EXPRESSION IN YEAST OF A *PLASMODIUM VIVAX* ANTIGEN OF POTENTIAL USE IN A HUMAN MALARIA VACCINE

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Plasmodium vivax, the agent of one of the tertian malarias, is widespread in Asia, Central and South America, and less frequent in Africa. Although infection with this parasite is rarely fatal, the course of the disease is characterized by frequent relapses, slow development of acquired immunity and the presence of dormant forms of the parasite in the liver, which can persist for several years (1).

Protective immunity against rodent, monkey (2), and human malaria (including *P. vivax*) (3) has been achieved by vaccination with gamma-irradiated sporozoites, the infective stage of parasite found in the salivary gland of the *Anopheles* mosquito vector. The antigen involved in protective immunity to sporozoites is the circumsporozoite (CS)[1] protein, a stage-specific membrane molecule with unusual structural and immunological properties (4). It contains a large central domain, occupying close to half of the polypeptide chain, formed by tandem repeats of 4–12 amino acids whose composition varies in different species (and sometimes strains) of malaria parasites (5). Most of the antibodies found in the serum of humans (6, 7) or of experimental animals inoculated with sporozoites, and all mAbs so far raised against this stage of the parasite, are directed against repetitive epitopes in this domain. Monovalent Fab fragments of mAbs against the repetitive epitopes neutralize parasite infectivity in vitro and in vivo in several experimental models of malaria (8, 9). Epidemiological evidence shows that acquired immunity and resistance to malaria infection in humans is age dependent and associated with the development of high levels of antibodies against the repeats (10, 11).

The *P. vivax* CS protein gene has been recently cloned and sequenced (12, 13). It encodes a protein of 373 amino acids with general features resembling those of other members of the family (4, 5, 14). Here we report the results of

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[1] *Abbreviations used in this paper:* ADH-2, alcohol dehydrogenase; CS, circumsporozoite; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HF, hydrogen fluoride; HIV, human immunodeficiency virus; hSOD, human superoxide dismutase; IFA, indirect immunofluorescence; IRMA, immunoradiometric assay.

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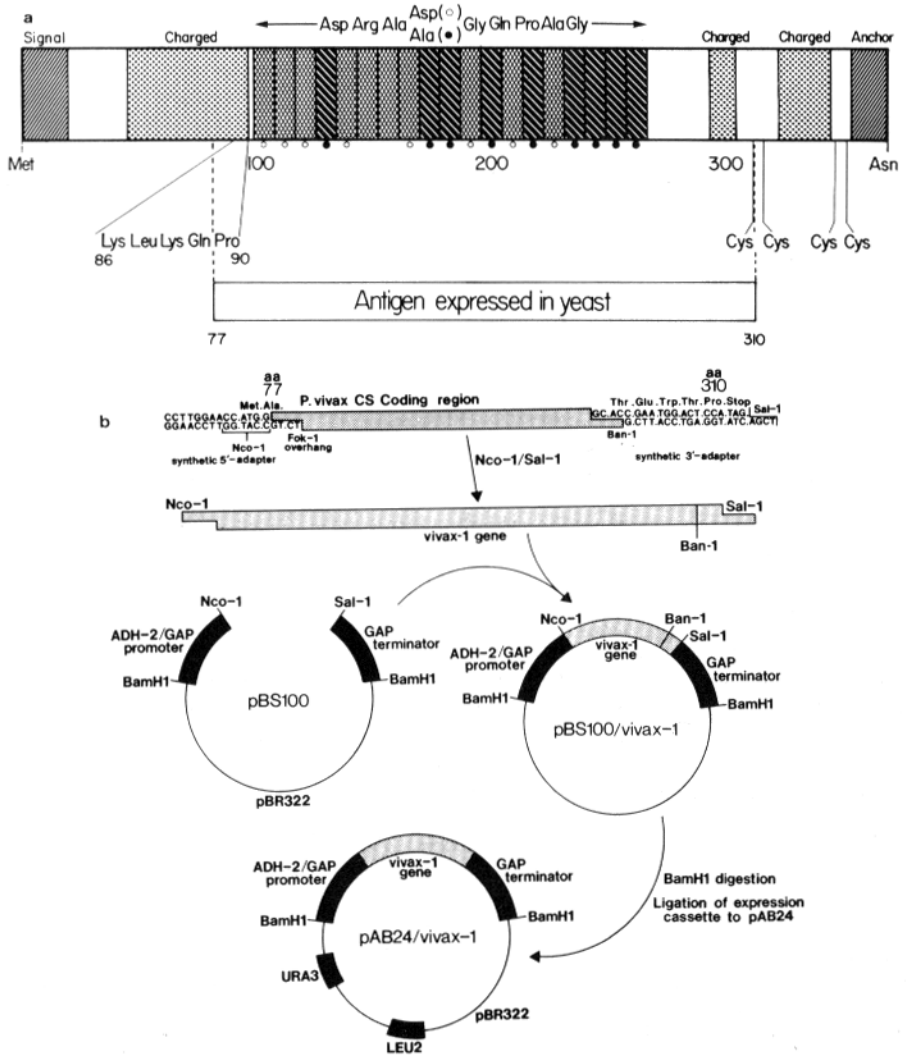

FIGURE 1. (*a*) Schematic representation of the CS protein gene and of the segment expressed in yeast. The assigned amino acid numbers correspond to those of Arnot et al. (12). In addition to signal and presumed anchor sequences, and areas containing large numbers of charged amino acids, a large portion of the CS molecule consists of tandem repeats of nine amino acids. As shown, repeats bearing alanine in the fourth position (●) predominate at one end of the repeat domain. (*b*) Plasmid constructions for the expression of *P. vivax* CS proteins in yeast as explained in the Materials and Methods section.

our effort to develop a recombinant DNA–derived vaccine against *P. vivax* consisting of portions of the CS polypeptide with potential immunoprophylactic value, including the repeat domain.

Materials and Methods

Plasmid Construction and Yeast Expression. A Fok-1/Ban-1 fragment encoding amino acids Ala 77 to Thr 306 of the *P. vivax* CS gene (Fig. 1 *a*) was excised from pUC9 plasmids

containing this region of DNA. Synthetic adapters containing in-frame initiation and termination codons (as shown in Fig. 1 *b*) were ligated to this fragment and the ligation product was digested with Nco I and Sal I before cloning in the vector pBS100. Oligonucleotides were synthesized by the phosphoramidite method using DNA synthesizers (No. 380A; Applied Biosystems, Inc., Foster City, CA). DNA cloning and linker ligations were carried out by standard techniques (15). The vector pBS100 contains ~1 kb of the regulatory region of the *Saccharomyces cerevisiae* alcohol dehydrogenase 2 (ADH-2) promoter (Cousens, L. S., J. R. Shuster, C. Gallegos, L. Ku, M. M. Stempien, M. S. Urdea, R. Sanchez-Pescador, A. Taylor, and P. Tekamp-Olson, manuscript submitted for publication; and Shuster, J., unpublished data) and ~200 bp of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter (16). This hybrid promoter, together with the GAPDH transcriptional terminator, flanks a region of the human immunodeficiency virus (HIV) envelope gene in pBS100 (Barr, P. J., K. S. Steimer, E. A. Sabin, et al., manuscript in preparation). Replacement of the HIV sequences with the *P. vivax* Nco-1/Sal-1 fragment gave the plasmid pBS100/vivax-1. The promoter-gene-terminator expression cassette was excised from pBS100/vivax-1 as a Bam HI fragment and cloned into Bam HI and phosphatase-treated pAB24. pAB24 (provided by Dr. A. J. Brake, Chiron Corp.) is an autonomously replicating yeast plasmid containing pBR322 sequences for amplification in *Escherichia coli*, and URA3 and LEU2 markers for selection of *ura* or *leu* yeast auxotrophs in uracil- or leucine-deficient media. Yeast strain AB110 (16) was transformed with the expression plasmid, designated pAB24/vivax-1 and leucine prototrophs selected and grown in minimal media lacking leucine and containing 8% glucose. For induction and analysis of vivax-1 CS protein, yeast cells were diluted 1:20 into medium containing 1% glucose and were grown for 36 h at 30°C. A second plasmid encoding amino acids Ala 77 to Leu 335 was constructed using the Nco-1/Ban-1 fragment from vivax-1 together with synthetic DNA encoding amino acids Glu 307 to Leu 335. The resulting plasmids pBS100/vivax-2 and pAB24/vivax-2 (not shown) were used as described for vivax-1 for the production of the corresponding vivax-2 protein.

Synthetic Peptides. The 18-mer peptide $(DGQPAGDRA)_2$ was synthesized as described in (12). The tetradecapeptide CY(PKNPRGNKLKNP) was synthesized by Dr. D. H. Schlesinger on 1 g of a benzhydrylamine resin (0.67 mEg/g, Beckman Instruments, Inc., Palo Alto, CA) with Boc-L-amino acids (Bachem, Torrance, CA) using a modified solid-phase peptide synthesis (17, 18) on a Vega model 250c automated peptide synthesizer controlled by an Apple II C computer. The following side chain protecting groups were used: Cys, 4 methoxybenzyl; Tyr, O-2,6-dichlorobenzyl; Lys, 2-chlorocarbobenzoxy; Asn and Gln, nitrophenyl ester; Arg, tosyl; Glu, *o*-benzyl. The cysteine and tyrosine residues are not part of the CS protein sequence and were added to facilitate radiolabeling and coupling to carrier proteins. After completion of synthesis the peptide resin was treated with anhydrous hydrogen fluoride (HF) (10 ml/g peptide resin) at 0°C for 60 min in the presence of anisole as free radical scavenger to remove protecting groups and to cleave the peptide from the resin. The crude peptide was extracted using alternate washes of glacial acetic acid and water and then was lyophilized. The peptide was desalted and purified on a 100 × 1.9 cm column of Sephadex G-25 equilibrated with 1 N acetic acid. The final tetradecapeptide product gave the following amino acid composition: Asp (2), 1.77; Glu (2), 2.06; Pro (3), 3.10, Cys (1.0), 0.74; Leu (1), 1.06; Tyr (1), 0.84; Lys (3), 2.85; Arg (1), 1.02.

Radioimmunoassay (RIA). The purified engineered CS polypeptide was radiolabeled with ^{125}I to a sp act of 2×10^6 cpm/μg protein using Iodogen. A standard curve relating the dose of antigen with the specific counts per minute obtained in the RIA was included in every assay; 30 μl of mixtures containing a constant amount of radiolabeled antigen and increasing amounts of purified cold antigen were delivered to the bottom of wells of microtiter plates precoated with a mAb (2F2) against the repeats of CS protein of *P. vivax* (19) and then were saturated with BSA. After incubation at room temperature, the wells were washed and the radioactivity was counted in a gamma counter. As a negative control, 30 μl of the same mixtures were delivered to wells coated with BSA alone, and the RIA was performed as above. The counts per minute observed in the controls were subtracted

from the corresponding experimental values. The cold antigen inhibited the binding of the hot antigen to the immobilized 2F2 antibody in a dose-dependent manner. All dilutions were performed in a PBS containing 1% BSA and 0.1% sodium azide. To determine the antigen concentration in fractions obtained during the purification procedure, duplicate samples were mixed with the labeled-purified antigen, and the RIA was performed as above. The concentrations of cold antigen were calculated by referring the mean of the results of the duplicate measurements to the standard curve. The counts per minute in the duplicates did not differ by >10% from the mean values. The RIA detected antigen at concentrations as low as 5 ng/ml.

Immunoradiometric Assay (IRMA). Wells of microtiter plates were coated with purified engineered polypeptide or with synthetic peptides at a concentration of 10 μg/ml in PBS. The wells were saturated with 1% BSA and incubated with 30 μl of increasing dilutions in PBS–1% BSA of the immune sera. After incubation for 1 h at room temperature the wells were washed and reincubated with 5–10 ng of radiolabeled, affinity-purified goat antibodies to mouse Igs (2×10^7 cpm/μg protein). After a second incubation of 1 h at room temperature, the wells were washed and counted in a gamma counter.

NH_2-Terminal Sequencing Analysis. This was performed by Dr. D. H. Schlesinger in a gas-phase sequencer (No. 470 A; Applied Biosystems, Inc.) using a polybrene-treated glass filter that had been conditioned by several cycles of the Edman degradation.

Immunization of Mice. The purified antigen was dissolved in water and adsorbed onto aluminum hydroxide. 10 mice were injected intramuscularly with 0.7 mg of the adjuvant coated with the antigen in a volume of 100 μl. Booster injections were given 3 and 6 wk afterwards. Serum samples were obtained 10 days after each antigen injection.

In Vitro Assay for Sporozoite Neutralization. This was performed as described in (9).

Results and Discussion

For expression of the *P. vivax* CS antigen in yeast, we constructed plasmids containing DNA coding for 234 amino acids (Ala 77 to Pro 310) of the *P. vivax* CS protein (Fig. 1 *a*). If the 39 amino acids representing the hydrophobic leader and presumed anchor sequences are excluded, this represents ~70% of the amino acids encoded by the *P. vivax* CS gene, and most likely an even larger proportion of the amino acids found in the mature, membrane form of the CS protein. The DNA encoded all amino acids from the repeat domain (19 tandem sequences DRA^D_AGQPAG), 15 amino acids preceding the repeats, and 48 amino acids flanking the repeats at their COOH-terminal end.

Although the NH_2-terminal amino acid of the genuine processed membrane form of the CS protein is unknown, immunological evidence suggests that it comprises several amino acids upstream of the repeat domain (20). We have selected Ala 77 as the NH_2-terminal of the expressed antigen because, immediately preceding it, the CS protein gene codes for a cluster of basic residues that could be the site for proteolytic processing of the intracellular precursor and generation of the membrane form. The chosen DNA fragment included the codons for the highly conserved sequence KLKQP found in all CS proteins sequenced to date (5, 21).

For expression in yeast we chose to use a hybrid promoter based on the strong yeast glyceraldehyde-3-phosphate dehydrogenase promoter (16) and the glucose regulatable alcohol dehydrogenase-2 (ADH-2) promoter. Fusion of this promoter to heterologous genes allows the growth of yeast cultures to high density using glucose as a carbon source. Depletion of glucose in the medium during fermentative growth gives concomitant induction of expression of the heterologous protein. Translation frames encoding *P. vivax* CS antigens were fused to this

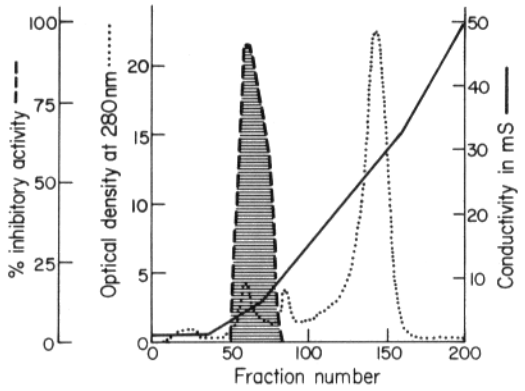

FIGURE 2. Extracts from pelleted yeast from 20 liters of yeast culture were prepared in a bead beater, diluting the yeast in an equal volume of 0.1 M sodium phosphate buffer, pH 7.3, 0.1% Triton-X, 1 mM EDTA, 1 mM PMSF, and 1 μg per ml of pepstatin. The extract (370 ml) was added to 200 ml of boiling water containing 1 ml of PMSF and 1 μg of leupeptin. The mixture was brought to 100°C and kept for 10 min with constant stirring at this temperature. The mixture was cooled to 0°C and centrifuged at 18,000 rpm in a Ultracentrifuge Rotor TI-19 (Beckman Instruments, Inc.) for 15 min. The supernatant was then lyophilized.

The dry material was dissolved in 120 ml of water, centrifuged to remove a small residual amount of insoluble material, dialyzed extensively against distilled water for 48 h, and lyophilized again. The powder was dissolved in 3 mM sodium potassium phosphate buffer, pH 7.5, and the conductivity was adjusted with water to 0.58 mS. The solution was centrifuged to remove insoluble materials and subjected to anion-exchange chromatography in a DEAE-Sephacel (Pharmacia Fine Chemicals, Piscataway, NJ) column (5 cm × 24 cm) equilibrated in the same buffer. The flow rate was adjusted to 100 ml/h and 21 ml per tube were collected. The column was then washed with 500 ml of the same buffer. The elution continued with a buffer formed in a linear gradient in which 1,500 ml of the initial buffer were mixed with 1,500 ml of the same buffer containing 0.75 M NaCl.

The positive fractions in the RIA (10) eluted between fraction nos. 50–80, corresponding to a relatively small optical density peak, at conductivities between 2 and 10 mS. The pooled fractions 50–80 were lyophilized, redissolved in 60 ml of 0.3 M NaCl, and dialyzed against 0.3 M NaCl at room temperature for several hours. The dialyzate was centrifuged to remove a small amount of insoluble material and one-third, i.e. 20 ml, was subjected to molecular-sieve chromatography on Sephadex G-200 (Pharmacia Fine Chemicals) equilibrated with sodium chloride 0.3 M. The Sephadex was superfine and the column was 5 cm in diameter and 100 cm long. Samples of 21 ml per tube were collected from the column. The CS polypeptide eluted in a sharp symmetrical peak with an M_r of ~50,000. However, CS antigen with higher and lower molecular weight in smaller amounts was found in several tubes preceding and following this peak. The contents of tubes in the main peak of antigen were pooled, dialyzed extensively against distilled water, and lyophilized. A total amount of 89 mg of CS antigen was recovered.

promoter using synthetic oligonucleotide adapters. Incorporation of these constructions into high copy number, autonomously replicating yeast plasmids and transformation of yeast cells, generated strains that could express high levels of CS antigens upon induction.

The isolation of the CS polypeptide antigen from the yeast extracts transformed with this plasmid was monitored by a solid-phase competitive RIA. The first steps of purification consisted of heating the extracts at 100°C for 10 min and removing the large amounts of denatured insoluble materials by centrifugation (see legend of Fig. 2 for details). This approach was based on our prior observations that the antigenic reactivity of the repetitive epitopes of CS proteins

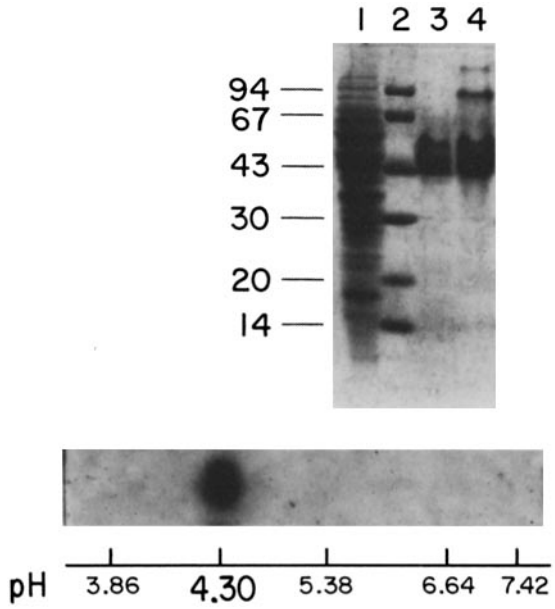

FIGURE 3. (*Top*) SDS-PAGE analysis under reducing conditions in 7.5% acrylamide gels of (*1*) total yeast extracts, (*2*) molecular weight markers, (*3*) purified CS protein (2 mg/ml) obtained after anion-exchange and molecular sieve chromatography, (*4*) supernatant obtained after heating the extracts at 100°C for 10 min. (*Bottom*) Isoelectric focusing under denaturing conditions of the purified CS antigen (2 mg/ml). The gels were dried and stained with Coomassie blue.

was unaltered by this treatment (22). The supernatant of the boiled yeast extract was subjected to ion-exchange chromatography and eluted with a salt gradient. The positive fractions, found in a symmetrical optical density peak with conductivities between 2 and 10 mS (Fig. 2), were pooled, concentrated, and subjected to molecular-sieve chromatography. Most of the CS polypeptide eluted in a sharp peak with an apparent M_r between 40,000 and 60,000, but reactive molecules with higher and lower M_r were found in tubes preceding and succeeding the major peak of inhibitory activity in the RIA.

All of the engineered CS polypeptide was recovered in the yeast extract supernatant obtained after boiling, which was devoid of most yeast proteins (Fig. 3). The few remaining contaminants as well as large amounts of nucleic acids were removed in the subsequent steps of purification. In three separate runs, the yields of purified antigen were between 15 and 25 mg per liter of culture. The purified CS antigen was soluble in water at concentrations >50 mg/ml. Its extinction coefficient at 280 nm at a concentration of 1 mg/ml (determined by Kjeldahl nitrogen analysis) was 0.2. The SDS-PAGE analysis revealed a doublet with 43,000–45,000 M_r. This is a much higher M_r than that expected from the calculated molecular weight of 22.7 kD. However, several CS proteins or engineered repeat domains migrate abnormally in SDS-PAGE (23, 24), probably due to the low proportion of hydrophobic residues. Indeed, by isoelectric focusing under denaturing conditions, a single band was detected with a pI of 4.3. The purity of the preparation was also assessed by immunoprecipitation of a radiolabeled sample by the mAb 2F2 and capture of the immune complexes with Sepharose–protein A. In several experiments, 80 ± 5% of the labeled protein was specifically recognized by the antibody.

A sample of the protein was subjected to six steps of NH_2-terminal sequencing analysis and the major peaks of amino acids were AEPRNP. This sequence

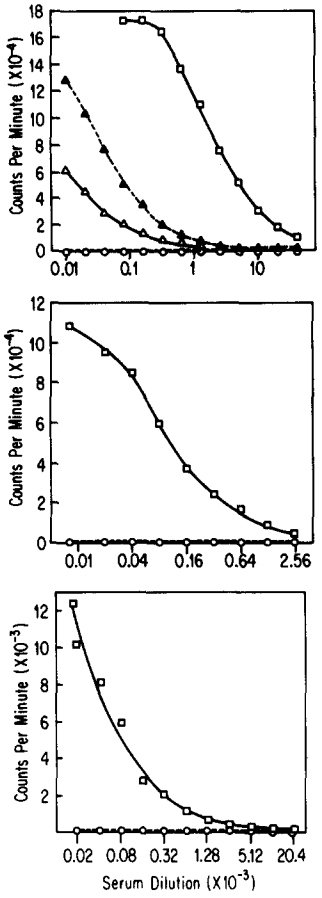

FIGURE 4. Antibody response of Swiss-Webster mice to the yeast-engineered *P. vivax* CS polypeptide. Antibodies were detected by solid phase IRMA. Assays were run in duplicate and the averages did not differ by more than 15% from the experimental values. Shown are antibody titrations against the engineered CS protein (*top*), the Pro79-Pro87 peptide (*middle*) and the repeat peptide (*bottom*). Symbols represent pooled sera from bleeding obtained after the first antigen injection (△), first booster (▲), and second booster (□), and normal mouse serum (○).

corresponds to that of the predicted NH_2-terminal of the expressed polypeptide, except that the engineered NH_2-terminal methionine is absent, probably due to the presence in the yeast extracts of a specific aminopeptidase. This is not unprecedented, since the HIV reverse transcriptase has been shown to be thus processed (25). Also, recombinant human superoxide dismutase (hSOD), a protein that is normally acetylated in human cells, is appropriately modified by methionine removal and acetylation in yeast (26).

Three groups of 10 female Swiss-Webster 8–12-wk-old mice were injected intramuscularly three times at 3 wk intervals with 50, 100, or 250 μg of the purified recombinant protein adsorbed onto aluminum hydroxide. Preceding each booster, and 10 days after each booster injection, the mice were bled and the sera were assayed for the presence of antibodies using a solid-phase IRMA assay.

3 wk after the initial injections of antigen, all mice already had serum antibodies against the recombinant protein. After the second booster, reciprocal titers were ≥20,000 (Fig. 4). We found no significant difference in the geometrical means of serum titers between the groups of mice receiving increasing antigen doses. To define the domains of the recombinant antigen recognized by the antibodies,

TABLE I
Neutralization of the Infectivity of P. vivax Sporozoites In Vitro*

Origin of serum	Dilution	EEF‡	Inhibition§
			%
Preimmune	1:20	122 ± 12	
Immune	1:50	12, 10	91.0
	1:250	12, 11	90.6
	1:1,000	33, 27	75.4
	1:5,000	74, 68	41.8

* 5.10^5 ONG strain *P. vivax* sporozoites added to each culture.
‡ EEF, number of intracellular (exoerythrocytic) forms. In the case of preimmune sera, the numbers represent the mean ± SD of the EEF seen in four coverslips incubated with a 1:20 dilution of serum; the numbers that follow are the numbers of EEF in two coverslips incubated with various dilutions of immune sera.
§ Calculated as 100 − [(mean experimental values/mean of controls) × 100].

we performed IRMAs using as antigen two synthetic peptides, one representing the repeat domain $(DGQPAGDRA)_2$ and the other representing amino acids Pro 79 through Pro 87 of the protein encoded by the CS gene. All sera reacted with the two peptides, but the titers were significantly higher against the repeat peptide.

In other experiments we found that ~50% of the reactivity of the pooled sera with the engineered protein was inhibited by the synthetic repeat peptide. We first incubated samples of a 1:3,000 dilution of the pooled serum from mice (obtained after the second booster injection) with increasing concentrations of the 18-amino-acid repeat peptide. As a control, samples of serum were incubated with the same concentration of another 24-mer peptide with a different and unrelated sequence of amino acids representing the repeat peptide from the *Plasmodium knowlesi* CS protein (23). After a 1-h incubation at room temperature, 30 μl of the mixtures (or of controls containing no peptides) were added to wells of plates precoated with the engineered CS polypeptide. After 1 h of incubation the wells were washed, incubated for 1 h with radiolabeled goat anti–mouse Ig, washed again, and counted. The results demonstrated that the repeat peptide (but not the control peptide) inhibited in a dose-dependent fashion the reaction between the antibodies and the engineered CS protein. A maximum of 50% inhibition was obtained with a 10^{-3} M concentration of the synthetic peptide (not shown).

A pool of sera from mice receiving the lowest dose of antigen and obtained 10 days after the second booster injection was assayed both for reactivity with *P. vivax* sporozoites by indirect immunofluorescence (IFA) of glutaraldehyde-fixed parasites and for the ability to neutralize the parasite infectivity in vitro. The pooled sera reacted strongly with *P. vivax* sporozoites, as shown by IFA (reciprocal titers of 1,000) and effectively blocked invasion of a hepatoma cell line in vitro (Table I). Significant inhibition of invasion was detected at dilutions of 1:5,000. As mentioned above, the sera also contained antibodies to the sequence

of amino acids Pro 79 to Pro 87 found upstream and immediately flanking the repeats. Although this sequence includes five amino acids that are conserved in all CS proteins sequenced to date, the antibodies did not react with glutaraldehyde-fixed sporozoites from other rodent or human malaria parasites. Additional experiments are necessary to determine the fine specificity of these antibodies and to study their possible role in the serum-mediated neutralization of the *P. vivax* sporozoites.

We show here that large amounts of a homogeneous *P. vivax* CS antigen can be isolated from the engineered yeast cultures. This high yield, the simplicity of the purification procedure, the ease with which yeast grows in large volumes of inexpensive media, and the convenient induction of expression of the polypeptide at the fermentation level, suggest that this system is amenable to large-scale production of a malaria vaccine. Yeast expression systems have demonstrated applicability to the production of heterologous proteins with clinical uses (16, 27, 28). Indeed, yeast-derived recombinant hepatitis B vaccines have been shown to be a safe and effective vaccine in humans (29). The convenient construction scheme described here can be used to express other portions of the CS molecule. For example, we also produced large quantities of a protein (vivax-2) encompassing Ala 77 to Leu 335 of the CS polypeptide sequence in an attempt to raise antibodies to conserved COOH-terminal regions of the molecule.

Ideally, a sporozoite malaria vaccine should contain both B and T cell CS protein epitopes to stimulate the production of neutralizing antibodies and also of CS-specific memory Th cells. The expansion of the population of T cells would insure that a secondary immune response would be triggered upon encounter with the invading sporozoites, and that IFN-γ would be produced to inhibit the development of the remaining exoerythrocytic, intrahepatic forms (30, 31). In mice, however, T cell recognition of the repeats of the *P. falciparum* CS protein is severely restricted genetically. Among 14 inbred strains, with 9 different H-2 haplotypes, only mice bearing H-2^b were responsive (32–34). This finding raises the possibility that a vaccine containing only repeats may not be recognized by the T cells of all humans. Therefore, at least until the structures representing the T cell epitopes of CS protein are known and can be engineered into subunit vaccines, these should contain as wide, and as faithful a representation of the native CS molecules as possible (35).

The yeast antigen here described contains large segments of the CS protein of *P. vivax* in addition to the repeat domain, and no other covalently bound extraneous materials. Mice produced high titers of antibodies against the repeats and another highly conserved region of the CS protein of *P. vivax*, after administration of the protein adsorbed onto alum, an adjuvant that can be used in humans. The antibodies recognized the authentic CS protein and at high dilutions they inhibited the invasion of hepatocytes by *P. vivax* sporozoites in vitro. Moreover, T-dependent secondary antibody responses in vitro were obtained by incubation of lymphoid cells from these mice with *P. vivax* sporozoite extracts (Nardin, manuscript in preparation). It appears, therefore, that the yeast antigen has potential advantages over other types of subunit vaccines consisting of recombinant fusion proteins, or of synthetic peptides coupled to nonrelevant carrier molecules. It remains to be determined whether this type of vaccine will

stimulate the production of antibodies and of CS protein–specific T cells in a human population.

Summary

DNA coding for 234 amino acids of the circumsporozoite (CS) protein of *Plasmodium vivax* was incorporated into yeast expression vectors. The DNA encoded all the repeat domain and codons for a highly conserved sequence, KLKQP, found in CS proteins from all malaria parasites. Yeast cells transformed with these autonomously replicating plasmids expressed, upon induction, high levels of the CS polypeptide. The malaria antigen was purified in good yields from yeast extracts and was injected into mice using alum as adjuvant. The antibodies recognized the authentic CS protein, and at high dilutions, they inhibited the invasion of hepatocytes by sporozoites in vitro.

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