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RESEARCH ARTICLE

Expression of *Plasmodium falciparum* Circumsporozoite Proteins in *Escherichia coli* for Potential Use in a Human Malaria Vaccine

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Abstract. *The circumsporozoite (CS) protein of the human malaria parasite* Plasmodium falciparum *may be the most promising target for the development of a malaria vaccine. In this study, proteins composed of 16, 32, or 48 tandem copies of a tetrapeptide repeating sequence found in the CS protein were efficiently expressed in the bacterium* Escherichia coli. *When injected into mice, these recombinant products resulted in the production of high titers of antibodies that reacted with the authentic CS protein on live sporozoites and blocked sporozoite invasion of human hepatoma cells in vitro. These CS protein derivatives are therefore candidates for a human malaria vaccine.*

The feasibility of immunization against the sporozoite stage of malaria has been established. Irradiated sporozoites have been used to immunize and protect both man and animals (*1*). This protection is correlated with antibody to a protein on the surface of the sporozoite—circumsporozoite (CS) protein (*2–6*). Monoclonal antibodies (Mab's) to the CS protein block infection with sporozoites in vitro and protect animals in vivo (*3, 4, 6*).

Recently, Dame *et al.* (*7*) cloned and sequenced the complete CS gene of the human malaria parasite *Plasmodium falciparum*. The gene encodes a protein of 412 amino acids. This protein has a sequence typical of a membrane protein with an NH_2-terminal signal peptide and a COOH-terminal anchor domain. The most striking feature of this polypeptide is a large central repeat domain composed of 37 tetrapeptides with the sequence Asn-Ala-Asn-Pro interspersed with four tetrapeptides with the sequence Asn-Asp-Val-Pro. This general structure is analogous to that of the CS protein of the simian malaria parasite *P. knowlesi* (*8*), although the overall sequence homology between the CS protein of *P. falciparum* and *P. knowlesi* is very low. In fact, only two regions of approximately 15 amino acids each, in the charged sequences flanking the repeat domain, are conserved (*7*).

Protection by Mab's to the CS protein is both species- and stage-specific and, in the case of *P. knowlesi*, Mab's react with the 12-amino-acid repeat region of the CS protein (*9*). These Mab's also bl the binding of polyclonal antisera to CS protein in a radioimmunometric say (*10*). Thus, Zavala *et al.* (*10*) p posed and Dame *et al.* (*7*) confirmed t the repeat domain was the immu dominant region of the CS protein. F different Mab's to the CS protein of *falciparum* recognized synthetic p tides of various lengths corresponding portions of the repeat region (*7*). T immunodominant repeat region mi thus form the basis for a malaria vacc (*7, 11*). That such a vaccine would be widespread use is indicated by the fi ing that the CS gene is highly conser in *P. falciparum* isolates from many g graphic areas (*12*). Here we describe efforts to develop a vaccine against sporozoite stage of *P. falciparum* us proteins containing tandem repeats the CS tetrapeptide sequence produ in *Escherichia coli*.

Expression of the P. falciparum *protein in* E. coli. A recombinant p mid (pUC8 clone 1) containing the I RI insert from λmPf1 (*7*) was the sou of the gene encoding the *P. falcipar* CS protein. This 2337 base pair (fragment contains the entire CS g (Fig. 1A). Since the sequence of the f 16 amino acids of the CS protein characteristic of a cleaved signal pept (*7*), these amino acids are presuma absent from the mature CS protein sporozoites. Restriction endonucle Stu I cleaves the CS gene in the 1 codon of the sequence. Thus, a 1216 Stu I–Rsa I fragment from pUC8 clon (Fig. 1A) should encode all but the f two amino acids of the mature CS p tein predicted from the sequence This fragment was isolated and liga into the λPL *E. coli* expression plasm pAS1 (Fig. 1B) (*13, 14*), which had b cut with Bam HI and treated with D polymerase to create a blunt-end. In resulting plasmid, pCSP, the coding gion of the CS protein is fused, in fra to the translation initiation codon a cent to the Bam HI site in pAS1 (*13,*

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The protein predicted by this construction contains 394 amino acids of the mature CS protein fused to three amino acids (Met-Asp-Pro-) at its NH_2-terminus which are derived from the vector sequence (CSP, Fig. 2).

This construct was examined for production of the CS protein. Expression was monitored by immunoblot analysis (*15*) with a pool of five monoclonal antibodies specific for the CS protein of *P. falciparum* (*7*). The immunoreactive species obtained ranged in molecular weight from 30,000 to 65,000 (Fig. 3A, lane 2) indicating that the CS protein (i) is unstable when produced in *E. coli* and (ii) migrates in an anomalous fashion on sodium dodecyl sulfate–polyacrylamide gels (SDS-PAGE), since the expected molecular weight of the full-length CS protein is approximately 44,000. The results were similar when we used a λgt11 expression system (*7*). None of these individual products were present in amounts sufficient to allow their isolation from bacterial extracts.

We then prepared fragments in which only the repeat sequence would be expressed because (i) immunogenicity studies with synthetic peptides corresponding to the repeat region or to the conserved sequences [see regions I and II in (*7*)] of the CS protein showed that only antibodies to the repeat region peptides had biological activity (*16*), (ii) heterogeneity and low levels of expression were observed for the full-length product, and (iii) the immunodominant epitope (or epitopes) of the CS protein resides in the repeat domain (*7, 10*). We excised a 192-bp Xho II fragment from the CS gene (Fig. 1A) coding for 16 tetrapeptide repeats, and ligated it into the Bam HI site of pAS1 (Fig. 1B). Three clones were isolated—pR16tet$_{86}$, pR32tet$_{86}$, and pR48tet$_{86}$—which had inserts of one, two, or three Xho II fragments, respectively. The proteins synthesized from these plasmids, R16tet$_{86}$, R32tet$_{86}$, and R48tet$_{86}$, would have 15, 30, or 45 (Asn-Ala-Asn-Pro) tetrapeptide repeats and 1, 2, or 3 (Asn-Val-Asp-Pro) tetrapeptide repeats, respectively (Fig. 2). In each case, the repeat segment was fused to a downstream open reading frame of 86 amino acids in the tetracycline resistance (Tet^r) region of the plasmid (Fig. 2). Thus, the expected molecular weights of the three products were approximately 17,000 for R16tet$_{86}$, 24,000 for R32tet$_{86}$, and 31,000 for R48tet$_{86}$.

The immunoblot analysis of the proteins expressed by these clones again revealed a heterogeneous set of products, the largest of which was greater in size than that expected in each instance (Fig. 3A). The sizes of the smallest immunoreactive degradation products appears to be proportional to the number of tetrapeptide repeats in each of the clones. Therefore, we reasoned that the instability of these proteins was due to degradation of the 86 amino acid COOH-terminus fused to the CS portion. To produce a more homogeneous product, we removed most of this large terminal region by deleting a 14-bp Ban II fragment (Fig. 1B) from the Tet^r region in the pR16tet$_{86}$, pR32tet$_{86}$, and pR48tet$_{86}$ constructions. This resulted in a shift of the reading frame at that point and the intro-

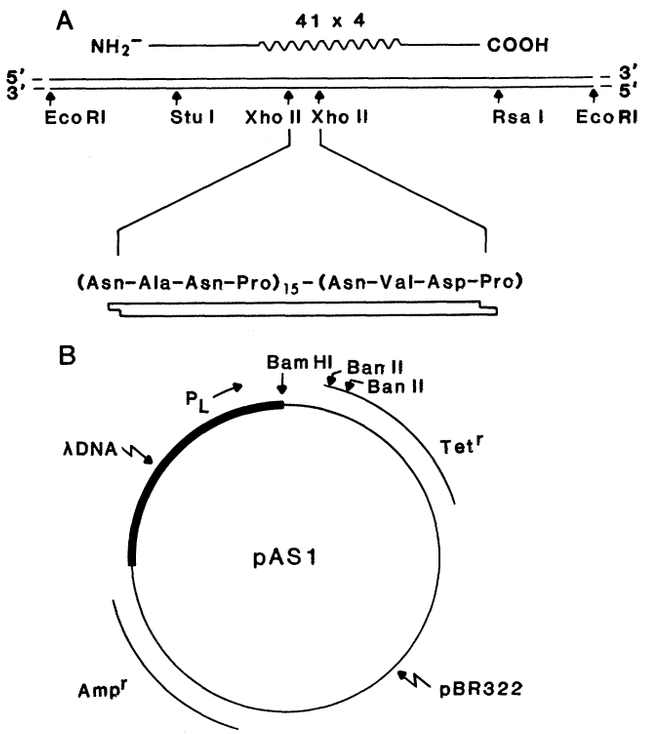

Fig. 1. (A) Schematic representation of the Eco RI insert from λmPf1 (*7*) containing the CS gene and a diagram of the protein encoded by this gene showing the 41 tetrapeptide repeats as a wavy line. The 192-bp Xho II fragment codes for 15 Asn-Ala-Asn-Pro tetrapeptide repeats and one Asn-Val-Asp-Pro repeat, as shown. (B) Diagram of the pAS1 *E. coli* expression plasmid (*13, 14*). The segments of DNA derived from λ phage and pBR322 are indicated. The complete ampicillin resistance region (Amp^r) and part of the Tet^r are from pBR322. Expression of genes inserted into the Bam HI site is under control of the λPL promoter, whose transcription is controlled by a cI repressor protein. Vector constructs are therefore transformed and maintained in a cI$^+$ lysogenic host (MM294cI$^+$) to prevent expression of protein that may be toxic to the host strain. To control expression, the plasmid is transferred into a lysogen carrying a temperature-sensitive cI857 repressor (*22*). Bacteria can then be grown in the absence of expression (32°C, repressor active) and then shifted to 42°C to inactivate the repressor and turn on transcription from the P_L promoter (*23*).

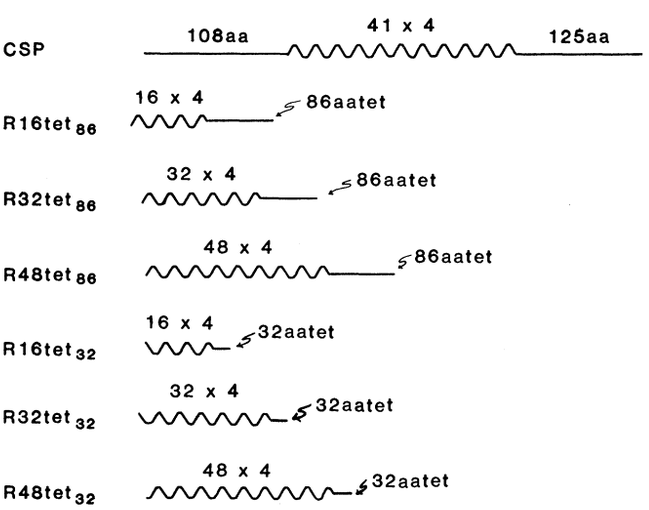

Fig. 2. Summary of CS protein derivatives produced in *E. coli*. The wavy lines represent the immunodominant tetrapeptide repeat unit and the number of repeats in each construct is indicated over this line. The number of amino acids flanking the repeat units is also indicated. The CSP construct contains 108 amino acids NH_2-terminal to the repeats, three of which are derived from the vector and 105 of which are from the CS gene. The COOH-terminal 125 amino acids are also derived from the CS gene. The R16tet$_{86}$, R32tet$_{86}$, and R48tet$_{86}$ constructs contain 16, 32, or 48 tetrapeptide repeats, respectively, fused to 86 amino acids derived from an open reading frame in the Tet^r region. Similarly, R16tet$_{32}$, R32tet$_{32}$, and R48tet$_{32}$ contain 16, 32, or 48 repeats repsectively, fused to 32 amino acids from this same open reading frame in the Tet^r region.

duction of a termination codon, in frame, 33 codons beyond the CS coding region. These truncated constructs, pR16tet$_{32}$, pR32tet$_{32}$, and pR48tet$_{32}$, should now have COOH-terminal tails of only 32 amino acids derived from the plasmid sequences (Fig. 2).

We examined the expression of these new constructs in *E. coli* strain N5151. In contrast to our previous results, we observed a single major immunoreactive species for each construct (Fig. 3A). The mobilities of these proteins were similar to the smallest species in the corresponding constructs with 86 amino acid tails. This suggested that our hypothesis concerning the instability of the 86 amino acid tail was correct. Although the observed molecular weights on SDS-PAGE were still approximately twice that expected, the migration of each of the proteins with the 32 amino acid tail was again proportional to the number of tetrapeptide repeat units.

Table 1. Circumsporozoite reactivity of antisera to R16tet$_{32}$, R32tet$_{32}$, and R48tet$_{32}$. Circumsporozoite precipitin reactions were performed essentially as described by Vanderberg *et al.* (*2*). Salivary gland sporozoites from *P. falciparum* (500 to 1000 sporozoites, suspended in Medium 199, total volume 5 μl) were mixed with 5 μl of serum on a microscope slide, sealed under a cover slip rimmed with petroleum jelly, and incubated at 37°C for 1 hour. Reactions were evaluated by phase-contrast microscopy at ×400 magnification. Twenty-five random sporozoites were examined for each serum sample and the number of CSP positive organisms are indicated. The degree of CSP reactivity as described by Vanderberg *et al.* (*2*) is shown in parentheses (−, no CSP reactivity detectable; 2+, a granular precipitate on the surface of the sporozoites; 4+, a long, threadlike filament at one end of the sporozoites). Normal mouse serum, or serum from mice immunized with CFA alone, produced no detectable CSP reactivity in parallel assays.

Adjuvant	Reactivity of antisera to		
	R16tet$_{32}$	R32tet$_{32}$	R48tet$_{32}$
None	0/25 (−)	17/25 (2+)	21/25 (4+)
CFA	23/25 (4+)	21/25 (4+)	21/25 (4+)
Aluminum hydroxide	25/25 (4+)	25/25 (4+)	16/27 (2+ to 4+)

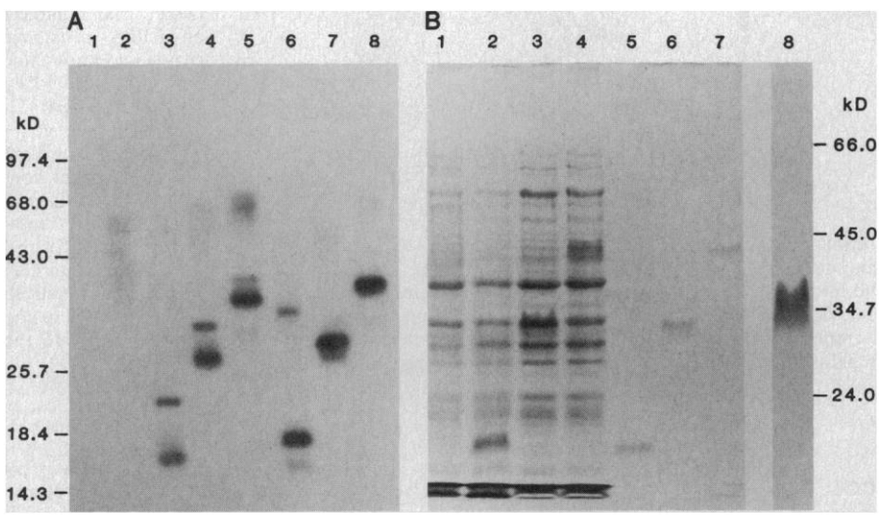

Fig. 3. (A) Western blot analysis of CS protein products synthesized in *E. coli* (*15*). A pool of five monoclonal antibodies reactive with the tetrapeptide repeat domain of the *P. falciparum* CS protein was used (*7*). *Escherichia coli* strain N5151 (cIts857) carrying the constructs (see text and Fig. 2) were grown at 32°C to an absorbance at 650 nm of 0.6 and temperature induced for expression as described (*23*). Aliquots (1 ml) of cells were centrifuged and the pellets were resuspended in lysis buffer [10 m*M* tris-HCl, *p*H 7.8, 25 percent glycerol (by volume), 2 percent 2-mercaptoethanol, 2 percent sodium dodecyl sulfate (SDS), and 0.1 percent bromophenyl blue] and incubated at 105°C for 5 minutes. Proteins were separated by SDS-PAGE (13 percent acrylamide with acrylamide and bis-acrylamide in a 30:0.8 ratio). Total cell extracts were prepared before temperature induction of the *E. coli* host containing the CSP construct (lane 1) and 2 hours after temperature induction of the strains carrying the constructs described in Fig. 2. Lane 2, CSP; lane 3, R16tet$_{86}$; lane 4, R32tet$_{86}$; lane 5, R48tet$_{86}$; lane 6, R16tet$_{32}$; lane 7, R32tet$_{32}$; lane 8, R48tet$_{32}$. The positions of prestained molecular weight markers (Bethesda Research Laboratories) are indicated. (B) Accumulation of CS proteins in *E. coli*. Temperature inductions, preparation of total cell extracts, and SDS-PAGE analysis of proteins were as described above. Proteins were stained with Coomassie Brilliant Blue R-250. Lane 1, total cell extract of an uninduced *E. coli* strain containing the pR16tet$_{32}$ construct. Lanes 2 to 4, total cell extracts of induced cultures of pR16tet$_{32}$, pR32tet$_{32}$, and pR48tet$_{32}$, respectively. To isolate the CS proteins, induced *E. coli* were lysed and total soluble proteins were collected after centrifugation. The CS protein derivatives were selectively precipitated with ammonium sulfate and purified by standard chromatographic techniques including butyl reversed-phase high-performance liquid chromotography (*18*). Lanes 5 to 7, 1 μg each of purified R16tet$_{32}$, R32tet$_{32}$, and R48tet$_{32}$, respectively. Lane 8, 20 μg of R32tet$_{32}$. The positions of molecular weight markers (Sigma) are indicated.

That these species accumulated in *E. coli* was shown by staining with Coomassie blue (Fig. 3B). We estimated that these products represent approximately 5 percent of total cell protein. It is interesting that the high level of expression of these proteins in *E. coli* was obtained in spite of the unusual amino acid composition of the products—approximately 50 percent asparagine, 25 percent alanine, and 25 percent proline. These results demonstrate that *E. coli* can accommodate this highly repetitive codon usage. The unusual amino acid content probably also accounts for the anomalous migration of these proteins on SDS-PAGE. Similar artifacts in gel mobility have been observed for other proteins with a high proline content (*17*).

The R16tet$_{32}$, R32tet$_{32}$, and R48tet$_{32}$ polypeptides were purified to homogeneity from bacterial extracts at a yield of approximately 60 mg of protein from 30 g (wet weight) of induced *E. coli* (Fig. 3B) (*18*). Amino acid composition determinations on these products were consistent with the expected values (*19*). The purified proteins were then used for immunogenicity studies.

Products of the E. coli *recombinant elicit high titers of antibody to the CS protein.* The purified recombinant protein constructs R16tet$_{32}$, R32tet$_{32}$, and R48tet$_{32}$ were inoculated into C57BL/6 mice to evaluate their immunogenicity. Proteins were injected alone, with aluminum hydroxide, or emulsified with complete Freund's adjuvant (CFA). Serum samples from these immunized animals were screened in an enzyme-linked immunosorbent assay (ELISA) in which we used a synthetic 16 amino acid peptide, with the sequence (Asn-Ala-Asn-Pro)$_4$, conjugated to bovine serum albumin (*16*). It was shown previously that Mab's recognizing the CS protein also react with synthetic peptides corresponding to the repeat domain (*7*, *16*). The R16tet$_{32}$, R32tet$_{32}$, and R48tet$_{32}$ proteins all resulted in the production of antibody reacting with the synthetic peptide in the ELISA detectable at 7 days after the primary immunization. A booster dose of immunogen was given 4 weeks after the primary injection and serum samples were collected 1 week later and tested in the ELISA (Fig. 4). The R32tet$_{32}$ and R48tet$_{32}$, even when admin-

istered without adjuvants, were very immunogenic and produced high titers of antibody, whereas R16tet$_{32}$ was less effective in eliciting a response (Fig. 4). As expected, both alum and CFA enhanced the immunogenicity of the three proteins, and antibody was detected at reciprocal titers up to 102,000 in at least one regimen (Fig. 4). Similar results were observed with BALB/c mice.

These antisera also reacted strongly with authentic CS protein on *P. falciparum* sporozoites when tested in an indirect immunofluorescent antibody assay (IFA) (Fig. 5). Reactivity against *P. knowlesi*, *P. cynomolgi*, *P. vivax*, and *P. gallinaceum* was not detected by IFA, although weak reactivity of the antisera to R32tet$_{32}$ was seen with *P. berghei* (data not shown). This cross-reactivity between antisera to *P. falciparum* repeat regions and *P. berghei* sporozoites is consistent with previous data showing that some Mab's to the *P. falciparum* repeating epitope react with *P. berghei* sporozoites by IFA (*11*).

Antibodies to recombinant products possess biological activity associated with protective immunity. Protection associated with sporozoite-induced immunity to malaria is correlated with the circumsporozoite precipitin (CSP) reaction in sera (*1, 2*). In this assay, antisera are mixed with live sporozoites and, after incubation, the sporozoites are examined under a microscope. The appearance of a precipitate at the surface of the parasite indicates the presence of CSP reactive antibodies. When sera from mice immunized with R16tet$_{32}$, R32tet$_{32}$, or R48tet$_{32}$ were examined in this assay, strong CSP positive reactions were observed (Table 1). Even when administered without adjuvant, R32tet$_{32}$ and R48tet$_{32}$ produced antibody which gave positive CSP reactions, whereas, when given with CFA or aluminum hydroxide, all three constructs induced antibodies with strong CSP reactivity.

A second test that has been correlated with immunity to sporozoites is an in vitro assay which measures the inhibition of hepatoma cell invasion by live sporozoites (*20*). Hollingdale *et al.* used this assay to demonstrate that sera from immune humans and other animals block sporozoite invasion of human hepatoma cells in vitro. By means of this assay we showed that, even in the absence of adjuvant, the R32tet$_{32}$ and R48tet$_{32}$ proteins induced antibodies with strong blocking activity (Table 2). The R16tet$_{32}$ protein was less efficient in eliciting blocking antibodies except when administered with alum or CFA.

Taken together, the results from the

Table 2. Percentage inhibition of *P. falciparum* sporozoite invasion of HepG2-A16 human hepatoma cells in vitro. The assay was performed as described (*20*). The sera obtained from mice immunized with the R16tet$_{32}$, R32tet$_{32}$, and R48tet$_{32}$ constructs were tested for their ability to inhibit invasion of cultured cells by *P. falciparum* sporozoites. Briefly, the sera were diluted in culture medium and added to HepG2-A16 cell cultures to yield a final dilution of 1:20 (by volume). Cultures were received 12,000 to 40,000 salivary gland sporozoites and were incubated at 37°C in a 5 percent CO_2 atmosphere for 3 hours, rinsed with Dulbecco's phosphate-buffered saline (PBS), fixed in methanol, and rinsed twice with PBS. Sporozoites that had entered cells were visualized by an immunoperoxidase antibody assay (IPA) (*20*). The IPA was carried out by first treating the fixed cultures with a Mab to *P. falciparum* [2F1.1, see (*7*)], then incubating them with rabbit antiserum to mouse immunoglobulin G conjugated with horseradish peroxidase and staining them with 3,3-diaminobenzidine. The number of sporozoites that invaded cultured cells was determined by counting the intracellular parasites present in the entire preparation on a Nikon microscope at ×200 with a dark blue filter. Experiments were carried out either in duplicate or in triplicate and each cell culture within an experiment received an equal number of sporozoites. Inhibition was the percentage reduction of sporozoite invasion brought about by antisera to the protein construct compared to normal mouse serum controls where CS reactive Mab 2F1.1 (*7, 11*) gave 100 percent inhibition of invasion at dilutions of 1:20. Assays were run in duplicate and the mean and standard error of the mean (S.E.M.) were calculated. In all instances the S.E.M. was less than 10 percent.

Adjuvant	Percentage of inhibition by antisera to		
	R16tet$_{32}$	R32tet$_{32}$	R48tet$_{32}$
None	46	95	92
CFA	76	92	94
Aluminum hydroxide	100	100	96

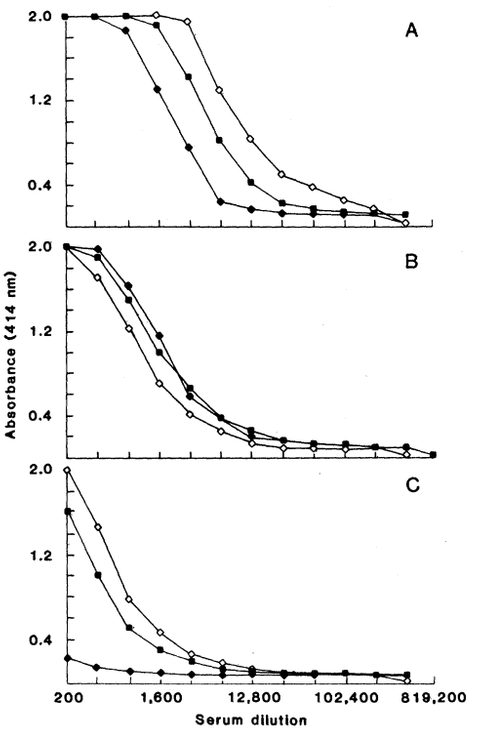

Fig. 4. Antibody responses of mice to recombinant *P. falciparum* CS protein. Purified R16tet$_{32}$, R32tet$_{32}$, or R48tet$_{32}$ were dialyzed against PBS and stored at −80°C. Proteins were mixed with PBS, aluminum hydroxide, or CFA to yield a 0.5-ml dose containing 50 μg of protein. CFA (Gibco) plus antigen in PBS were emulsified in a 1:1 ratio by agitation for 30 minutes. Aluminum hydroxide gel was diluted in PBS. Antigen was absorbed to the aluminum hydroxide at 4°C for 12 hours. After being allowed to settle for 12 hours, sufficient supernatant was discarded to yield 0.80 mg of aluminum hydroxide and 50 μg of recombinant protein per dose. C57BL/6 mice (6 to 8 weeks old) were immunized both subcutaneously and intraperitoneally with a total of 50 μg of protein (five animals per group). Booster injections were given 4 weeks later according to the same protocol as was used for the first injections, except that the group that initially received the immunogens in CFA received booster injections of the proteins emulsified in incomplete Freund's adjuvant. One week later, whole blood from all animals within a group was pooled, clotted overnight at 4°C, and centrifuged to separate the serum which was then stored at −80°C. An ELISA was used to test the pooled sera for their ability to react with a 16 amino acid synthetic peptide antigen consisting of four repeats of the *P. falciparum* CS protein (Asn-Ala-Asn-Pro)$_4$ (*7, 16*). The antigen (50 μl diluted to 20 μg/ml with 0.01*M* PBS, *p*H 7.4) coupled to bovine serum albumin was coated onto the wells of microtiter plates (*16*) and held overnight at room temperature. Well contents were then aspirated, filled with blocking buffer (1.0 percent BSA, 0.5 percent casein, 0.005 percent thimerosal, and 0.0005 percent phenol red in PBS) and held for 1 hour. Mouse sera were diluted serially in blocking buffer and 50 μl was added to each well. After 2 hours, the wells were washed three times with PBS containing 0.05 percent Tween 20 (PBS-Tw 20). Fifty microliters of goat antiserum to mouse immunoglobulin G (H + L chains) (Bio-Rad) conjugated to horseradish peroxidase and diluted 1:500 with 10 percent heat-inactivated human serum in PBS was added to each well. After 1 hour the wells were washed three times with PBS-Tw 20, and 150 μl of substrate [1 mg of 2,2′-azino-di(3-ethyl-benzthiazoline sulfonic acid-6) per milliliter of 0.1*M* citrate-phosphate buffer, *p*H 4.0, with 0.005 percent hydrogen peroxide] was added to each well. Absorbance at 414 nm was determined 1 hour later with an ELISA plate reader (Titertek Multiskan). Assays were run in triplicate and the means and S.E.M. were calculated. In all instances the S.E.M. was less than 0.09. Symbols for the antisera are: ◆, R16tet$_{32}$; ◇, R32tet$_{32}$; ■, R48tet$_{32}$. Animals were immunized with (A) proteins in CFA; (B) proteins with aluminum hydroxide; or (C) proteins in PBS without adjuvant.

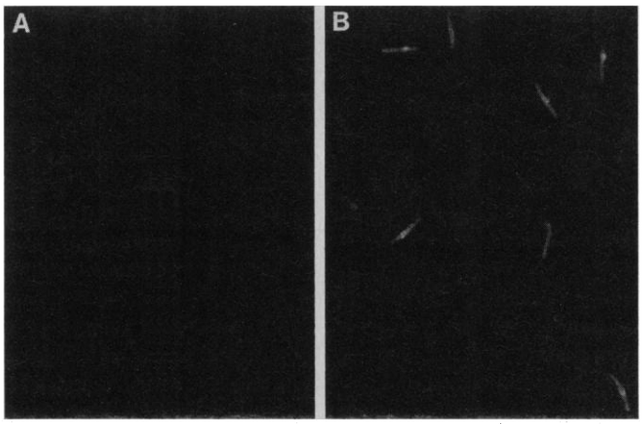

Fig. 5. Immunofluorescence antibody (IFA) assays. Sporozoites from the salivary glands of mosquitoes (*24*) were diluted in saline or Medium 199 containing 0.5 percent BSA, counted with a hemacytometer, and diluted to 2000 to 5000 sporozoites per 10 μl. Aliquots (10 μl) spread onto wells of multiwell printed IFA slides were air-dried at room temperature and stored at −80°C. The IFA's were initiated by spreading 20-μl volumes of serum, diluted 1:100 with blocking buffer (see Fig. 4), onto the dried sporozoites. After 20 minutes of incubation in a moist chamber at room temperature, the serum solutions were aspirated and the spots washed with two drops of PBS. Aliquots (20 μl) of goat antiserum to mouse antibody conjugated with fluorescein isothiocyanate and diluted 1:40 with blocking buffer were then added to each spot. After 20 minutes of incubation the spots were again washed with PBS, mounted in glycerol, and examined under ultraviolet light at ×500 magnification for fluorescence. (A) Nonimmune serum; (B) pooled sera from mice immunized with $R32tet_{32}$.

ELISA, the immunofluorescence assay, the CSP assay, and the in vitro inhibition assay all demonstrate the strong immunogenic potential of these *E. coli*–produced CS protein derivatives.

Discussion. Development of an efficacious sporozoite vaccine has not been feasible because of the inability to culture sporozoites in vitro for use in an attenuated vaccine or of isolating adequate amounts of CS protein for a subunit vaccine. The cloning of the *P. falciparum* CS gene (*7*) made possible the production of CS protein that might be used for vaccine preparation. Of the seven constructs containing the repeat region of the CS protein that we produced in *E. coli*, four of them, CSP, $R16tet_{86}$, $R32tet_{86}$, and $R48tet_{86}$, were synthesized by the bacteria but were unstable. Although total yields may have been adequate, the heterogeneity of the product precluded further development of the material. In contrast, $R16tet_{32}$, $R32tet_{32}$, and $R48tet_{32}$ were efficiently expressed in *E. coli* and sufficient quantities of a homogeneous product could be purified for immunogenicity studies.

Studies in mice showed that high titers of antibody to the $R32tet_{32}$ and $R48tet_{32}$ could be achieved even when the proteins were administered without adjuvants, whereas $R16tet_{32}$ gave a relatively weak response in the absence of adjuvants. These data suggest that the length of the immunogen may play a critical role in determining the level of response to the protein. This may be due to conformational differences among the molecules or simply to the number of epitopes per molecule. The design and flexibility of our construction scheme enables the insertion of additional Xho II fragments to generate proteins with greater numbers of tetrapeptide repeats. We recently produced proteins with up to 112 of these repeats to further examine the relation of size and conformation to immunogenicity. These data may have implications for the development of vaccines based on synthetic peptides, since molecules of this length cannot be produced easily by solid-phase synthesis.

In any synthetic vaccine, the antibody to the immunogen must both recognize the authentic molecule and possess the necessary biological properties to confer protection. That the antibodies produced against the recombinant CS protein derivatives in our experiments may be protective in vivo is indicated by the following. First, in both the immunofluorescence assay and the CSP reaction the antibody to the *E. coli* products reacted with the surface of the sporozoite and thus recognized the authentic CS protein. The presence of CSP antibody in humans and other animals is an important correlate of protective immunity (*1*, *4*, *6*). Second, antibodies to the protein constructs inhibited sporozoite invasion of human hepatoma cells in vitro. In studies with primary human hepatocyte cultures, up to 97 percent inhibition of sporozoite invasion was obtained with these same antisera (*21*).

The immune responses to these recombinant proteins as assessed by ELISA titer, surface reactivity (as shown by IFA and CSP), and blocking of sporozoite invasion were enhanced dramatically by use of either CFA or aluminum hydroxide. Complete Freund's adjuvant cannot be used in humans since it causes fever, produces granulomas, and results in tuberculin hypersensitivity. Aluminum hydroxide, however, has a history of safe use in humans. It is currently used as an adjuvant in established vaccines such as diphtheria and tetanus toxoid as well as one novel recombinant-based vaccine against hepatitis B. Thus our data show that polypeptides that contain tandem repeats of the CS protein can be expressed at high levels in *E. coli*, can be readily purified, and can induce high titers of antibody that are likely to confer protection in vivo. These recombinant proteins may be appropriate for development of a human malaria vaccine.

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