HEPATITIS B SURFACE ANTIGEN AS CARRIER MATRIX FOR THE REPETITIVE EPITOPE OF THE CIRCUMSPOROZOITE PROTEIN OF *PLASMODIUM FALCIPARUM*.

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The immune dominant repetitive epitope of the circumsporozoite protein of *Plasmodium falciparum* was fused to the pre-S2 region of the surface antigen of hepatitis B virus within an expression cassette. Yeast cells transformed with vectors carrying the fusions synthesized hybrid proteins that assembled into particles similar to those formed in human serum from the surface antigen alone. These hybrid protein particles expose the circumsporozoite epitope on their exterior and induce a high titer antibody response towards the circumsporozoite epitope in immunized animals. The antibodies show typical circumsporozoite precipitin reactions and inhibit invasion of hepatoma cells *in vitro*, both indicative of a protective immune response. This formulation could prove useful as a candidate sporozoite malaria vaccine for human clinical trials.

Recently, major steps have been taken towards the development of a vaccine against the sporozoite stage of *Plasmodium falciparum*, the parasite which causes the most severe form of human malaria.[1] The gene encoding the circumsporozoite (CS) protein of the parasite has been cloned and sequenced, revealing a protein containing a large central segment consisting of 37 repeats of the tetrapeptide Asn-Ala-Asn-Pro interspersed with three repeats of the variant sequence Asn-Val-Asp-Pro[2,3]. Antibodies raised against synthetic peptides or recombinant proteins expressed in *Escherichia coli*, derived from the amino acid sequence of this repeat region, have been shown to mediate the circumsporozoite pecipitin reaction (CSP) and inhibit sporozoite invasion of liver cells *in vitro*[4–6]. These biologic activities correlate with protection against sporozoite challenge[7–11] and indicate that this repeat region is a suitable target for vaccine development. Recently, two candidate sporozoite vaccines have been tested in man[12,13]. The vaccine used by Herrington et al.[12] is a synthetic peptide containing three tetrapeptide repeats, conjugated to tetanus toxoid. Ballou et al.[13] used a vaccine prepared by recombinant DNA consisting of 32 tetrapeptide repeats and a 32 amino acid-long tail encoded by the vector (R32tet32)[4]. Both studies indicated a correlation between the level of antibody directed against the repeat and protection against sporozoite challenge. However, high doses of these vaccines are required to obtain protection and there are no booster effects on repeated injections. An improved vaccine formulation both in terms of antigen presentation and carrier system to provide T-cell help, could lead to a more efficacious vaccine.

In this study we report the expression in yeast of a hybrid protein consisting of 64 amino acids from the repeat region of the *P. falciparum* CS protein fused to a 42 amino acid sequence from the pre-surface (pre-S2) region of hepatitis B surface antigen, followed by 226 amino acids from the HBsAg itself. We determined if such a hybrid protein would assemble into a particle similar to HbsAg and if the circumsporozoite epitopes would be exposed on such a particle. We have also conducted and report the results of immunogenicity studies designed to (1) determine if hybrid antigen would elicit antibodies to both the CS protein of *P. falciparum* and HBsAg, and (2) if such antibodies show typical CS precipitin reactions and inhibit sporozoite invasion of hepatoma cells, suggesting a protective immune response.

RESULTS

Insertion of CS sequences into the pre-S2 region of hepatitis B surface antigen. To create fusions between the CS repetitive epitope and the pre-S2 region of the HBsAg protein, Sau3A DNA fragments of 24 and 192 bp were cloned into the BamHI site of the expression cassette of pRIT10911 (Fig. 1A). The cassette consists of a yeast TDH3 promoter fragment fused to 42 codons of the HBsAg pre-S2 sequence and the 226 codons of the HBsAg coding sequence (adw serotype) followed by a transcription termination region from the ARG3 gene.

The BamHI site in the cassette, located at the TDH3 Met initiation codon (ATGGATCC), is in phase with the Sau3A sites in the repetitive CS epitope so as to give translational fusions with the pre-S2 region. The cassettes with the CS-HBsAg fusions were then recloned as HindIII fragments on the yeast vector YEp13[14] to give recombinant plasmids pRIT12573 and pRIT12574 (Fig. 1.A), which were transformed into the recipient *Saccharomyces cerevisiae* strain 10S44C (pep4-3, leu 2-3, leu2-112).

The subsequent hybrid protein R2HBsAg, synthesized from pRIT12573, would then contain 277 amino acids, 8 from the CS repeat region (one tetrapeptide repeat Asn-Ala-Asn-Pro), 42 from the pre-S2, and 226 amino acids from the HBsAg gene. The protein R16HBsAg, synthesized from pRIT12574 would contain 333 amino acids and is similar to R2HBsAg with the exception that it contains 15 tetrapeptide repeats (Fig. 1B). In both hybrid proteins one tetrapeptide repeat Asn-Val-Asp-Pro is also present resulting from the fusion of the CS sequences (-Asn-Val-) to the pre-S2 sequences (-Asp-Pro-).

Identification of the hybrid proteins. Expression of the hybrid proteins from pRIT12573 and pRIT12574 was monitored by immunoblot analysis of cellular proteins using a pool of five monoclonal antibodies specific for the tetrapeptide repeat domain of the CS protein of *P. falciparum*[2] and a monoclonal antibody specific for the denatured and reduced yeast derived HBsAg monomer. A protein of molecular weight of about 30K, R2HBsAg, reacting with both anti-CS and anti-HBsAg antibodies was detected among the proteins from cells containing pRIT12573 (Fig. 2, lane 3), and a 37K protein, R16HBsAg, in cells containing pRIT12574 (Fig. 2, lane 4). In the latter case, bands of lower molecular weight were also detected, suggesting some degradation of the hybrid protein. Control samples of cells containing plasmid pRIT10172, which does not contain any CS or HBsAg sequences showed no specific immune reaction (Fig. 2, lane 1) while analysis of cells carrying a plasmid containing the native HBsAg gene resulted in a band reacting only with anti-HBsAg (Fig. 2, lane 2).

These results demonstrate that hybrid proteins of the expected molecular weights containing both CS and HBsAg sequences are synthesized in yeast. Furthermore, while the band intensity in the anti-HBsAg reaction was about equal for the samples from pRIT12573 and pRIT12574 transformed cells, the intensity of the anti-CS reaction was much higher in the sample derived from cells containing the longer CS protein construct, pRIT12574, than the one from cells containing the small fusion protein, pRIT12573. This demonstrates a quantitative difference in the way the two proteins respond to CS antibodies and correlates with the fact that the R16HBsAg protein, contains more of the repetitive epitope of the CS protein than the hybrid protein, R2HBsAg.

Hybrid protein particles exposing the CS epitope. HBsAg synthesized in yeast is found in the form of particles[15] similar to the 22 nm particles isolated from human serum. These lipoprotein particles have a buoyant density in CsCl of 1.18 g/cm³. To examine the physical properties of the hybrid proteins synthesized in yeast, crude extracts of pRIT12573 and pRIT12574 transformed cells were subjected to CsCl equilibrium centrifugation. HbsAg and CS antigenic activities in the CsCl fractions were measured in an ELISA assay in which the antigen was first bound to immobilized anti-HBsAg and afterwards assayed for either anti-HbsAg or anti-CS binding capacity. The HBsAg and CS antigenic activities were found to coequilibrate in the gradients of cell extracts derived from both pRIT12573 and pRIT12574 transformed cells (Fig. 3B and C). The buoyant density of the hybrid proteins was found to be slightly higher (ρ= 1.20 g/cm³) than that for native yeast HBsAg (Fig. 3A) suggesting that the R2HBsAg and R16HBsAg hybrid proteins assemble into particles, as does the native HBsAg protein synthesized in yeast. These hybrid particles expose the CS sequences on their exterior as demonstrated by their capacity to react with anti-CS antibodies when bound to immobilized anti-HBsAg. Further evidence of the particulate structure of the hybrid protein was obtained by electron microscopy of purified antigen, which revealed particles of the same approximate size and shape as described for HBsAg[15] (Fig. 4).

As the antibody recognition of HBsAg is strongly dependent on conformational epitopes on the HBsAg particle, the ELISA assay would not detect free unassembled hybrid protein. To estimate efficiency of the assembly of hybrid proteins, an immunoblot analysis of the gradient fractions was performed, utilizing a monoclonal antibody that recognizes a denaturation- and reduction- resistent epitope on HBsAg monomer. The immunoblot analyses followed the ELISA profile accurately. No free HBsAg related monomer was found at the density for protein of about 1.3 g/cm³ indicating that the hybrid proteins R2HBsAg and R16HBsAg assemble as efficient as native HBsAg and that assembly is complete in the crude cell

TABLE 1 AUSRIA activity in crude cell extracts

Plasmid	Gene coding for	Protein (mg/ml)	HBsAg(RIA) (ng/ml)	HBsAg as ng/mg of total protein in extract
pRIT10172	–	5.6	0	0
pRIT12329	HBsAg	3.3	5700	1730
pRIT12573	R2HBsAg	3.6	4000	1110
pRIT12574	R16HBsAg	2.8	450	160

Preparation of crude cell extracts, protein determination and AUSRIA assay were performed as described in the Experimental Protocol.

TABLE 2 Development of anti-HBsAg titers in mUI/ml (AUSAB test) upon inoculation of R16HBsAg hybrid protein particles.

Animal	Adjuvant	Week 1	4	8	12
Mice	None	32	2	2	4
Mice	Alum	41	1	3	19
Control mice (PBS only)	Alum	0	0	0	0
Rabbit #36	Alum	14	454	4,471	3,382
Control rabbit (PBS only)	Alum	10	4	1	1

TABLE 3 Circumsporozoite precipitin (CSP) reactivity, and percent inhibition of sporozoite invasion (% ISI) of HepG2-A 16 hepatoma cells.

Animal(s) 1 week post 2nd boost	CSP Reactivity* (4+	2+	0)	Percent ISI**
Pooled mouse sera	14/25	9/25	2/25	91
Rabbit #36	22/25	3/25	0/25	96

* On 25 randomly selected sporozoïtes
Degree of CSP reactivity is shown in parenthese[11]: 0—No CSP reactivity detectable, 2+—granular precipitate on surface of sporozoite, 4+—threadlike filament from end of sporozoite.
** Intracellular sporozoïtes were visualized by a monoclonal antibody immunoperoxidase assay. The percentage inhibition of sporozoïte invasion was calculated as ([control-test]/control) × 100.

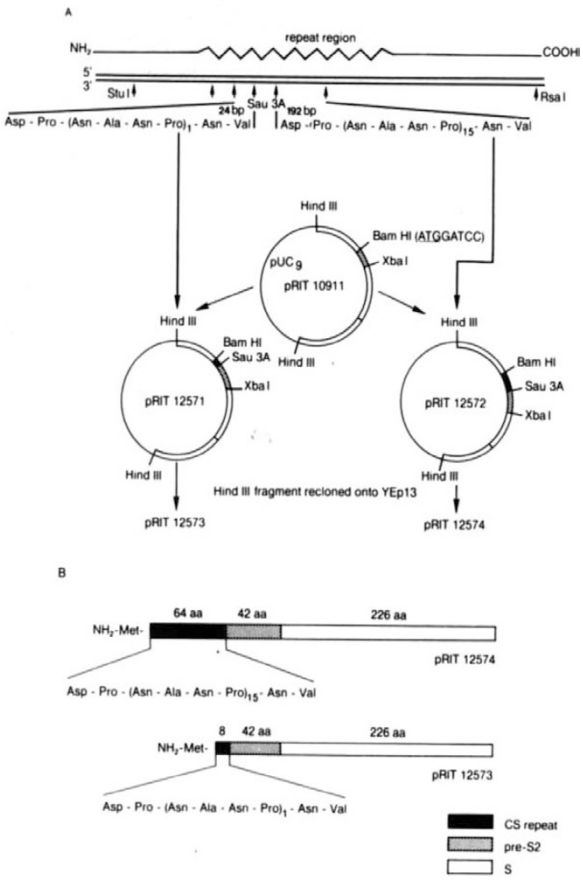

FIGURE 1 (A) Construction of plasmids for the synthesis of hybrid CS-HBsAg particles in yeast. The source of DNA encoding the repeat region of the CS protein was a 2.3 Kb EcoRI fragment from λ mpfl[2] recloned into pUC8. A 1215 bp StuI-RsaI sub-fragment was purified from a polyacrylamide gel and digested with Sau3A. The Sau3A digest was ligated into the BamHI site of the vector pRIT10911. pRIT10911 contains a 1050 bp HindIII-BamHI yeast glyceraldehyde-3P-dehydrogenase (TDH3) promoter fragment fused at the BamHI site to a 935 bp BamHI-HpaI fragment of HBV DNA encoding 42 aa of the pre-S2 region, 226 aa of the S gene and 128 bp of 3′ non-coding DNA. The S gene is flanked by a 1150 bp yeast DNA fragment carrying the ARG3 transcription terminator. Recombinant plasmids were screened for the presence of either a 411 bp or a 243 bp BamHI-XbaI fragment, indicative of insertion of the 192 bp (pRIT12572) or the 24 bp (pRIT12571) Sau3A fragments, respectively. The HindIII cassette fragments of pRIT12572 and pRIT12571 were then recloned onto the yeast vector YEp13[14], to give respectively plasmids pRIT12574 and pRIT12573. Plasmid DNA of pRIT12574 and pRIT12573 was introduced into yeast strain 10S44C (pep4-3, leu2-3, leu2-112) by transformation of lithium acetate treated cells[20] and selection for leucine independence. (B) Expected hybrid proteins.

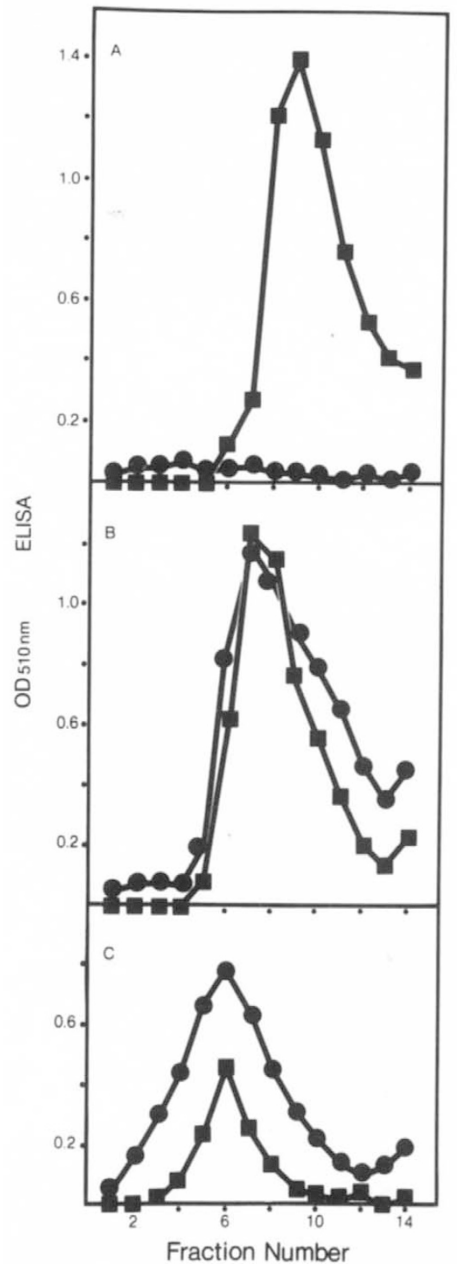

FIGURE 3 CsCl equilibrium contrifugation of crude cell extracts. 1.0 ml of crude extract was mixed with 1.5 M CsCl, 50 mM sodium phosphate buffer, pH 7.4 and centrifuged in a SW 50.1 rotor at 40,000 rpm for 40 hours. Left side of figure corresponds to the bottom of the tube. Anti-HBsAg (■—■) and anti-CS (●—●) reactivities were measured by ELISA assays as described under Experimental Protocols. A: pRIT12329 (HBsAg) B: pRIT12573 (R2HBsAg) C: pRIT12574 (R16HBsAg).

extracts.

Antigenicity of hybrid protein particles. Crude cell extracts containing hybrid protein particles were assayed for HBsAg antigenicity in an AUSRIA test and their ability to be recognized by anti-HBsAg antibodies was compared to that of native HBsAg synthesized in yeast from a comparable expression plasmid (pRIT12329). Table 1 shows that the HBsAg antigenicity, expressed per mg of total protein in the extract, of the R2HBsAg and R16HBsAg hybrid protein particles is about 60% and 10% respectively, of the value found for native HBsAg particles. This could be due to a lower expression level of the hybrid proteins compared to HBsAg or to a less efficient particle assembly process. The CsCl gradient analysis described above indicated that the vast majority of HBsAg related protein is found in particles and electron microscopical observation showed particles of approximate the same size and shape as native HBsAg. Therefore, differences in assembly seem not to be responsible for the observed difference in antigenicity. To compare the expression level of the hybrid proteins in respect to HBsAg, the crude extracts were subjected to a quantita-

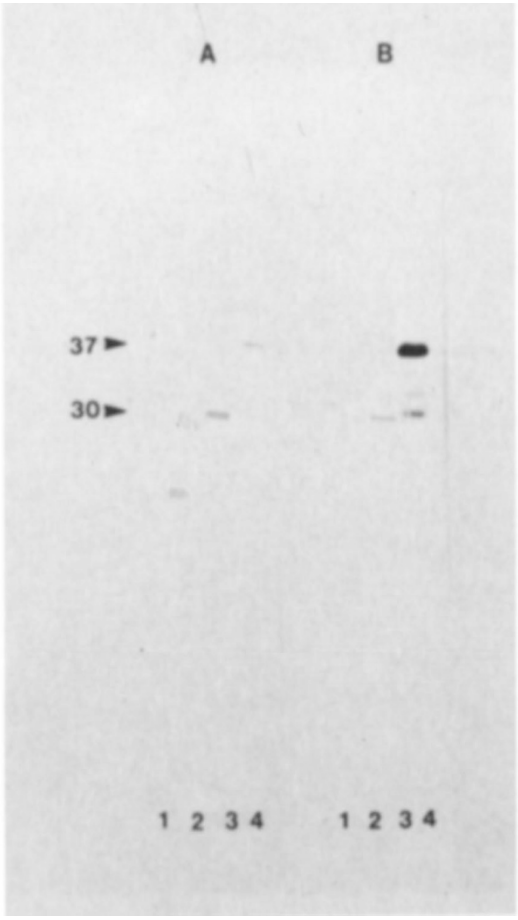

FIGURE 2 Immuno blot detection of hybrid proteins in transformed cells after SDS gel electrophoresis Panel A, reaction to anti-HBsAg antibodies Panel B, reaction to anti-CS antibodies. Lane 1: pRIT10172 (control plasmid, no CS or HBsAg sequences), lane 2: pRIT12329 (plasmid containing sequences coding for the native HBsAg protein), lane 3: pRIT12573 (plasmid coding for R2HBsAg), lane 4; pRIT12574 (plasmid coding for R16HBsAg).

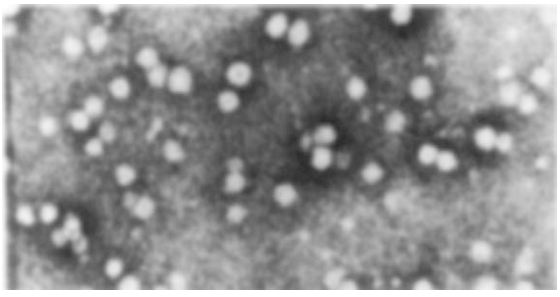

FIGURE 4 Electron micrograph of R16HBsAg hybrid protein particles. Particles were purified by CsCl centrifugation and visualized by negative straining with uranyl acetate.

tive immunoblot analysis. Equivalent amounts of HBsAg related proteins were present in the three extracts. Given the accuracy limits of the analysis method used (see Experimental Protocol), the lower HBsAg antigenicity in the extract containing R2HBsAg hybrid protein particles as compared to the extract containing native HBsAg particles is not significant. In contrast, the lower HBsAg antigenicity in the R16HBsAg extract is significant and indicates that the R16HBsAg hybrid protein particles are less antigenic as concerns HBsAg epitopes in the radioimmune assay than either native HBsAg particles synthesized in yeast or R2HBsAg hybrid protein particles.

Apparently, the presence of CS sequences on the exterior of the R16HBsAg particles partially obstructs the accessibility of the *a* determinant of HBsAg (the major epitope responsible for AUSRIA activity) to the anti-HBsAg antibodies in the AUSRIA assay. Alternatively, the presence of CS sequences may induce some conformational change in the HBsAg moiety and thereby alter the *a* determinant.

In contrast, when antigen present in crude extracts was reacted first with immobilized anti-HBsAg and then assayed by ELISA for anti-CS reactivity, the R16HBsAg antigen reacted at a much greater level than did the R2HBsAg antigen (results not shown). This is consistent with the higher relative proportion of Asn-Ala-Asn-Pro repeat contained on the R16HBsAg hybrid protein.

Immunogenicity of hybrid protein particles. To evaluate the immunogenicity of purified R16HBsAg particles, 1 μg doses were inoculated without adjuvant or after adsorption to aluminum hydroxide into C57BL/6 mice. Booster doses of immunogen were given at weeks 3 and 7. Both inocula elicited high levels of antibodies which reacted with the bacterially synthesized R32tet32 antigen in an ELISA test. Figures 5A and B show the development of an anti-R32tet32 antibody response to R16HBsAg particles administered without adjuvant and alum-adjuvated, respectively. Alum enhances the immunogenicity and in both regimen the response was augmented by boosting. However, both groups of mice failed to develop appreciable antibody response to HBsAg epitopes despite the repeated boosting (Table 2). In contrast, rabbits inoculated according to the same inoculation scheme with 10 μg doses, developed antibodies to both CS (Fig. 5C) and HBsAg (Table 2) epitopes. Interestingly, the anti-CS respone of R16HBsAg particles in rabbits was not enhanced by adsorption to alum (data not shown). Anti-HBsAg titers augmented upon boosting but no booster effect was observed on the anti-CS titers. Anti-CS titers dropped considerably after week 4 despite the booster at week 7.

Sera from both mice and rabbits all reacted strongly in the CSP reaction as well as inhibited sporozoite invasion of hepatoma cells *in vitro* (Table 3). Finally, antisera from all animals recognized CS protein as determined by Western blot analysis of NP40 extracts of *P. falciparum* sporozoite surface antigen (Fig. 6).

DISCUSSION

Valenzuela et al.[16], reported expression and assembly of hepatitis B surface antigen-herpes simplex gD hybrid protein particles in yeast. Although speculation has been that such particles would be improved immunogens for the foreign epitopes presented, no experimental data have been provided. To test this hypothesis we have used the hepatitis B surface antigen particle as a carrier matrix for the receptive epitope of the circumsporozoite protein of *Plasmodium falciparum*. We constructed two fusion proteins, containing either 8 or 64 amino acids of the CS repeat sequence. Both were fused at the N-terminus of the pre:-S2 region of HBsAg. In each case, the particle assembly naturally undergone by HBsAg was not interfered with by the presence of the CS sequences. The particles formed by both density and EM criteria are essentially identical to those obtained with HBsAg. Recognition of the hybrid protein particles by anti-CS antibodies indicates that the CS epitopes are well exposed on this matrix. The incorporation of CS sequences into the HBsAg matrix however did alter the antigenicity of the HBsAg moiety as determined by radioimmunoassay. This effect was most pronounced in the case of the R16HBsAg hybrid protein containing the larger number of tetrapep-

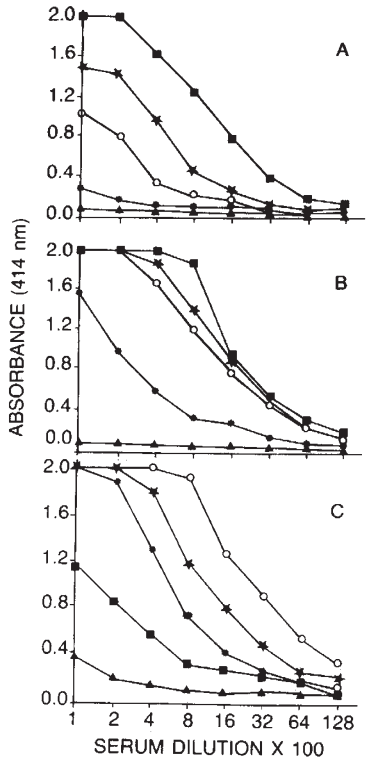

FIGURE 5 Development of CS-specific antibody response upon injection of R16HBsAg hybrid protein particles. (A) particles without adjuvant in mice (B) particles with aluminum hydroxide in mice (C) particles with aluminum hydroxide in rabbit #36. Week 1 (●—●) week 4 (○—○) week 8 (★—★) week 12 (■—■) control animals inoculated with PBS (▲—▲).

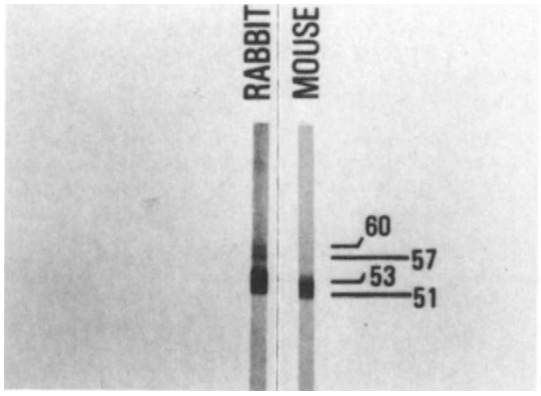

FIGURE 6 Western blot analysis of R16HBsAg antisera from rabbit and mice against intact *P. falciparum* circumsporozoite protein extracted as described by Dame et al.[2]

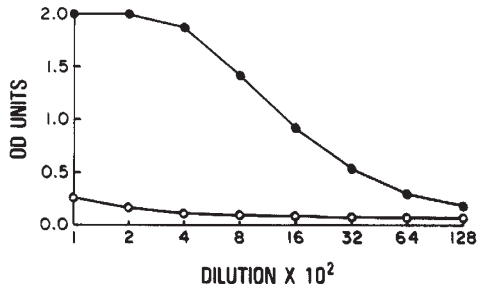

FIGURE 7 Comparative immunogenicity of 1 μg of R16tet32 peptide and 1 μg R16HBsAg protein particles adsorbed to alum in mice. Antisera to R16tet32 (○); antisera to R16HBsAg (●).

tide repeats. These CS sequences may either partially cover the major HBsAg epitopes or modify the natural epitopes by inducing a conformational change in the structure of HBsAg. Both the HBsAg antigenic determinants and the T cell epitopes appear to be partially masked or altered. This indicates that a partial loss of CS sequences from the particles might have a distinct influence on the accessibility of the HBsAg epitopes and consequently on the antigenicity and immunogenicity of the HBsAg moiety of the particles. In this respect, it should be noted that during extraction and purification of the hybrid protein particles some degradation of the hybrid protein can occur. Cleavage appears to happen preferentially at a protease sensitive site in the preS2 portion of the hybrid protein thus separating the CS sequences from the HBsAg sequences (results not shown).

In spite of the effects on the HBsAg epitopes, the 64 amino acids CS fragment-HBsAg hybrid particle is a potent CS immunogen, and induces high titer anti-CS antibodies in mice and other animals. The effectiveness of this molecule to act as an immunogen is best demonstrated by its comparison to the immunogenicity of the identical CS sequence expressed as a monomeric protein in *E. coli* (R16tet32)[4]. As seen in Figure 7 the monomeric protein is not effective in eliciting an antibody response. The biological properties of the induced antibodies, as measured by CSP reaction and inhibition of sporozoite invasion of hepatoma cells, suggest that the hybrid protein particle is able to induce protective immunity against *P. falciparum*. The formation of anti-sporozoite antibodies requires T-help and genetic restriction of immune responses to the repeat have been shown in congenic mice[17–19]. The failure to boost antibody titers to the repeat in humans[12,13] may reflect insufficient T-helper epitopes in the candidate vaccines used. Immunization experiments in monkeys (*Cercopithecus aethiops*) showed high anti-CS antibody titers increasing upon booster injections for the hybrid protein particles while R32tet32 is poorly immunogenic in this species (Hauser et al., manuscript in preparation). This may reflect a lack of T-helper epitopes recognizable in both monkeys and humans in the R32tet32 molecule while adequate T-helper epitopes are provided by the HBsAg carrier in the hybrid protein particles. Our data suggest that hybrid protein particles have an improved CS antigen presentation and may have sufficient T-helper epitopes to be an efficacious vaccine.

This report demonstrates the utility of recombinant technology in yeast to produce a CS-HBsAg fusion protein capable of producing high titer anti-sporozoite antibody that recognizes native CS protein and is biologically active. Our data suggests that this fusion protein may possibly be used at lower doses than anti-sporozoite vaccines currently in production and may offer the potential of multiple epitope/disease/vaccines, if a suitable combination of epitopes can be engineered.

EXPERIMENTAL PROTOCOL

Immunoblot analysis. Two ml cells, grown in YNB to an OD_{620} of 0.2 were collected by centrifugation and resuspended in 0.125 M tris-HCl pH6.8 containing 20% glycerol, 4% SDS, 6 M urea and 10% 2-mercaptoethanol. Samples were heated for 5 min. at 100°C, divided in two and both aliquots electrophoresed through a 12.5% separating gel, 5% stacking gel[21]. Proteins were electroblotted onto nitrocellulose[22] and one half of the sheet was incubated with a mixture of 5 monoclonal antibodies specific for CS protein[2], the other half with a monoclonal antibody against yeast derived HBsAg that had been shown to be capable of reacting with reduced and denatured HBsAg. Detection of the immuno-

reaction was by the biotin-streptavidin peroxidase method (Amersham). Quantitative blot analyses were made from serial 2-fold dilutions of crude cell extracts, which were first normalized in respect to total protein content. Estimations of relative amounts of HBsAg related proteins in crude extracts have an error factor of 2.

Preparation of crude cell extracts. Cells were grown in defined medium to the end of exponential phase, collected by centrifugation and resuspended in 50 mM sodium phosphate buffer, pH7.4 containing 0.5% Tween 20, 1 mM PMSF and 2.5% isopropanol. The cell suspension was passed twice in a French Press at 20,000 psi and centrifuged for 30 min at 30,000 g. The supernatant liquid was taken as crude cell extract. Total protein concentration was measured by the method of Lowry[23].

Immune assays. HBsAg antigenicity was measured by a commercially available radioimmune assay, AUSRIA® II from Abbott Laboratories, or by an ELISA assay (Enzygnost-HBsAg® micro from Behringwerke). The availability of CS sequences on the HBsAg particles to an immune reaction was tested in the ELISA assay above. Appropriate dilutions of the samples were bound overnight at room temperature to the solid-phase anti-HBsAg. The bound HBsAg particles wre incubated for one hour at 37°C, with a mixture of 5 anti-CS monoclonal antibodies[2] as the first antibody and biotinylated anti-mouse Ig (Amersham) as the second (1 hour, 37°). Detection was by the streptavidin-biotinylated horseradish peroxidase complex (Amersham), 30 min. at 37°, and chromogen from the above mentioned ELISA kit. Between incubations, the trays were washed four times with washing solution from the ELISA kit.

Animal vaccination. Animals were immunized with the purified particles of hybrid protein R16HBsAg administered in phosphate buffered saline or adsorbed to alum. C57BL/6 mice (6–8 weeks), in groups of 5, and rabbits, in groups of 2, were immunized subcutaneously and intraperitoneally on week 0, 3 and 7. Mice received 1 μg total protein in 0.5 ml doses, and rabbits 10 μg protein in 1.0 ml doses at each immunization. Animals were bled on weeks 1, 4, 7 and 12. Blood was allowed to clot at 4°C overnight and sera were separated and stored at −70°C until used. Animals immunized with HBsAg (Heptavax-B® Merck Sharp and Dohme), 10 μg per dose, using a similar schedule and nonimmunized animals served as controls.

Characterization of antibodies. For determination of antibody responses, sera from mice were pooled for each group while rabbit sera were assayed individually. Antibody responses to sporozoite antigens were determined by ELISA using the recombinant CS protein R32tet32 as antigen as previously described[4]. Assays were run in triplicate and the mean and standard error of the mean were calculated. In all instances the standard error of the mean was less than 0.1. Antibodies to HBsAg were determined by using the commercially available AUSAB® kit (Abbott Labs) and the World Health Organization standard. Titers were calculated using the Hollinger formula[24]. Appropriate sera were then selected for CSP reaction assay performed as described by Young[4], as well as for determination of percent inhibition of *P. falciparum* sporozoite invasion of HepG2-A16 human hepatoma cells *in vitro* (ISI)[4,7]. Briefly, sporozoites were added to hepatoma cell cultures containing serum (1/20 dilution) and incubated at 37°C. Intracellular sporozoites were visualized by a monoclonal antibody immunoperoxidase assay. Non-specific inhibitory activity was calculated as the mean minus 2 SD of triplicate counts of internalised sporozoites incubated with control serum samples. The percentage inhibition of sporozoite invasion was calculated as ([control-test]/control) × 100.

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