Single-Chain Antibody Fragment Specific for *Plasmodium vivax* Duffy Binding Protein

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Malaria caused by *Plasmodium vivax* is responsible for substantial morbidity in Asia and Central and South America (19). Merozoites of *Plasmodium* must attach to and invade red blood cells (RBCs) to begin asexual reproduction of the parasite, making this brief event a critical phase in the parasite life cycle. Invasion occurs quickly through a complex, multistep process that follows a distinct sequence of events involving numerous molecules expressed on the surface of the merozoite and in the apical organelles (1, 4, 6, 7). This cascade of events represents potential targets for reducing or eliminating the blood stages of malarial parasites (21, 25, 31).

The Duffy binding protein (DBP) of *P. vivax* interacts with Duffy antigen receptor for chemokines (DARC) on the RBC during junction formation between the merozoite and RBC (1, 2, 16, 34). The *P. vivax* DBP (PvDBP) is a 140-kDa protein that belongs to a family of erythrocyte-binding proteins characterized by a functionally conserved cysteine-rich region (1, 6, 12). This cysteine-rich region is in DBP region II (DBP II), which contains the binding motifs necessary for adhering to DARC on the erythrocyte surface (9, 10, 29). The critical binding motif has been mapped to a 170-amino-acid segment between cysteines 4 and 8 in the cysteine-rich region (26, 28, 29). Studies have shown that although the cysteine residues are conserved, other regions of DBP II are highly polymorphic (3, 32, 36). However, the hypervariable region of DBP II is located on the sites remote from the DARC-binding site and does not alter the capacity of the protein to bind DARC-positive erythrocytes (28, 33).

Phage display antibodies offer a way to produce high-affinity single-chain variable fragment (scFv) derivatives of human antibodies of “natural host” origin (8). Our goal was to produce human monoclonal antibodies against the DARC-binding region of DBP II of *P. vivax* (PvRII). To do so, we constructed a combinatorial phage display library using peripheral blood mononuclear cells from three patients infected naturally with *P. vivax*. Subsequently, anti-PvRII human scFvs that had neutralizing activity against DBP binding to erythrocytes were identified. These neutralizing scFvs should prove valuable for developing both passive and active immunization strategies based on DBP.

MATERIALS AND METHODS

Constructing the human scFv phage display library. Peripheral blood mononuclear cells were collected from three patients infected by a Korean strain of *P. vivax* using Ficoll-Paque. Total RNA was extracted from each sample using TRIzol (Gibco-BRL/Life Technologies, Gaithersburg, MD). First-strand cDNA was generated using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Light- and heavy-chain genes were cloned using PCR with the primers described by Barbos et al. (5). The variable regions of the light (V<sub>L</sub>) and heavy (V<sub>H</sub>) chains were amplified separately from each cDNA and recombined in a second round of PCR. A pool of gene fusions that encoded scFvs of the V<sub>L</sub>-spacer-V<sub>H</sub> sequence was assembled. Following overlap PCR and gel purification, the amplified products were cloned into the phagemid vector. The ligated mixtures were electroporated into *Escherichia coli* ER2537 cells using Gene Pulser II (Bio-Rad Laboratories, Munich, Germany). Library phages were harvested from the culture supernatant of recombinant *E. coli* and precipitated with 20% polyethylene glycol-2.5 M NaCl, as described previously (24). The phage pellet was reconstituted in 2 ml of 1% (wt/vol) bovine serum albumin in Tris-
The scFvs were diluted with running buffer, HBS-EP buffer (0.01 M HEPES [pH 7.4], 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20 [Biacore AB]), and were injected at 30 μl/min. The rate constants of association (k_on) and dissociation (k_off) were obtained at five different scFv concentrations (range, 1 to 100 μg/ml). All assays were repeated at least three times. The dissociation constant (K_D) was calculated from the ratio of the rate constants of association and dissociation (k_on/k_off). The sensorgrams were analyzed using BIAevaluation 3.2 software. The raw data was removed with 50 mM NaOH after each measurement.

Production of soluble scFv antibody fragments. We induced scFv expression as described previously (22). Briefly, E. coli Top10F' cells were infected with selected plagues after the cells were grown to the logarithmic phase and induced with 0.1 mM IPTG overnight to produce soluble scFvs. The expressed scFvs were purified using immobilized metal affinity chromatography with Ni2+ charged HP chelating column, following the manufacturer’s protocol (Amersham-Pharmacia). The plasmid DNA used for transfection was amplified as a free plasmid MidPrep kit (QIAGEN) from inclusion bodies that had been solubilized with 8 M urea. The DNA sequencing of full-length inserts from 40 clones using PCR and BstNI digestion showed that 70% had inserts, and there were 108 independent clones. The investigators admitted to Busan Paik Hospital in August 2001. The peripheral blood mononuclear cells from P. volvax-infected patients were used to analyze the kinetic properties of the scFvs. PvRII was immobilized on a 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride-N-hydroxysuccinimide-activated CM5 sensor chip by injecting 10 μg/ml PvRII in 10 mM sodium acetate (pH 4.0) to obtain 1,000 resonance units (RU). The scFvs were diluted with running buffer, HBS-EP buffer (0.01 M HEPES [pH 7.4], 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20 [Biacore AB]), and were injected at 30 μl/min. The rate constants of association (k_on) and dissociation (k_off) were obtained at five different scFv concentrations (range, 1 to 100 μg/ml). All assays were repeated at least three times. The dissociation constant (K_D) was calculated from the ratio of the rate constants of association and dissociation (k_on/k_off). The sensorgrams were analyzed using BIAevaluation 3.2 software. The raw data was removed with 50 mM NaOH after each measurement.

Surface expression of PvDBP II in COS-7 cells. The surface expression of region II of PvDBP was carried out as reported previously, with some modifications (14). Briefly, PbDBP II was amplified by PCR using the primers PbDBP (5'-ACAATTTGGTAATGTTAGAT-3') and PbDBPR (5'-TGAATTTTCCAATGACCATC-3') and cloned into the pDE vector using the BglII and SacII sites (pDE-PvDBP). The plasmid DNA used for transfection was purified using an endonuclease-free plasmid MidiPrep kit (QIAGEN). 1 μg of DNA was used to transfect COS-7 cells in six-well plates (approximately 5 x 10⁶ cells/well) using FuGene6 (Roche). After 2 days, the transfected COS-7 cells were used for fluorescence and erythrocyte binding-inhibition assays (EBIA). Fluorescence was detected using confocal microscopy (510Meta; Carl-Zeiss, Germany).

EBIA. The EBIA was performed as described previously (14). After 48 h of transfection, COS-7 cells were incubated with various concentrations of purified scFvs for 2 h at 37°C. After washing with phosphate-buffered saline, a 10% human erythrocyte suspension of type O blood was added to each well, incubated for an additional 2 h, and washed three times with phosphate-buffered saline. Binding was quantified by counting the rosettes observed over 20 fields of view at a >100 magnification. Each experiment was performed in triplicate, and the data shown are from at least two separate experiments.

RESULTS

A human scFv antibody library was constructed using peripheral blood mononuclear cells from P. volvax-infected patients admitted to Busan Paik Hospital in August 2001. The library contained 7.85 x 10⁶ independent clones. The investigation of full-length inserts from 40 clones using PCR and BstNI digestion showed that 70% had inserts, and there were no identical digestion patterns in the inserted clones (data not shown).
In *E. coli* BL21(DE3), the recombinant PvRII accumulated mostly in inclusion bodies (Fig. 1). Purified recombinant PvRII showed a single 20.4-kDa band (Fig. 1C). The recombinant PvRII reacted with the pooled sera of the six patients with *P. vivax* malaria but didn’t react with an irrelevant antibody, anti-hepatitis B virus (HBV) pre-S1 scFv (22). After the fourth round of selection, 96 clones were analyzed for PvRII binding using ELISA (Fig. 2). Clones which show a higher optical density (OD) than anti-HBV pre-S1 scFv at least three times were considered positive. Twelve clones were selected as positive, and these were categorized into three groups according to absorbance: low, middle, and high. *E. coli* Top10F’ (amber nonsuppressant) was infected with three clones (one from each group: SFDBII12, SFDBII58, and SFDBII92), and these were expressed as soluble scFv forms by IPTG induction for affinity measurements and EBIA. The soluble scFvs were purified using the IMAC system and were about 34 kDa, as expected (Fig. 3A). A comparison with the sequences of the germ line VH genes shows that the clones use a VH III family-derived germ line segment. Alignment with the VL germ line sequences showed that these clones use VH9260 I, VH9260 III, and VH9261 I family segments. The kinetic parameters of these clones were measured using the BIAcore instrument (Fig. 3B). SFDBII92 had the greatest affinity ($K_D = 3.62 \times 10^{-9}$ M versus $K_D = 1.54 \times 10^{-7}$ M for SFDBII12 and $K_D = 1.26 \times 10^{-7}$ M for SFDBII58).

![FIG. 2. Selection of binders to PvRII. Each well of the ELISA plate was precoated with recombinant PvRII (1 μg/well), and then antibodies from each phage (10¹⁰ CFU/well) were added to each well. Bound phages were detected with horseradish peroxidase-conjugated anti-M13 mouse antibody. Anti-HBV pre-S1 scFv (22) was used as an irrelevant antibody, and pooled sera of the six patients with *P. vivax* malaria were used for the reactivity of the purified recombinant PvRII. The results are the average of triplicate assays.](image)

![FIG. 3. Purification and analysis of the binding kinetics of soluble scFvs. (A) scFvs were expressed in the form of soluble proteins without a p3 phage protein fused to the C-terminal portion of scFv. Monomeric scFvs were purified with an Ni-NTA affinity column using fast-protein liquid chromatography and subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. (B) The binding kinetics of the scFvs were measured using surface plasmon resonance on a BIAcore biosensor instrument. The graph shows an overlay plot of the sensorgrams obtained for SFDBII92 at six different concentrations (0.5 to 1 μM) against immobilized PvRII.](image)
To evaluate the biological effects of anti-PvRII scFvs, region II of PvDBP was expressed on the surface of COS-7 cells in the form of an enhanced green fluorescent protein (EGFP) fusion protein. Following transfection, we confirmed the cell surface localization of green fluorescence protein and rosette formation under a confocal microscope (Fig. 4). The erythrocyte-binding activity to the transfected cells was measured by counting the number of rosettes. The rosette count of transfected cells was 53.6 ± 3.5. Next, to determine whether the anti-PvRII scFvs blocked the adhesion of the PvDBP molecules to human erythrocytes, transfected COS-7 cells were treated with anti-PvRII scFvs and reacted with erythrocytes to form rosettes. All anti-PvRII scFvs showed inhibitory activity, and the number of rosettes decreased as the amount of scFv was increased (Fig. 5). For a negative control, anti-pre-S1 human scFv (22) was used and had no inhibitory activity. At 90 μg/ml of scFvs, SFDBII92 showed the highest inhibition (91.6% for SFDBII92, 88.4% for SFDBII12, and 80.9% for SFDBII58). The 50% inhibitory concentrations (IC50s) were 2.9 μg/ml for SFDBII12, 4.0 μg/ml for SFDBII58, and 4.5 μg/ml for SFDBII92.

**DISCUSSION**

The invasion of erythrocytes by *P. vivax* merozoites is thought to be as complex a process as the invasion of erythroy-
creased continuously with each panning. After the fourth pan-
to affect the binding affinity of PvDBP to RBCs (17).
area of endemicity of South Korea. Comparing Sal-1, Belem, dbp
have been reported using the MSP protein of Plasmodium
also been examined, including merozoite surface protein 1, reticulocu-
the DBP, which have high binding affinity to erythrocytes (9, 10, 14, 26). For P. vivax malaria, DBP is con-
sidered an important vaccine candidate antigen due to its high
binding affinity and its strong antigenicity in humans (20, 36).
The cysteine-rich region of DBP II, especially in the region
between C4 and C8, has the binding motifs necessary for ad-
herence to DARC on the erythrocytes; this region of the dbp
gene is much more variable than other regions (32). Using
site-directed mutation analysis, the DARC-binding site of DBP
II consists of conserved amino acids (33) and is located on the
surfaces distant from the hypervariable region (28). Therefore,
we decided to make neutralizing antibody fragments against
PvRII from an scFv library of naturally infected malaria pa-

tients.
Some human recombinant antibodies against Plasmodium
have been reported using the MSP protein of Plasmodium falci-
parum or the AMA protein of Plasmodium chabaudi as
targets (13, 18, 27, 30). We constructed a phage display library
from the peripheral blood of human patients, as reported pre-
viously. Roef et al. (27) showed their biological activity and
invasion inhibition using competitive ELISA with neutralizing
antibodies. Lundquist et al. (18) showed the neutralizing ac-
tivity with an antibody-dependent cellular assay. Here, we
demonstrated the neutralizing activities of recombinant anti-
body fragments using EBIA.

Two genotypes of P. vivax (SK-1 and SK-2) coexist in the
area of endemicity of South Korea. Comparing Sal-1, Belem,
PNG (Papua New Guinea), and COLV and COLT (Colombia)
isolates, DBP variations of the SK-1 strain are considered not
to affect the binding affinity of PvDBP to RBCs (17).

Using conventional panning, the output/input ratio in-
creased continuously with each panning. After the fourth pan-
ning, 96 clones were tested with phage ELISA to determine
whether they recognized the PvRII protein. Twelve positive
clones showed OD values between 0.2 and 0.8. We grouped
these positive clones into three groups, selected three clones
(SFDBII12, SFDBII58, and SFDBII92), and analyzed their
antibody and functional characteristics.

All of the heavy-chain genes of the scFvs were derived from
the V\textsubscript{H} III germ line gene, whereas the light chains were
derived from the kappa I, III, and lambda I germ line genes
(Table 1). Surface plasmon response measurements showed
that the three antibodies had affinities (K\textsubscript{D}) in the nanomolar
range. Previous work on an invasion-inhibiting circumc-
osporoite antibody (K\textsubscript{D} \(\approx\) 300 nM) (35), HBV neutralizing
antibodies (K\textsubscript{D} \(\approx\) 160 nM) (22), and human immunodeficien-
cy virus neutralizing antibodies (K\textsubscript{D} \(\approx\) 4.6 nM) (11) showed
that the SFDBII antibody series described here indeed has affinities
within a biologically relevant range. Hans et al. (15) estimated
that the binding constant of the PvRII-DARC is 8.7 nM. The
affinity of the SFDBII antibodies is lower than that of the
PvRII-DARC interaction. If an SFDBII antibody recognizes
the binding site of DARC, the affinity can be increased using
phage display techniques.

The EBIA results showed that the erythrocyte-binding inhibi-
tion activities of the anti-DBP II scFvs were correlated with
their affinities. SFDBII92 had the greatest inhibition activity
(IC\textsubscript{50} \(\approx\) 2.9 \(\mu\)g/ml versus 4.0 \(\mu\)g/ml for SFDBII12 and 4.5
\(\mu\)g/ml for SFDBII58). The neutralizing activities of SFDBIIs
are correlated with ELISA signal and affinity. These suggest
that in PvRII, the C4-to-C7 region of DBP II is a binding motif
as previously reported.

Our anti-DBP antibodies can be candidates for passive im-
munization to prevent or treat P. vivax infection. Unfortu-
nately, we cannot estimate the amount of scFv showing a neu-
tralizing effect in vivo. For therapeutic purposes, SFDBII92
should have greater affinity, producing an effective neutralizing
activity against P. vivax malaria, which can be facilitated by
using error-prone PCR, chain shuffling, or complementarity
determining region (CDR) mutagenesis of the variable regions
of SFDBII92 (23, 24).

We successfully made human antibody fragments that neu-
tralized DBP binding to the erythrocyte surface and demon-
strated the neutralizing activity of these antibodies in vitro
using an erythrocyte-binding inhibition assay.

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