropriate hosts for expression of the CSP from *P. falciparum*. These results encourage the testing of recombinant BCG vaccines of CSP, possibly with additional *Plasmodium* antigens, in appropriate animal models for protective immunity and further in clinical trials of immunotherapy and immunoprophylaxis in humans.

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