Vaccine 29 (2011) 7811–7817

Contents lists available at ScienceDirect

Vaccine

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

A polyvalent hybrid protein elicits antibodies against the diverse allelic types of block 2 in *Plasmodium falciparum* merozoite surface protein 1

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ARTICLE INFO

Article history:
Received 23 December 2010
Received in revised form 18 July 2011
Accepted 23 July 2011
Available online 4 August 2011

Keywords:
Antigenic polymorphism
Plasmodium falciparum
Malaria vaccine
Merozoite

ABSTRACT

Merozoite surface protein 1 (MSP1) of *Plasmodium falciparum* has been implicated as an important target of acquired immunity, and candidate components for a vaccine include polymorphic epitopes in the N-terminal polymorphic block 2 region. We designed a polyvalent hybrid recombinant protein incorporating sequences of the three major allelic types of block 2 together with a composite repeat sequence of one of the types and N-terminal flanking T cell epitopes, and compared this with a series of recombinant proteins containing modular sub-components and similarly expressed in *Escherichia coli*. Immunogenicity of the full polyvalent hybrid protein was tested in both mice and rabbits, and comparative immunogenicity studies of the sub-component modules were performed in mice. The full hybrid protein induced high titre antibodies against each of the major block 2 allelic types expressed as separate recombinant proteins and against a wide range of allelic types naturally expressed by a panel of diverse *P. falciparum* isolates, while the sub-component modules had partial antigenic coverage as expected. This encourages further development and evaluation of the full MSP1 block 2 polyvalent hybrid protein as a candidate blood-stage component of a malaria vaccine.

1. Introduction

Complex antigenic polymorphisms present a significant challenge for design of a vaccine against the malaria parasite *Plasmodium falciparum*. Although partial protection offered by the current leading malaria vaccine candidate RTS,S appears not to be compromised by limited polymorphism in the pre-erythrocytic circumsporozoite protein [1], the problem of polymorphism is likely to be more important for vaccines based on blood-stage parasite proteins that are targets of naturally acquired immunity [2,3]. The extracellular merozoite that invades erythrocytes is an important target of immunity [4], and a leading vaccine candidate is the most abundant surface component, merozoite surface protein 1 (MSP1) which is expressed as a large ~200 kDa precursor that needs to be proteolytically processed to allow merozoite maturation [5]. Antibodies to several parts of the protein can inhibit this processing [6], but most research has focused on the C-terminal region, particularly the 19 kDa C-terminal fragment MSP1-19 [7–10].

Although the N-terminal region of MSP1 has received less attention, it contains the most highly polymorphic 'block 2' sequences that group into three major allelic types (K1-like, MAD20-like, and R033-like) [11–16], with hybrid alleles occurring rarely [17,18]. The allele frequencies in endemic populations appear to be under balancing selection [12], and antibodies against the sequences have been associated with protection from malaria [11,12,14,19]. Allele-specific growth inhibition has been reported with an antibody-dependant cellular inhibition (ADCI) assay [13], although antibodies alone are not inhibitory except for a report of activity with one monoclonal antibody [20].

Previously, we demonstrated how an epitope mapping approach could be used to characterize the complex antigenic polymorphism seen in the K1-like block 2 repeat sequences, and employed this in the design of a single synthetic sequence termed the K1 Super Repeat (K1SR) [15]. Immunization of mice with this K1SR antigen elicited a broad antibody repertoire against *P. falciparum* isolates bearing diverse K1-like allelic types. Here we present the design and characterization of a polyvalent hybrid protein incorporating the K1SR sequence together with K1-like flanking block 2 sequences, T helper cell epitope sequences near the junction of blocks 1 and 2, and MAD20-like and R033-like block 2 allele sequences. To investigate the immunogenic contributions

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doi:10.1016/j.vaccine.2011.07.106

of each module that made up the final construct, five other subcomponent constructs were designed and tested for comparative immunogenicity. Antibody responses were largely dependent on the presence of the T helper cell epitopes, and showed expected combinations of allele specificity. Antibodies to the full polyvalent hybrid protein raised in both mice and rabbits displayed a broad repertoire with serological coverage against isolates of all allelic types.

2. Materials and methods

2.1. Construction of sequences encoding MSP1 block 2 polyvalent hybrid proteins

Six recombinant antigens were constructed, five of which were designed as comparative reagents (antigens 1–5, Fig. 1A and Supplementary Fig. 1) to validate the final candidate immunogen (+)T-K1SR-R033-Wellcome (antigen 6, Fig. 1A and Supplementary Fig. 1). The DNA sequence encoding each antigen was generated using a modular construction, with each module separated by restriction enzyme sites (Supplementary Fig. 1).

For constructs incorporating the K1-like 3D7 module (antigens 1 and 3, Fig. 1A), PCR products were amplified from 3D7 parasite genomic DNA using the primer pair KTPfK1F1*Bam*H1 (5′-GGGGATCCGTAACACATGAAAGTTAT-3′) and KTPfR1*Sac*1M1 (5′-GGGAGCTCGCTTGCATCAGCTGGAGG-3′). This module also included the sequence for a conserved T-cell epitope within MSP1 block 1 (T1, amino acid position 20–39: VTHESYQELVKKLEALEDAV) and a polymorphic T-cell epitope (T2, amino acid position 44–63: GLFHKEKMILNEEEITTKGA) [21], spanning the junction of blocks 1 and 2. The R033-type block 2 module was amplified from R033 parasite genomic DNA using the primer pairs KTPfR033F1*Sac*1M2 (5′-GGGAGCTCAAGGATGGAGCAAATACT-3′) and KTPfR033R1*Kpn*1M2 (5′-GGGGTACCACTTGAATCATCTGAAGG-3′). The Wellcome (MAD20-type) module was amplified from Wellcome parasite genomic DNA using the primer pair KTPfWellF1*Kpn*1M3 (5′ GGGGTACCAATGAAGGAACAAGTGGA-3′) and KTPfWellR2*Sma*1M3 (5′-GGCCCGGGTTAACTTGAATTATCTGAAGG-3′). All PCR amplifications were performed using Accuzyme High Fidelity DNA Polymerase (Bioline Ltd, London, UK) on *P. falciparum* genomic DNA isolated from cultured parasites using the QIAamp DNA blood minikit following manufacturer's instructions (Qiagen, WestSussex, UK).

The remaining three modules were commercially synthesised (GeneArt, Germany) as codon optimized sequences for *E. coli* expression and cloned into the pG4 shuttle vector. These were: (i) a 3D7 allelic block 2 module that lacked the N-terminal T cell epitopes (in antigen 4, Fig. 1A and Supplementary Fig. 1); (ii) the K1SR module [15] also lacking the N-terminal T1/T2 T-cell epitopes (in antigen 5, Fig. 1A and Supplementary Fig. 1); (iii) the K1SR module [15] integrating the N-terminal T-cell epitopes (in antigen 6, Fig. 1A and Supplementary Fig. 1).

2.2. Plasmid cloning and recombinant protein expression

All synthetic DNA products were first cloned into the pGEM-T Easy cloning vector plasmid (Promega, UK). Sequence verified DNA was excised from the relevant clones using module specific restriction sites and ligated into pGEM-T Easy vector to derive the completed recombinant constructs. The commercially synthesised modules were excised using module specific restriction sites directly from the pG4 shuttle vector and cloned onto the pGEM-T backbone to derive the relevant polyvalent constructs. All constructs were sequenced at each stage to ensure fidelity of the cloned products with ABI BIGDYE terminator v3.1 chemistry using an ABI 3730xl electrophoresis system (Applied Biosystems, UK).

Each completed coding region was excised using *BamHI*/*KpnI* restriction sites for the full polyvalent hybrid protein sequence (antigen 6), and *BamHI*/*SmaI* for the remaining 5 modular polyvalent sequences (Fig. 1A), before cloning into complementary digested sites in the pQE30 His-tag expression vector (Qiagen) for antigens 1–3 or the pET15b His-tag expression vector (Novagen) for antigens 4–6 (Fig. 1A). Each cloned recombinant plasmid was transformed into M15 [pREP4] host *E. coli* strain (Qiagen) for the pQE30 cloned products or BL21 (DE3) (Stratagene) for the pET15b cloned products. All constructs were sequenced to ensure complete fidelity.

For protein expression, isopropyl-ß-D-thiogalactopyranoside (IPTG) was added to each culture to a final concentration of 1 mM following bacterial culture growth to OD_{600} of 0.6–1.0. Bacterial cells were pelleted, resuspended in BugBuster protein extraction reagent (Novagen, Merck Chemicals International) and incubated at room temperature for 20 min on a rolling platform. Cellular debris was pelleted by centrifugation, and the histidine-tagged protein purified from each supernatant following Nickel His-tag affinity chromatography using Ni-NTA agarose (Qiagen). The stability of 50 μg batches of lyophilized full polyvalent hybrid protein was tested by incubation at −20, 4, 37 and 56 °C for a period of three weeks.

2.3. SDS PAGE and Western blot analysis

The purified polyvalent hybrid proteins were separated under reducing conditions by 12% Tris–glycine–SDS PAGE and electrophoretically transferred to nitrocellulose membrane (Whatman, UK). Western blots were probed using murine sera raised to recombinant proteins based on the individual MSP1 block 2 types [11,15]. Bound antibody was detected with horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody (DAKO), and bands visualized using 5 ml per blot of stabilized TMB (3,3′,5,5′-tetramethylbenzidine) substrate (Promega, UK).

2.4. Murine polyclonal sera

Groups of five CD-1 outbred mice were immunized (Northwick Park Institute for Medical Research, UK) with each antigen formulated in the ImjectAlum adjuvant (Perbio Science, Cheshire, UK). Each polyvalent hybrid protein was diluted with phosphate-buffered saline (PBS) to a concentration of 1 mg ml^{-1}, and 3 volumes of Imject Alum added and allowed to mix for 30 min at room temperature. Each antigen–adjuvant mixture was administered intra-peritoneally, each mouse receiving 50 μg protein per dose in a final volume of 200 μl. Three doses were administered at monthly intervals, and blood was collected before immunization and 2 weeks after each dose (on days 14, 42, and 70).

2.5. Rabbit immunizations

The purified polyvalent hybrid antigen (+)T-K1SR-R033-Wellcome (antigen 6, Fig. 1A) was used to immunize New Zealand white rabbits (Pettingill Technology Limited, UK). Five rabbits received 200 μg of purified protein intramuscularly at days 0, 14, 28, 42, 56 and 70 following a 77 day protocol with one rabbit receiving adjuvant with PBS only as a control (Freund's complete adjuvant was used on day 0 immunization, Freund's incomplete adjuvant for boosting immunizations). Test bleeds were taken on days 35, 49 and 63, final bleeds were collected on day 77.