

## Single-Chain Antibody Fragment Specific for *Plasmodium vivax* Duffy Binding Protein<sup>∇</sup>

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**Phage display of single-chain variable fragment (scFv) antibodies is a powerful tool for selecting important, useful, and specific human antibodies. We constructed a library from three patients infected with *Plasmodium vivax*. Panning on recombinant PvRII enriched a population of scFvs that recognized region II of the *P. vivax* Duffy binding protein (DBP). Three clones of scFvs that reacted with PvRII were selected, and their biological functions were analyzed. These scFvs inhibited erythrocyte binding to DBP. Clone SFDBII92 had the greatest affinity (dissociation constant =  $3.62 \times 10^{-8}$  M) and the greatest inhibition activity (50% inhibitory concentration  $\approx 2.9$   $\mu$ g/ml) to DBP. Thus, we demonstrated that human neutralizing antibody could be made from malaria patients using phage display and that these neutralizing scFvs should prove valuable for developing both passive and active immunization strategies based on DBP.**

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 Barbas et al. (5). The variable regions of the light ( $V_L$ )- and heavy ( $V_H$ )-chain genes were amplified separately from each cDNA and recombined in a second round of PCR. A pool of gene fusions that encoded scFvs of the  $V_L$ -spacer- $V_H$  sequence was assembled. Following overlap PCR and gel purification, the amplified products were cloned into the phagemid vector. The ligation 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-

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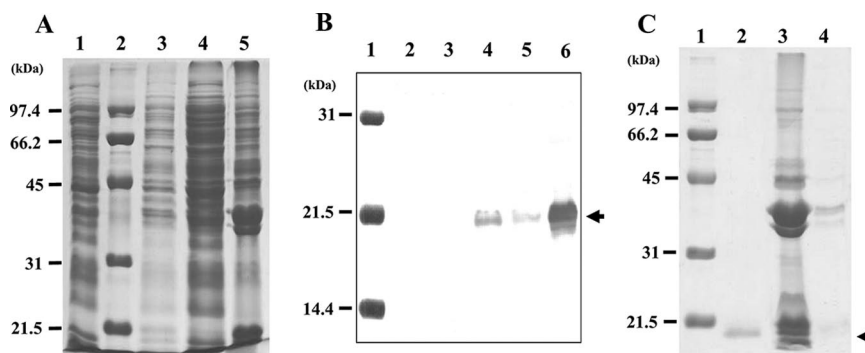


FIG. 1. Expression and purification of recombinant PvRII protein. (A) Recombinant PvRII was expressed as an insoluble protein in *E. coli* BL21(DE3) and electrophoresed using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Lanes: 1, BL21(DE3) with pet28a; 2, Bio-Rad low-range protein marker; 3, whole lysates; 4, soluble fraction; 5, insoluble fraction of pet28a-PvRII. (B) The expression of PvRII was confirmed by Western blotting (15% SDS-PAGE) with anti-His monoclonal antibody and horseradish peroxidase-conjugated anti-mouse immunoglobulin G. Lanes 1, Bio-Rad low-range protein marker; 2, BL21(DE3); 3, BL21(DE3) with pet28a; 4, pet28a-PvRII; 5, the soluble fraction of pet28a-PvRII; 6, the insoluble fraction of pet28a-PvRII. (C) Expressed PvRII was purified from the insoluble fraction with an Ni-NTA column under 8 M urea denaturing conditions. Lanes: 1, Bio-Rad low-range protein marker; 2, purified PvRII; 3, insoluble fraction of PvRII; 4, flowthrough. Arrows in panels B and C indicate the 20.4-kDa PvRII.

buffered saline (TBS; 50 mM Tris-HCl, 150 mM NaCl [pH 7.5]) before being filtered through a 0.45- $\mu$ m filter.

**Recombinant PvRII molecules.** Recombinant PvRII (C4-to-C7 cysteine-rich region of PvDBP II) protein of SK-1 strain isolated in South Korea was produced using a previously reported method (17). Briefly, the PvRII gene was amplified by PCR with C4-7-F (5'-CGAAGATATGAATTCTGTATGAAGGAAGCTT-3') and C4-7-R (5'-ATTGATTTCTCGAGCACATTTTCTTTCAG-3') and cloned in the *E. coli* expression vector pET28a+. The expression constructs were transformed in *E. coli* BL21(DE3). The expression of recombinant PvRII was induced with isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) in shake flask cultures and purified by metal affinity chromatography using Ni-nitrilotriacetic acid (NTA) matrix (QIAGEN) from inclusion bodies that had been solubilized with 8 M urea. The recombinant proteins purified under denaturing conditions were refolded by rapid dilution and purified to homogeneity using ion-exchange chromatography with Toyopearl-SP (Sigma) and gel filtration chromatography with Superdex-75 (Amersham-Pharmacia).

**Panning the scFv library to select PvRII binder.** The phage library was panned for binders using 96-well enzyme-linked immunosorbent assay (ELISA) plates (Costar) coated with PvRII (1  $\mu$ g/100  $\mu$ l) at 4°C overnight. Blocking and negative-selection well coating were performed with 10% fetal bovine serum in TBS. Antigen-binding clones were eluted by enzyme digestion with 10 mg/ml trypsin in TBS at room temperature for 30 min. The eluted phages were then allowed to infect *E. coli* ER2537 host cells to amplify the selected phages binding to PvRII. After amplification, phages were selected for three additional rounds using the same protocol. An aliquot of each of the polyclonal phages obtained after each round of selection was stored at 4°C until required. After four rounds of selection, 96 single clones were screened for binding to PvRII using ELISA.

**ELISA.** Microtiter wells were coated with PvRII, as described previously, and blocked with 10% fetal bovine serum in TBS for 1 h at 37°C. Phages were incubated in the wells for 1 h at 37°C and then washed four times with TBS-0.05% Tween 20. Bound phages were detected with a 1:3,000 dilution of horseradish peroxidase-conjugated mouse anti-M13 antibody (Amersham-Pharmacia) for 30 min at 37°C and detected by adding ABST substrate (Kirkegaard and Perry).

**Production of soluble scFv antibody fragments.** We induced scFv expression as described previously (22). Briefly, *E. coli* Top10F' cells were infected with selected phages before 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 an Ni<sup>2+</sup>-charged HP chelating column, following the manufacturer's protocol (Amersham-Pharmacia).

**DNA sequencing.** The DNA that encoded the scFv was sequenced using a BigDye terminator ready reaction kit (Applied Biosystems), and the products were analyzed on an ABI 3130xl (Applied Biosystems). The sequences of the V<sub>H</sub> and V<sub>L</sub> genes were compared with the sequences present in the V Base Sequence Directory to determine the closest germ line counterpart.

**Surface plasmon resonance.** A BIAcore 2000 instrument (Biacore AB, Uppsala, Sweden) was 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  $\mu$ g/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  $\mu$ l/min. The rate constants of association ( $k_{on}$ ) and dissociation ( $k_{off}$ ) were obtained at five different scFv concentrations (range, 1 to 100  $\mu$ g/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 residual scFv 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, PvDBP II was amplified by PCR using the primers PvDBPF (5'-ACAATTGGTAATGTTAGAT-3') and PvDBPR (5'-TGATTTCCATT TGACCATC-3') and cloned into the pDE vector using the BglIII and SacII sites (pDE-PvDBP II). The plasmid DNA used for transfection was purified using an endotoxin-free plasmid MidiPrep kit (QIAGEN); 1  $\mu$ g of DNA was used to transfect COS-7 cells in six-well plates (approximately  $5 \times 10^4$  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  $\times 100$  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. vivax*-infected patients admitted to Busan Paik Hospital in August 2001. The library contained  $7.85 \times 10^8$  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).

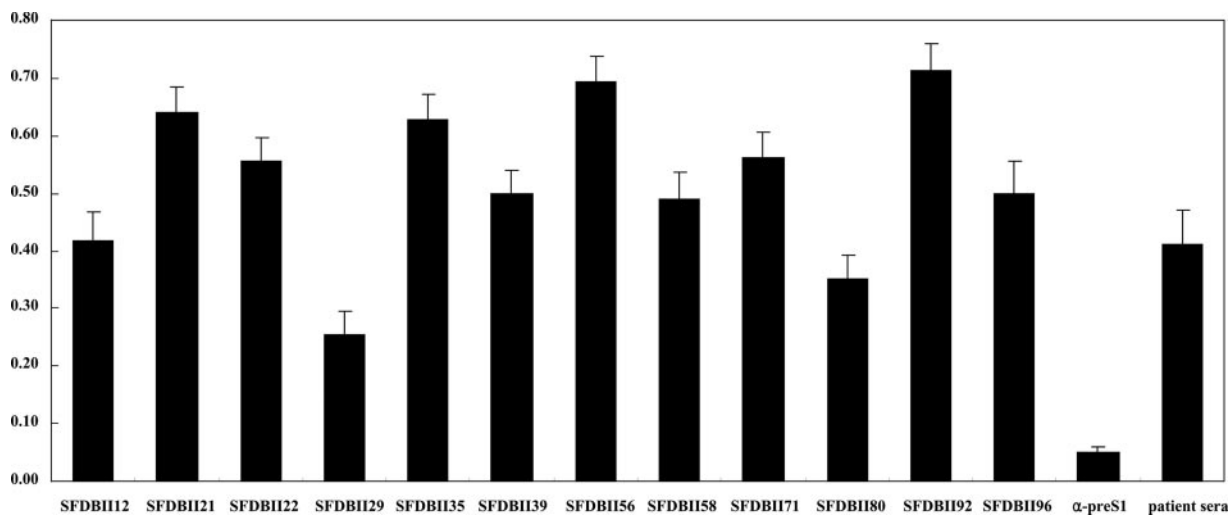


FIG. 2. Selection of binders to PvRII. Each well of the ELISA plate was precoated with recombinant PvRII (1  $\mu$ g/well), and then antibodies from each phage ( $10^{10}$  CFU/well) were added to each well. Bound phages were detected with horseradish peroxidase-conjugated anti-M13 mouse antibody. Anti-HBV 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.

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 (Fig. 2) (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). In addition, the three clones were sequenced (Table 1). A comparison with the sequences of the germ line  $V_H$  genes shows that the clones use a  $V_H$  III family-derived germ line segment. Alignment with the  $V_L$  germ line sequences showed that these clones use  $V_{\kappa}$  I,  $V_{\kappa}$  III, and  $V_{\lambda}$  I family segments. The kinetic parameters of these clones were measured using the BIAcore instrument (Fig. 3B). SFDBII92 had the greatest affinity ( $K_D = 3.62 \times 10^{-8}$  M versus  $K_D = 1.54 \times 10^{-7}$  M for SFDBII12 and  $K_D = 1.26 \times 10^{-7}$  M for SFDBII58).

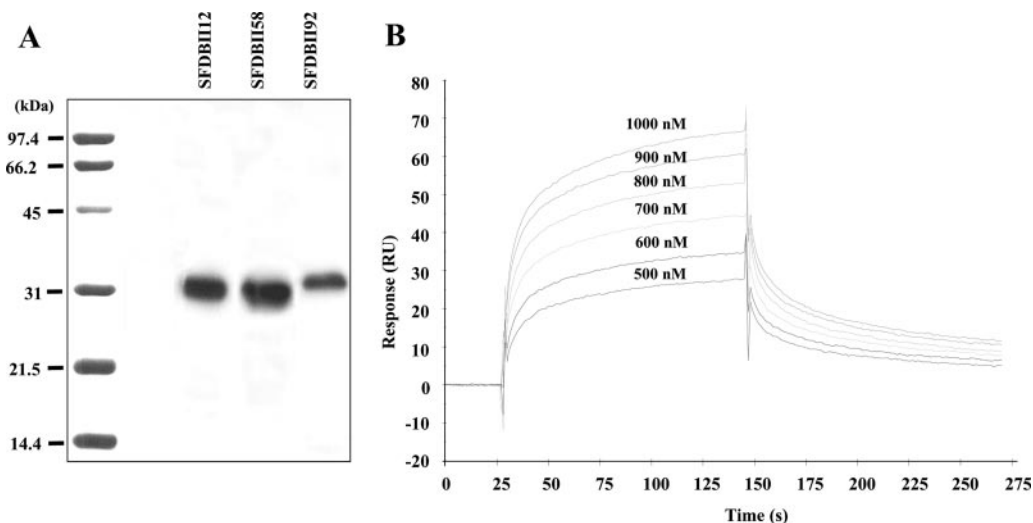


FIG. 3. Purification and analysis of the binding kinetics of soluble scFvs. (A) ScFvs were expressed in the form of soluble proteins without a p3 phage protein fused to the C-terminal portion of scFv. Monomeric scFvs were purified with an Ni-NTA affinity column using fast-protein liquid chromatography 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  $\mu$ M) against immobilized PvRII.

TABLE 1. Analysis of the CDR and human subgroup assignments for the three anti-PvR11 scFvs in this study<sup>a</sup>

Clone and region	Sequence for:			Family
	CDR1	CDR2	CDR3	
V <sub>L</sub>				
SFDBII12	GFTFGDYAMETH	GISWNSGSIG	GMETAANYYYYYGMETDV	V <sub>κ</sub> III
SFDBII58	GFTFGDYGMETH	GISWNSGSIG	DIYSSSWYAAFDI	V <sub>κ</sub> I
SFDBII92	GFTFSSYWNETS	NIKQDGESEKY	DCSSTSCYGYYYYGMETDV	V <sub>λ</sub> I
V <sub>H</sub>				
SFDBII12	RASQSVSSSYLA	GASSRAT	QQYGSSRLT	V <sub>H</sub> III
SFDBII58	RASQSISYHLN	AASTLOS	QKYNAPLT	V <sub>H</sub> III
SFDBII92	SGSSNIGSNTVN	SNNQRPS	AAWDDSLNGWV	V <sub>H</sub> III

<sup>a</sup> V<sub>H</sub> and V<sub>L</sub> were searched against the Kabat database using the BLAST algorithm, and the scFv groups were analyzed via the site at <http://acrmwww.biochem.ucl.ac.uk/abs/chothia.html>.

To evaluate the biological effects of anti-PvR11 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  $53.6 \pm 3.5$ . Next, to determine whether the anti-PvR11 scFvs blocked the adhesion of the PvDBP molecules to human erythrocytes, transfected COS-7 cells were treated with anti-PvR11 scFvs and reacted with erythrocytes to form rosettes. All anti-PvR11 scFvs showed inhib-

itory 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  $\mu\text{g/ml}$  of scFvs, SFDBII92 showed the highest inhibition (91.6% for SFDBII92, 88.4% for SFDBII12, and 80.9% for SFDBII58). The 50% inhibitory concentrations ( $\text{IC}_{50}$ s) were 2.9  $\mu\text{g/ml}$  for SFDBII12, 4.0  $\mu\text{g/ml}$  for SFDBII58, and 4.5  $\mu\text{g/ml}$  for SFDBII92.

## DISCUSSION

The invasion of erythrocytes by *P. vivax* merozoites is thought to be as complex a process as the invasion of erythro-

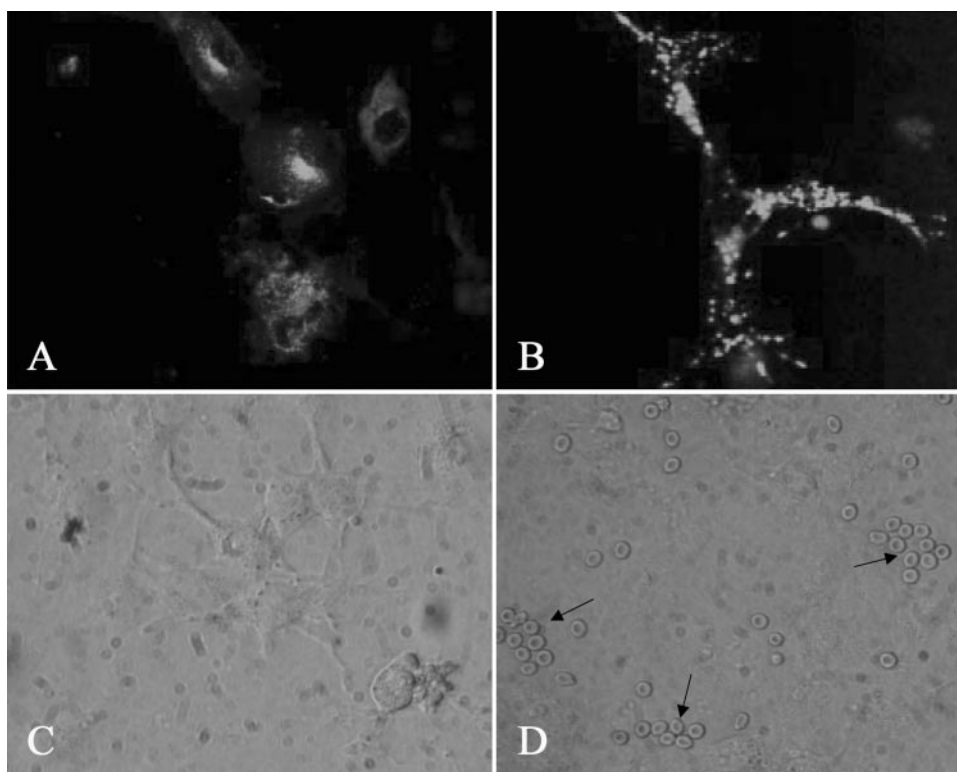


FIG. 4. Erythrocyte-binding assay. PvDBP11 was expressed on the surface of COS-7 cells using the pDE-GFP surface display system. Transfected COS-7 cells were observed under confocal microscopy and reacted with a 10% erythrocyte suspension for rosette formation (A and C, pDE; B and D, pDE-PvDBP11). The microscope magnification is  $\times 200$ . Interactions with the receptors of erythrocytes and PvDBP11 were observed as rosettes (arrows).

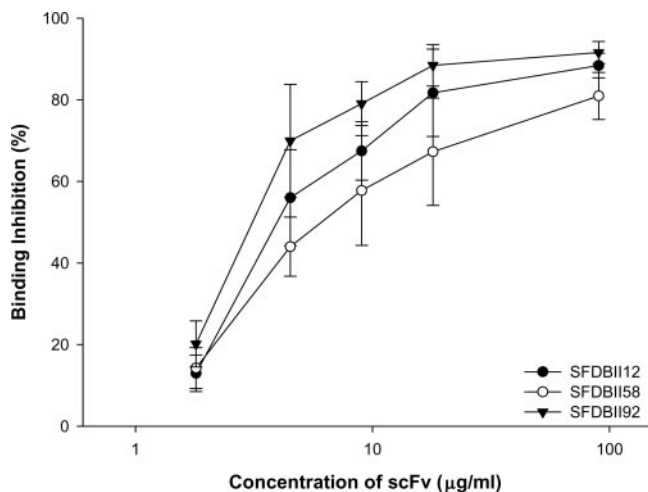


FIG. 5. EBIA. Transfected COS-7 cells with pDE-PvDBP II were incubated with various concentrations of soluble scFvs (2 to 100 µg/ml) for 2 h at 37°C. After washing, a 10% erythrocyte suspension was added for rosette formation. The binding inhibition activity was scored after counting the rosettes in 20 fields at a magnification of  $\times 100$ . An irrelevant scFv was used as a negative control. The error bars correspond to standard errors.

cytes by other *Plasmodium* species, such as *P. falciparum*. The erythrocyte-binding capabilities of several proteins have been examined, including merozoite surface protein 1, reticulocyte binding protein, and DBP, which have high binding affinity to erythrocytes (9, 10, 14, 26). For *P. vivax* malaria, DBP is considered 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 adherence 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 patients.

Some human recombinant antibodies against *Plasmodium* have been reported using the MSP protein of *Plasmodium falciparum* 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 previously. Roeffen et al. (27) showed their biological activity and invasion inhibition using competitive ELISA with neutralizing antibodies. Lundquist et al. (18) showed the neutralizing activity with an antibody-dependent cellular assay. Here, we demonstrated the neutralizing activities of recombinant antibody 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 increased 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<sub>H</sub> 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 ( $K_D$ ) in the nanomolar range. Previous work on an invasion-inhibiting anticircumsporozoite antibody ( $K_D \approx 300$  nM) (35), HBV neutralizing antibodies ( $K_D \approx 160$  nM) (22), and human immunodeficiency virus neutralizing antibodies ( $K_D \approx 4.6$  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 inhibition activities of the anti-DBP II scFvs were correlated with their affinities. SFDBII92 had the greatest inhibition activity ( $IC_{50} \approx 2.9$  µg/ml versus 4.0 µg/ml for SFDBII12 and 4.5 µg/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 immunization to prevent or treat *P. vivax* infection. Unfortunately, we cannot estimate the amount of scFv showing a neutralizing 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 neutralized DBP binding to the erythrocyte surface and demonstrated the neutralizing activity of these antibodies in vitro using an erythrocyte-binding inhibition assay.

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