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SAFETY AND IMMUNOGENICITY OF A *PLASMODIUM VIVAX* SPOROZOITE VACCINE

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Abstract. A recombinant DNA *Plasmodium vivax* sporozoite vaccine containing the repeating region of the Salvador I strain circumsporozoite (CS) protein was produced in *Escherichia coli*. This vaccine was tested in 13 naive volunteers at doses of 10–1,000 μg. No serious adverse reactions were noted. None of 4 volunteers receiving the 10 μg dose developed antibodies measurable by ELISA. Six of 9 volunteers in the other dose groups developed measurable antibodies: 5 of 5 volunteers receiving 100 μg and 1 of 4 receiving 1,000 μg. Antibody responses measured by immunofluorescence assays paralleled those seen by ELISA. None of the volunteers developed antisera that inhibited sporozoite invasion of human hepatoma cells in vitro. Lack of a classical anamnestic response and lack of a typical dose response to increasing amounts of antigen suggests the possible presence of an immunosuppressive epitope in the repetitive region of the CS protein.

The immunodominant protein covering the surface of malaria sporozoites, the circumsporozoite (CS) protein, continues to be a major target in the development of an effective antisporozoite malaria vaccine. Animals and humans immunized with irradiated sporozoites develop antibodies directed against the respective CS protein and are protected against infection when challenged with sporozoites administered either iv or by mosquito bite.[1–3] Passive transfer of monoclonal antibodies (Mabs) directed against the immunodominant repeat region of the *Plasmodium berghei* CS protein can confer protection, in a dose dependent fashion, to recipient animals subsequently challenged with viable sporozoites.[4,5] These data have provided the impetus to develop and test subunit vaccines designed to elicit antibodies to the repeating NANP/NVDP epitope found in the *P. falciparum* CS protein.[6,7] The gene which codes for the CS protein of *P. vivax* has also been cloned and sequenced, and the amino acid sequence of the sporozoite surface protein deduced.[8,9] The CS protein of *P. vivax* is analogous to other CS proteins and contains a highly charged region near the amino terminus followed by an area of repeating amino acids. In *P. vivax*, the immunodominant region consists of 20 tandem repeats of the 9 amino acid sequence Gly-Asp-Arg-Ala-(Asp/Ala)-Gly-Gin-Pro-Ala (GDRA D/A GQPA). A fragment of the gene encoding the 20 tandem repeats (V20) has been cloned into *Escherichia coli*[10] and expressed as a fusion protein with $NS1_{81}$ (81 amino acids from the N-terminus of the nonstructural protein NS1 of influenza A virus). $NS1_{81}$ was chosen as part of the fusion protein with the *P. vivax* repeat region because it may provide T cell epitopes important in the induction of cell mediated immunity.[10] The $NS1_{81}V20$ fusion protein, which is expressed at high levels and readily purified, has been shown to induce significant antibody responses in New Zealand White rabbits (G. Wasserman, personal communication) and *Saimiri sciureus boliviensis*.[11]

Studies in our laboratory with a related fusion protein, $R32NS1_{81}$ (composed of 32 tetrapeptide repeats from the *P. falciparum* repeat region expressed as a fusion protein containing the same 81 amino acids from NS1), showed that BALB/c mice, which are nonresponders to R32 when expressed as $R32NS1_{81}$, are capable of developing high titers of antibodies to the *P. falciparum* repeat epitopes after they have been exposed to and have seroconverted to the A/PR/8/3 strain of influenza A. This suggests that prior infection with influenza A may enhance the antibody response to the malaria repeat epitopes presented with $NS1_{81}$. Preliminary safety, immunogenicity, and efficacy studies in humans indicated that

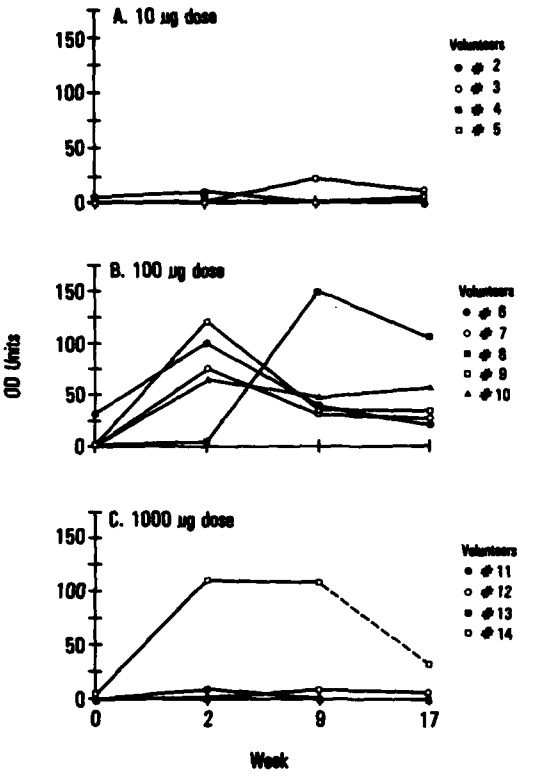

FIGURE 1. Antibody response to PVSV-1 as measured by ELISA. Results are reported in OD units, i.e., that serum dilution that would result in an absorbance of 1 at 414 nm in our standard ELISA. Doses of vaccine were administered at 0, 7, and 15 weeks.

R32NS1$_{81}$ was safe, well tolerated, and able to induce partial protection against sporozoite challenge as manifested by a delay in the pre-patency period in individuals exposed to *P. falciparum* using a laboratory developed challenge model (J. E. Egan, WRAIR, personal communication).

Based on the above information, we considered NS1$_{81}$V20 to be a good candidate for a human *P. vivax* sporozoite vaccine. We now report the results of a study in human volunteers to evaluate the safety and immunogenicity of alum-adsorbed NS1$_{81}$V20, referred to as *P. vivax* sporozoite vaccine 1 (PVSV-1).

MATERIALS AND METHODS

Study subjects

Volunteers were recruited under a protocol approved by The Army Surgeon General's Human Subjects Research Review Board. Healthy men and women, ages 18–50 years, underwent a medical history (with special attention to a previous history of malaria or splenectomy), physical examination, and routine standard laboratory tests, including complete blood count, serum biochemistries (serum creatinine, blood urea nitrogen, bilirubin, alanine aminotransferase, and aspartate aminotransferase), urinalysis, serum β-HCG for females, hepatitis B surface antigen, serologic test for HIV infection, 2 malaria antibody tests, sporozoite antibodies as measured by ELISA, and blood stage antibodies measured by an indirect fluorescent antibody (IFA) test using red blood cells infected with *P. cynomolgi* as antigen. All tests were performed within 2 weeks before immunization. Subjects were excluded from participation if they had been in a malarious area within 1 year of the start of the study, if they had previously had vivax malaria as determined by history, if the malarial antibody tests were positive, if they had had a splenectomy, or if they had had any cardiovascular, hepatic, renal, or immunological illness, or were taking any immunosuppressive medications. Thirteen volunteers, 7 males and 6 females, meeting the above criteria gave informed consent and entered this study.

Vaccine preparation

NS1$_{81}$V20 is a 26,692 dalton, single chain polypeptide of 265 amino acids with the following sequence:

MDPNTVSSFQ VDCFLWVRKR
VADQELGDAPF
LDRLRRDQKS LRGRGSTLGL
DIETATRAGKQ
IVERILKEES DEALKMTMQI P
(GDRADGQPA)$_4$ (GDRAAGQPA)$_1$
(GDRADGQPA)$_4$ (GDRAAGQPA)$_2$
(GDRADGQPA)$_1$ (GDRAAGQPA)$_1$
(GDRADGQPA)$_1$ (GDRAAGQPA)$_1$
(GDRADGQPA)$_1$ (GDRAAGQPA)$_4$.

NS1$_{81}$V20 was produced by inserting a BstNI-MnlI restriction enzyme fragment encoding the repeat domain of the *P. vivax*, Salvador I (Sal-1) strain, CS protein coding sequence into a vector which expresses 81 amino acids of the NS1 non-structural protein of A/PR/8/34 (H1N1) influenza virus, creating the plasmid pNS1$_{81}$V20. An *E. coli* expression strain, a derivative of the standard NIH *E. coli* K12 strain N99 (F$^-$su$^-$galK21acZ$^-$thr$^-$), was then transformed

with $pNS1_{81}V20$ and used to produce the initial cell paste. Subsequently, crude $NS1_{81}V20$ was obtained, following the mechanical disruption of the cells, by a series of precipitation steps with polyethyleneimine, ammonium sulfate, and acidic pH conditions. Further purification of $NS1_{81}V20$ consisted of ion exchange chromatography on sulfopropyl-sepharose, size exclusion chromatography on superose 12, and reversed-phase high performance liquid chromatography on Vydac C-4 packing. Homogeneity of the final product was confirmed by immunoblot analysis with antisera directed against NS1 and a Mab specific for the *P. vivax* repeat region, analytical size exclusion, and RP-HPLC.

$NS1_{81}V20$ was supplied by SmithKline Beecham Pharmaceuticals, Swedeland, PA, as a 4-vial multi-pack designated *P. vivax* sporozoite vaccine 1 (PVSV-1). Vial no. 1 contained 0.4 ml of the $NS1_{81}V20$ antigen solution at 7 mg/ml in a 50 mM acetate buffer, pH 5.5. Vials nos. 2A and 2B contained the diluent (50 mM acetate buffer, pH 5.5) supplied in 2 sizes, 1.8 ml/vial (no. 2A) and 19.8 ml/vial (no. 2B). Vial no. 3 contained the adjuvant suspension consisting of 0.5 ml of 4.4 mg/ml aluminum oxide (2.2 mg/ml Al^{+++}) in 50 mM acetate buffer, pH 5.5, with 0.005% w/v thimerosal as a bactericide. The system is such that a 0.5 ml dose containing either 10, 100, or 1,000 μg of $NS1_{81}V20$ and 2.2 mg of aluminum oxide could easily be made just prior to administration. All components of PVSV-1 were stored at 4°C prior to use.

Immunization schedule

Volunteers were randomly assigned to 1 of 3 groups and received either 10, 100, or 1,000 μg of $NS1_{81}V20$ as PVSV-1 im at week 0, with identical booster doses being given at 7 and 15 weeks. Volunteers were observed for 20 min after each dose for immediate reactions and evaluated at 24 and 48 hr for symptoms of headache, fever, chills, malaise, local pain, fever, erythema, warmth, induration, lymphadenopathy, and other complaints. Prior to and again 2 days after each dose of vaccine, each volunteer had blood and urine collected for evaluation. Serum samples were obtained each week for 3 weeks after each dose. Whole blood was allowed to clot at room temperature, serum was separated and stored at −70°C until analyzed.

Serological assays

CS antibodies were measured using a standard ELISA technique[12,13] except that a recombinant protein, referred to as VIVAX-1, which contained the entire repeat region of *P. vivax* flanked by the 15 amino acids amino terminal and the 48 amino acids carboxy-terminal of the repeats, was used as the capture antigen.[14] This molecule does not contain the $NS1_{81}$ segment present in PVSV-1. Horseradish-peroxidase conjugated rabbit anti-human IgG (gamma chain specific) was used as the secondary antibody. Assays were run in triplicate and the mean absorbance and standard deviation was calculated for each dilution.

Immunofluorescence assays were performed as previously described.[12] Hand dissected salivary-gland sporozoites from the Sal-1 strain of *P. vivax* were suspended in Medium 199, containing 0.01% (v/v) bovine serum albumin, dotted onto multi-well immunofluorescence assay slides at 10,000 sporozoites/dot, air-dried at room temperature, and stored at −70°C until used. Antibodies reactive to sporozoites were detected with fluorescein-labeled anti-human IgG under ultraviolet light at 500× magnification. Fluorescence was graded from 0 to +4, where 0 indicates no fluorescence detectable and +4 indicates intense fluorescence over the entire surface of the sporozoite.

Percent inhibition of sporozoite invasion of HepG2A-16 human hepatoma cells in vitro was calculated according to Hollingdale.[15]

RESULTS

The 13 individuals (mean age 35 years, range 22–44 years), were randomly divided into 3 groups to receive PVSV-1. The vaccine was well-tolerated by all. The major side effect noted was minor discomfort at the injection site in all volunteers, which resolved within 24 hr. Two individuals in the 1,000 μg dose group noted the onset of malaise and generalized myalgias 6–9 hr after the administration of the second dose of vaccine. These symptoms resolved after 48 hr. One of these individuals continued in the study, receiving the third dose of vaccine as scheduled, and experienced no untoward reaction, suggesting that the previous reaction was not causally related to the vaccine. There were no abnormalities in blood chemistry, hematology profiles,

TABLE 1
IFA results

Volunteer no.	Week 2	Week 9	Week 17
6	25	0	0
7	100	50	50
8	50	400	200
9	100	50	100
10	100	100	100

Results represent the highest serum dilution which produced a 2+ IFA reaction.

or urinalysis attributable to vaccine administration.

Six of the 9 volunteers who received either the 100 or 1,000 μg dose of PVSV-1 developed antibodies to the vivax repeat epitopes as measured by ELISA (Fig. 1). Four of the individuals receiving the 100 μg dose had an initial response after the first dose of vaccine, but failed to demonstrate any boosting with subsequent doses. Volunteer no. 8 demonstrated a minor response to the first dose of vaccine, which returned to baseline by week 4. This volunteer demonstrated a response after the second dose of vaccine, but not after the third dose of vaccine. Only 1 individual (no. 14) in the 1,000 μg dose group seroconverted. This volunteer showed an initial response to the vivax epitope, but antibody levels failed to rise after the second dose of vaccine. She withdrew from the study because of malaise and generalized myalgias after the second dose of vaccine.

Sera from week nine from volunteers in groups A and C were negative by IFA, while the IFA results from volunteers in Group B paralleled those measured by ELISA (Table 1). None of the volunteers developed antisera capable of inhibiting sporozoite invasion of HepG2-A1G cells in vitro.

DISCUSSION

Mabs directed against the repeat region of CS proteins can passively transfer protection against sporozoite challenge. This fact provides compelling rationale for the development of anti-sporozoite malaria vaccines designed to induce high levels of anti-CS antibody. Early studies with *P. falciparum* subunit vaccines supported this approach, although the degree of protection achieved was suboptimal.[6,7] In an analogous manner, we have developed a recombinant *P. vivax* subunit vaccine designed to induce antibodies to the immunodominant repeat region of the *P. vivax* CS protein. This study demonstrates that the recombinant fusion protein $NS1_{81}V20$ is safe and well tolerated when administered to normal male and female volunteers in doses up to 1,000 μg. Somewhat surprising, however, was the lack of a classical anamnestic response to subsequent doses of the vaccine and the lack of a typical dose response with increasing amounts of antigen. The data obtained using $NS1_{81}V20$ suggest the possibility of an immunosuppressive epitope in the repeating (GDRA D/A GQPA) region of the *P. vivax* CS protein. The implication of less than optimal immune responses reflected in these data, combined with the recently reported discovery of *P. vivax* isolates from Thailand which produce sporozoites that possess a CS protein with significant differences in the repeat region,[16] may complicate efforts to develop a *P. vivax* anti-sporozoite vaccine based solely on the immunodominant region of the CS protein.

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