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Development and Evaluation of an Enzyme-Linked Immunosorbent Assay for *Plasmodium vivax*-VK247 Sporozoites

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ABSTRACT An enzyme-linked immunosorbent assay (ELISA) for the *Plasmodium vivax*-VK247 (variant) circumsporozoite (CS) protein was developed and evaluated using sporozoites produced by feeding mosquitoes on Thai patients with parasitologically confirmed *P. vivax* infections. The ELISA had a detection threshold of fewer than 50 sporozoites. Using this assay in conjunction with an ELISA for the VK210 polymorph, nearly 16% of the 235 *P. vivax* cases produced sporozoites positive only for the variant; 69% produced sporozoites positive only in the VK210 assay; and 15% were positive in both assays, indicating mixed infections. Twelve cases (5%) produced sporozoites negative in one assay and with unexpectedly low activity in the other ELISA, indicating the possibility of other CS protein polymorphs.

KEY WORDS *Plasmodium vivax*, circumsporozoite protein detection, ELISA

ENZYME-LINKED IMMUNOSORBENT assays (ELISAs) for the detection of *Plasmodium vivax* (Grassi & Feletti) and *P. falciparum* (Welch) circumsporozoite (CS) proteins in anopheline mosquitoes have become valuable tools to study malaria (Wirtz & Burkot 1991). The recent discovery of a *P. vivax* polymorph (VK247) that produces an immunologically distinct CS protein, not detectable with existing ELISAs (Rosenberg et al. 1989), has made it necessary to develop an assay for the variant sporozoites. The VK247 polymorph is widespread in Asia and Latin America, based on studies of *P. vivax* sporozoites (Rosenberg et al. 1989), blood-stage parasites (Kain et al. 1992), and detection of human antibody to the VK247 CS protein (Cochrane et al. 1990, Wirtz et al. 1990). We report herein the development and initial evaluation of an ELISA for the *P. vivax*-VK247 CS protein in anopheline mosquitoes.

Materials and Methods

Sporozoite Production. Details of sporozoite production have been described by Sattabongkot et al. (1991). Briefly, laboratory-reared *Anopheles dirus* Peyton & Harrison (Bangkok colony) were fed on volunteer patients who reported to malaria clinics and who were found to have at least one *P. vivax* gametocyte per 50 fields (700×) on a Giemsa-stained thick blood film. Mosquitoes were examined for oocysts 7–10 d after feeding, and positive groups were checked for salivary gland sporozoites 14–16 d after feeding. Blood slides were rechecked, and only mosquitoes that had fed on volunteers with confirmed, uncomplicated *P. vivax* infections were used for further study.

Salivary glands were dissected from infected mosquitoes and sporozoites were harvested by transferring the glands into 1.5-ml tubes containing 0.01 M phosphate-buffered saline (PBS), pH 7.4. Glands were ground gently with a pestle (Kontes, Vineland, NJ) to release the sporozoites, which were counted using a hemocytometer. Sporozoites used for monoclonal antibody (MAb) production and ELISA development were frozen at −70°C and then shipped on dry ice to the Walter Reed Army Institute of Research.

Sporozoites for evaluating the ELISA in Thailand were diluted to 1,600 sporozoites per 50 μl of 0.5% boiled casein blocking buffer containing 0.05% Nonidet P-40 (Sigma Chemical Co., St. Louis, MO). The blocking buffer was prepared by boiling 5 g of bovine milk casein (Sigma Chemical Co., catalog no. C-0376) in 100 ml of 0.1 M NaOH, adding 900 ml of PBS, adjusting the pH to ≈7.4 with HCl, and adding 0.01%

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thimerosal plus 0.002% phenol red. Samples not immediately tested were frozen at −70°C for later use. Serial (1:1) dilutions of sporozoites were tested in the VK210 and VK247 ELISAs. The picogram equivalents were determined, based on dilution curves of NS1V20 recombinant protein (Gordon et al. 1990) and synthetic peptide (Ala-Asn-Gly-Ala-Gly-Asn-Gln-Pro-Gly)$_3$ (Rosenberg et al. 1989) glutaraldehyde conjugated to boiled casein (VK247$_3$-BC) positive control CS antigens, respectively, run in the same microtiter plate.

Sporozoites used for MAb production were from mosquitoes fed on patients in Kanchanaburi or Ratchaburi provinces, in western Thailand where the VK247 strain was discovered (Rosenberg et al. 1989). Some sporozoites used to evaluate the ELISA also were produced from patients in Nakhon Si Thammarat and Trat provinces in southern and eastern Thailand, respectively (Sattabongkot et al. 1991).

Monoclonal Antibody Development. The MAbs were developed using the method described by Burkot et al. (1984). Hybridomas were produced from BALB/c mice given ≈200,000 sporozoites, which tested negative in the VK210 ELISA, in 100 μl PBS by intravenous tail vein injection on day 0, and 100,000 sporozoites per 50 μl on days 20 and 40. These mice also received 50 μg of VK247 synthetic peptide (Ala-Asn-Gly-Ala-Gly-Asn-Gln-Pro-Gly)$_3$ (Rosenberg et al. 1989) (University of Maryland Biopolymer Laboratory, Baltimore, MD), glutaraldehyde conjugated to keyhole limpet hemocyanin (Calbiochem, La Jolla, CA) in 100 μl of PBS on days 20 and 40. On day 60, 3 d before the fusion, mice received 100,000 sporozoites in 50 μl PBS. Hybridoma cell culture supernatants were screened for activity by indirect immunofluoroscence antibody assay (IFA), using air-dried sporozoites (Wirtz et al. 1991), or by antibody ELISA (Wirtz et al. 1990), using VK247$_3$-BC as the capture antigen in conjunction with peroxidase anti-mouse IgG (H+L) (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). Isotyping was conducted by Ouchterlony immunodiffusion or by antibody ELISA using IgG (γ) and IgM (μ) class-specific anti-mouse antibodies (Sigma Chemical Co.).

Ascitic fluid was produced from six cloned cell lines, purified by protein-A column chromatography (Ey et al. 1978) and evaluated by IFA. The two most promising MAbs were conjugated to horseradish peroxidase, aliquoted, and lyophylized (Kirkegaard & Perry Laboratories). Lyophylized reagents were dissolved in glycerol: distilled water (1:1) to yield working stock solutions of 0.5 mg MAb/ml.

Sporozoite ELISA Method. A two-site homologous "sandwich" ELISA was developed (Wirtz et al. 1991). Capture MAb diluted in PBS was incubated overnight in wells of polyvinyl chloride microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA). Wells were aspirated dry, filled with blocking buffer for 1 h, aspirated dry, and positive control antigen or sporozoites diluted in blocking buffer were added to the appropriate wells. After 2 h, wells were aspirated dry, washed twice with PBS–0.05% Tween 20, and the homologous peroxidase-conjugated MAb diluted in blocking buffer was added. After 1 h wells were aspirated dry, washed three times with PBS–0.05% Tween 20, and 100 μl of ABTS peroxidase substrate (Kirkegaard & Perry Laboratories) were added to each well. Absorbance (405 nm) values were recorded after 1 h with an ELISA plate reader (Molecular Devices Corporation, Menlo Park, CA). Assays were conducted at room temperature, and plates were covered during incubations to prevent evaporation.

Optimum VK247 ELISA capture and peroxidase-conjugated MAb concentrations were determined using serial (1:1) dilutions beginning at 0.4 μg/50 μl (8 μg/ml) and using VK247$_3$-BC as the positive control. The VK210 ELISA was conducted using capture and peroxidase-conjugated MAb concentrations of 0.025 and 0.05 μg per well (Wirtz et al. 1991), respectively, and NS1V20 recombinant protein, containing 20 Gly-Asp-Arg-Ala-(Ala/Asp)-Gly-Gln-Pro-Ala repeats (Gordon et al. 1990), as the positive control. The sporozoites used to evaluate the assays were produced by feeding mosquitoes on patients WR8 and VK744 (Rosenberg et al. 1989), respectively.

Results and Discussion

Four fusions were required to produce stable IgG-secreting cell lines. Two initial fusions resulted in unstable hybridomas or those that produced only IgM MAbs unacceptable because of low sensitivity and high background absorbance values (data not shown). Six IgG-secreting cell lines from two subsequent fusions were selected for ascites production, based on cell culture supernatant IFA and ELISA results. Comparative testing of these protein A purified MAbs resulted in the selection of two for peroxidase conjugation (182.1G12 and 182.2G5).

The optimum capture and peroxidase-conjugate concentrations for both 182.1G12 and 182.2G5 MAbs were 0.1 μg/50 μl PBS (2 μg/ml) and 0.2 μg/50 μl blocking buffer (4 μg/ml), respectively, when using VK247$_3$-BC synthetic peptide or Nonidet P-40 treated sporozoites. When testing sporozoite concentrations in the 50–400 per well range, lower (0.05 μg) or higher (0.2 μg) concentrations of capture 182.2G5 MAb resulted in 10–20% and 30–50% decreases in absorbance values, respectively. Using lower (0.1 μg) or higher (0.4 μg) peroxidase-182.2G5 MAb concentrations resulted in a 15–30% reduction or 10–15% increase in absorbance values, respectively. However, background absorbance

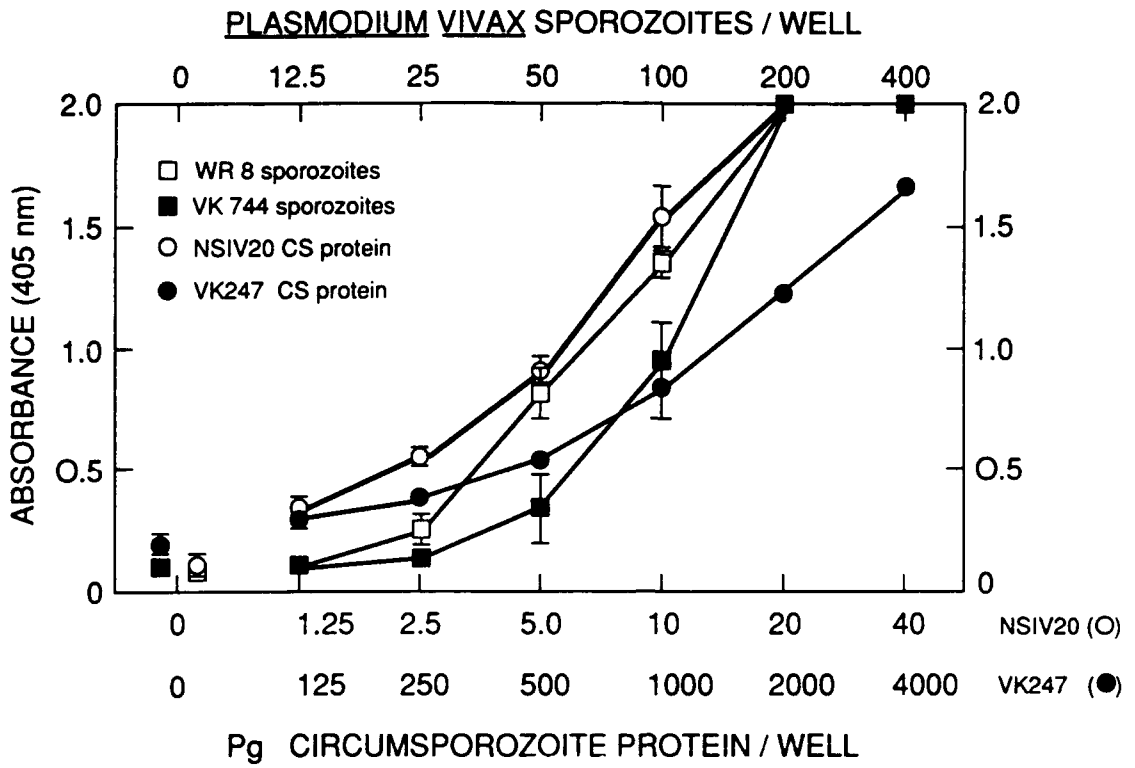

Fig. 1. Sensitivity of *Plasmodium vivax* VK247 (182.2G5) (closed symbols) and VK210 (NVS#3) (open symbols) ELISAs for Nonidet P-40-treated sporozoites, synthetic VK247, and recombinant VK210 circumsporozoite proteins (Monoclonal antibody concentrations: VK247-182.2G5 capture and peroxidase = 0.1 and 0.2 μg/50 μl per well; VK210-NVS#3 capture and peroxidase = 0.025 and 0.05 μg/50 μl per well; substrate reaction time = 1 h).

values were consistently higher when more enzyme-conjugate was used. Results with the 182.1G12 MAb were nearly identical.

The VK247-182.2G5 and VK210-NVS#3 ELISA CS antigen and sporozoite dilution curves are shown in Fig. 1. The lower detection limits of the VK247-182.2G5 and VK210-NVS#3 ELISAs are ≈50 and 25 sporozoites, respectively, using a cut-off value of two times the mean of six negative control wells containing blocking buffer (0.18 and 0.16, respectively). The 10^3 pg difference in sensitivity between the $VK247_3$-BC and NS1V20 antigens (Fig. 1; Table 1) can be attributed to their structures. The NS1V20 recombinant protein contains 20 nonapeptide repeats plus 81 N-terminal amino acids (Gordon et al. 1990). The VK247 synthetic peptide consists of only three nonapeptide repeats conjugated to boiled casein, which may reduce the number of functional epitopes. Similar differences in sensitivity were observed when these reagents were used as ELISA capture antigens to screen for anti-CS antibody in human sera (Wirtz et al. 1990).

The VK247 or VK210 CS protein equivalent of 1,600 sporozoites from each patient feed was used to construct Table 1. Thirty-six of the 235 *P. vivax*–infected patients (15.3%) produced sporozoites positive in the VK247 ELISA (>2,000 pg) and negative in the *P. vivax*–predominant (VK210) CS protein assay (<5 pg). This is similar to the initial report that 14.2% (23/162) of patients from the same geographic area produced sporozoites that did not react in the VK210 ELISA (Rosenberg et al. 1989). Thirty-six patients (15%) produced sporozoites positive in both assays, indicating concurrent VK247 and VK210 infections, or sporozoites containing both CS proteins; therefore ≈30% of all *P. vivax* cases were infected with the VK247 polymorph. The

Table 1. ELISA determination of *P. vivax* VK247 and VK210 circumsporozoite protein concentrations associated with 1,600 salivary gland sporozoites produced from each of 235 mosquito feeds on *P. vivax*–infected patients from Thailand

VK247 ELISA (pg × 10^3)	VK210 ELISA (pg)					
	0	>5–20	21–80	81–160	>160	Total (%)
>32	9	5	7	7	7	35 (14.9)
17–32	18	0	1	3	1	23 (9.8)
>2–16	9	0	0	3	2	14 (5.9)
0	0	3	18	50	92	163 (69.4)
Total	36	8	26	63	102	235 (100)
(%)	(15.3)	(3.4)	(11.1)	(26.8)	(43.4)	(100)

remaining 69% (163/235) of the cases produced sporozoites positive only in the VK210 assay. Three patients (1%) produced sporozoites that were negative in the VK247 assay and had unexpectedly low activity in the VK210 ELISA (>5–20 pg). In addition, sporozoites produced from nine patients (3.8%) were negative in the VK210 ELISA and had very low activity in the VK247 assay (>2,000–16,000 pg), indicating the possible presence of a CS protein repeat unit significantly different than the two published sequences. The CS genes of these parasites currently are being amplified for sequencing, and the VK247 ELISA is being used to screen *P. vivax* sporozoites from different geographic areas.

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