Phase I/IIa Safety, Immunogenicity, and Efficacy Trial of NYVAC-Pf7, a Pox-Vectored, Multiantigen, Multistage Vaccine Candidate for *Plasmodium falciparum* Malaria

Christian F. Ockenhouse, Pei-fang Sun, David E. Lanar, Bruce T. Wellde, B. Ted Hall, Kent Kester, Jose A. Stoute, Alan Magill, Urszula Krzych, Linda Farley, Robert A. Wirtz,* Jerald C. Sadoff, David C. Kaslow, Sanjai Kumar, L. W. Preston Church,* James M. Crutcher,* Benjamin Wizel,* Stephen Hoffman, Ajit Lalvani, Adrian V. S. Hill, John A. Tine, Kenneth P. Guito, Charles de Taisne, Robin Anders, Toshihiro Horii, Enzo Paoletti, and W. Ripley Ballou

Department of Immunology, Walter Reed Army Institute of Research, Washington, DC; Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, and Malaria Program, Naval Medical Research Institute, Bethesda, Maryland; Institute of Molecular Medicine, University of Oxford, Headington, Oxford, United Kingdom; Virogenetics Corp., Troy, New York; Connaught Laboratories, Inc., Swiftwater, Pennsylvania; Pasteur Mérieux Connaught, Marcy l'Étoile, France; Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia; Research Institute for Microbial Diseases, Osaka University, Osaka, Japan

Candidate malaria vaccines have failed to elicit consistently protective immune responses against challenge with *Plasmodium falciparum.* NYVAC-Pf7, a highly attenuated vaccinia virus with 7 *P. falciparum* genes inserted into its genome, was tested in a phase I/IIa safety, immunogenicity, and efficacy vaccine trial in human volunteers. Malaria genes inserted into the NYVAC genome encoded proteins from all stages of the parasite's life cycle. Volunteers received three immunizations of two different dosages of NYVAC-Pf7. The vaccine was safe and well tolerated but variably immunogenic. While antibody responses were generally poor, cellular immune responses were detected in >90% of the volunteers. Of the 35 volunteers challenged with the bite of 5 *P. falciparum*–infected *Anopheles* mosquitoes, 1 was completely protected, and there was a significant delay in time to parasite patency in the groups of volunteers who received either the low or high dose of vaccine compared with control volunteers.

Malaria is a major world-wide infectious disease problem, in which transmission by the *Anopheles* mosquito vector occurs throughout Africa, Asia, Oceania, and Latin America. It is estimated that >2 billion people live in malarious areas and that there were >489 million clinical cases of malaria during 1986 [1]. Of these, an estimated 234 million were due to *Plasmodium falciparum* infection and resulted in >2.3 million fatalities [1]. An effective malaria vaccine is urgently required in many areas of the world where drug resistance is becoming more frequent. Major obstacles that have impeded the development of an effective malaria vaccine include antigenic diversity and variation of parasites and the hosts' genetic nonresponsiveness to parasite antigens or epitopes. Previous clinical phase I studies have emphasized candidate malaria vaccine preparations designed to induce antibodies directed against the circumsporozoite (CS) protein present on the surface of the sporozoite of the parasite [2–4].

Immunization with combinations of antigens derived from all stages of the parasite's life cycle has been proposed as a way to overcome antigenic variability and HLA-restricted immune responses. Attenuated or inactivated strains of pathogenic viruses are suitable for the delivery of multiple genes and have been used successfully for vaccination against a variety of diseases. Advances in recombinant DNA technology have yielded means of developing live attenuated vaccines, which hold promise for overcoming the limitations of conventional approaches, including the effort and expense required to express and purify multiple protein antigens for polyvalent vaccines. Replication-competent animal viruses, such as poxvirus, have been engineered as delivery systems for vaccine candidate gene products [5, 6]. These vectors provide advantages over other approaches because the foreign gene product is expressed in infected cells, and the antigens may be processed and presented to the immune effector cells in a manner similar to that of natural infection, inducing functional antibody and cell-mediated responses.

Vaccinia virus, the prototypic orthopoxvirus, has been shown to induce a relatively long-lasting immunity and remain stable as a freeze-dried preparation. Low production costs and simple

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Written informed consent was obtained from all volunteers (recruitment of volunteers was noncoercive and in accordance with existing US Army regulations, USAMRDC reg. 70-2, ''Use of Volunteers as Subjects of Research''), and this study was conducted in accordance with guidelines established by and with the approval of the Institutional Review Board of Walter Reed Army Institute of Research.

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Reprints or correspondence: Dr. Christian F. Ockenhouse, Dept. of Immunology, Walter Reed Army Institute of Research, Washington, DC 20307 (LTC__Chris__Ockenhouse@wrsmtp-ccmail.army.mil).

* Present affiliations: Entomology Branch, Centers for Disease Control and Prevention, Atlanta, (R.A.W.); Michigan State University School of Medicine, Lansing (L.W.P.C.); Oklahoma State Department of Health, Oklahoma City (J.M.C.); University of Georgia, Athens (B.W.).

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administration are also advantages. The amount of exogenous DNA that can be inserted into the vaccinia virus genome without deleterious effects on virus growth approaches 50,000 bp, thus making it possible to create a recombinant vaccinia virus expressing multiple heterologous target epitopes. Such a polyvalent vaccine could provide several antigens for vaccination against a single disease, such as malaria [5]. The vaccine preparation in our study, NYVAC-Pf7, represents a new generation of multistage, multiantigen vaccines consisting of an attenuated NYVAC vaccinia virus strain that expresses genes coding for proteins expressed during the sporozoite (CSP, PfSSP2), liver (LSA1), blood (MSP1, SERA, AMA1), and sexual (Pfs25) stages of the parasite's life cycle [7]. In preclinical testing in rhesus monkeys, NYVAC-Pf7 was safe and well tolerated. Antibodies that recognize sporozoites, liver, blood, and sexual stages of *P. falciparum* were elicited [7]. The objective of the present study was to evaluate the safety and immunogenicity of NYVAC-Pf7 in human volunteers. We also evaluated the protective efficacy by experimental challenge with 5 *P. falciparum*–infected *Anopheles* mosquitoes in vaccinated volunteers.

Materials and Methods

Subjects. Fifty-nine malaria-naive male and female volunteers (age, 18–45 years) were recruited through the Walter Reed Army Institute of Research Clinical Center. Potential volunteers provided a medical history and underwent a physical examination with routine standard laboratory tests consisting of complete blood count (CBC), serum biochemistries, urinalysis, and serologic tests to exclude volunteers positive for human immunodeficiency virus, hepatitis C virus, or hepatitis B surface antigen. Women participating in this study had serum pregnancy tests (serum human chorionic gonadotropin [β-HCG]) completed within 48 h prior to receiving any of the vaccine doses. Volunteers were excluded from participation if they had any significant pulmonary, cardiovascular, hepatic, or renal functional abnormality, had previously undergone a splenectomy, had a positive β-HCG test, had known allergies to eggs or egg products, had previous allergy or anaphylactic reaction to any vaccine, or had previous malaria infection. Prior immunity to vaccinia was not an exclusionary criterion, and volunteers were stratified by history of smallpox vaccination.

Study design and vaccine formulation. This study was an open-label phase I/IIa trial of two dosages of NYVAC-Pf7 (vP1209) with saline control. The vaccine preparations for this study were manufactured in the United States under good manufacturing procedures by Connaught Laboratories. Two liquid preparations bottled in single-dose vials were prepared such that one dose of 1.0 mL contained either $\sim 10^7$ pfu (hereafter referred to as low dose) or $\sim 10^8$ pfu (hereafter referred to as high dose) of the NYVAC-Pf7 (vP1209) vector. The vaccine was stored frozen at −70°C until used. The vaccine was thawed for 30 min at room temperature prior to injection. Twenty-five volunteers were immunized with low-dose NYVAC-Pf7 vaccine intramuscularly in the deltoid region of alternate arms at 0, 4, and 26 weeks. Twenty-four volunteers were immunized on the same schedule with high-dose NYVAC-Pf7 ~12 weeks after the low-dose immunization schedule had begun. Ten control volunteers (divided into 2 groups of 5 individuals each) were given normal saline injections according to the same schedule as volunteers receiving the NYVAC-Pf7 vaccine.

Vaccine construction. NYVAC-Pf7 (vP1209) was developed under a collaborative agreement between the Walter Reed Army Institute of Research, Connaught Laboratories, and Virogenetics and consists of 7 *P. falciparum* full-length or nearly full-length genes inserted into the NYVAC vector, as described [7]. All antigens included in the vaccine have been shown to provide some level of protection in experimental models of malaria. The malaria genes inserted into the NYVAC vector are (1) sporozoite stage antigens: CS and sporozoite surface protein 2 (SSP2); (2) liver stage antigen: LSA1; (3) asexual blood stage proteins: MSP1, AMA1, and SERA; and (4) sexual stage antigen: Pfs25. All genes (except MSP1 and SERA) were isolated from the 3D7 strain of *P. falciparum.* MSP1 and SERA genes were cloned from the Palo Alto and FCR3 genes, respectively, and are very similar in sequence to the 3D7 genes.

Assessment of safety and reactogenicity. Subjects were observed for 20 min and evaluated at 1, 2, and 7 days after immunization. At each visit, reactogenicity was assessed by a physician and findings and complaints recorded and scored as follows: mild reactions (easily tolerated), moderate reactions (interferes with normal activity), or severe reactions (prevents normal activity and requires physician intervention). Safety was determined by serial laboratory evaluations at 2 days after immunization and 1 week prior to subsequent vaccine doses. Laboratory tests included CBC, serum biochemistries, and urinalysis.

Serologic assays. Blood was obtained from each volunteer before and 2 weeks after each immunization, and the serum was separated and stored frozen at −70°C until used. Vaccinia neutralization titers were obtained on pre- and postimmunization serum samples and were performed at the Salk Institute, Government Services Division (Swiftwater, PA) by the plaque reduction neutralization assay. Results are reported as the dilution of sera that gives 50% reduction of plaque-forming units.

Antibody responses to malaria antigens were measured by ELISA. Recombinant-expressed full-length antigens included Pfs25 [8] and AMA1, corresponding to the 3D7 ectodomain. Recombinant-expressed partial-length antigens included SERA (SE47′), encoding amino acid residues 17–382 [9]; LSA1, C-terminal fragment of LSA1 encoding amino acids 1628–1909 expressed as a [His]6-tagged protein in *Escherichia coli;* and the C-terminal 19-kDa fragment of MSP1 [10]. Synthetic peptides comprising the immunodominant epitopes of the CS protein (40-mer peptide composed of 10 repeating units of Asn-Asp-Asn-Pro amino acids [NANP]10) and SSP2 (SPNPEEGKGENPNGFDL-DENPENPPNPPNPPNPPNPPNPPN) were synthesized by standard solid-phase methods and purified to >90% by reverse-phase high-pressure liquid chromatography. Criteria for seroconversion required that the magnitude of the antibody response (kinetic measurement of optical density units per minute at A_{405} for 1 h) exceeded the mean plus 2 SD of baseline values (preimmunization sera) from triplicate determinations of serum samples diluted 1:100. Sera were analyzed by indirect IFA assay using air-dried sporozoites and air-dried and methanol-fixed schizont-stage, asexual, blood-stage parasites. End-point titers were read as the greatest dilution giving a 1+ reaction [11]. Functional activity of antibody

responses to Pfs25 was determined by transmission-blocking assays, as previously determined [8].

Cytotoxic lymphocyte (CTL) assay. CTL assays were performed using antigen-pulsed autologous Epstein-Barr virus (EBV)–transformed B lymphocytes as target cells to predict putative CTL activity in vivo against parasite-infected hepatocytes. All volunteers' peripheral blood mononuclear cells (PBMC) were tissue-typed for class I histocompatibility antigens. The methodology used to measure CTL responses induced by NYVAC-Pf7 immunization depended on HLA class I molecules, known CTL epitopes present within malaria antigens, the nature of the antigens or peptides used as stimulators, and the nature of the target cells used for measurement of lytic activity.

PBMC obtained before the first dose and ~2 weeks after each immunization were used to generate effector cells. PBMC were cultured in RPMI 1640 complete medium containing human AB serum and stimulated with either recombinant canarypox virus (ALVAC) constructs or defined malaria nonamer CTL peptide epitopes for 7 days before being assayed. The cultures were grown in the presence of 25 ng/mL recombinant interleukin (IL)-7 and 10 U/mL recombinant IL-2. Malaria-specific CTL were assayed in a standard chromium release assay. Target cells were prepared by three methods: (1) cells were infected 24 h prior to assay with recombinant vaccinia virus (WR strain) constructs containing individual malaria antigens CS, LSA1, and SSP2; (2) cells were transfected with a plasmid containing SSP2 (DNA-SSP2) or a control plasmid (DNA-neo); and (3) cells were peptide-pulsed with CS, SSP2, or LSA1 peptides (peptide sequences are available on request). In most cases, peptide-pulsed EBV-transformed cell lines were used as target cells to measure CTL activity and were selected depending on putative T cell epitopes corresponding to known class I HLA motifs. The cutoff value for positive CTL activity was set at 10% specific lysis above the value measured by lysis of control targets. Further controls included measuring CTL activity of PBMC collected from volunteers before immunization and processed in an identical manner as PBMC collected after vaccine immunization.

Lymphocyte proliferation assay. Lymphocyte proliferation was assayed as described [12]. Briefly, PBMC were cultured in triplicate (5×10^4 cells/well) in 96-well flat-bottomed microtiter plates in a final volume of 200 μL of RPMI 1640 supplemented with 15 m*M* HEPES buffer, 10% fetal bovine serum, 8 m*M* glutamine, 1 m*M* sodium pyruvate, 100 U/mL penicillin, 100 mg/mL streptomycin, and 50 m*M* 2-mercaptoethanol for 7 days at 37°C in a humidified atmosphere with 5% CO_2. The cells were stimulated with phytohemagglutinin (2 μg/mL), tuberculin purified protein derivative (2 mg/mL), CS or LSA1 peptide (0.1–10 μg/mL), or parasitized red blood cells ($0.01–1 \times 10^5$ parasites/mL). During the last 16 h of culture, 1 μCi/well [3H]thymidine was added. Cells were harvested with a cell harvester, and radioactive uptake was measured using a scintillation counter (LKB Instruments, Rockville, MD). Stimulation indices were calculated according to the following formula: counts per minute (mean of triplicate measurements)/counts per minute (mean of unstimulated control cells).

Malaria challenge. Vaccine efficacy was defined as no development of parasitologic or clinical evidence of *P. falciparum* infection following exposure to an infectious sporozoite inoculum that successfully infected 100% of nonimmunized control volunteers. Cloned *P. falciparum* 3D7 parasites were expanded from a master seed lot and used to infect laboratory-reared *Anopheles stephensii.* The malaria challenge phase of the study for the low- and high-dose vaccine groups was separated by ~11 weeks. Four weeks after the third inoculation, 19 low-dose subjects, 16 high-dose subjects, and 8 control volunteers consented to experimental infection. Challenge continued until 5 infected mosquitoes had successfully fed. Mosquitoes harvested from the same batch fed on a subject for 5 min, and insects with blood meals were dissected to quantify the presence of viable sporozoites. Sporozoite burdens were uniformly heavy throughout the challenge.

Volunteers were followed daily for symptoms and with blood smears between days 5 and 21 after challenge. Giemsa-stained thick smears from asymptomatic subjects were routinely screened for 200 high-power fields before being reported as negative. For symptomatic volunteers, smears were examined exhaustively. All subjects with positive malaria smears were treated with a standard oral regimen of chloroquine plus other medications as indicated by the symptoms and followed daily until three consecutive thick smears were negative and all symptoms had resolved.

Statistical methods. The primary analysis compared the antibody responses to different vaccine component antigens among volunteers receiving either the low or high dose of NYVAC-Pf7. In addition, antibody responses to parasite antigens were compared in volunteers who had prior immunity to vaccinia or were immunologically naive to vaccinia virus exposure as determined by history, physical examination, and preimmunization seroreactivity in plaque-neutralization assays. Mean (±SE) antibody responses after each vaccination and antibody titers at day 0 were compared by two-sided paired *t* tests. Between-group differences (vaccine vs. saline, low vs. high dose, vaccinia-immune vs. vaccinia-naive) were assessed by two-sided *t* tests. To assess the time to seroconversion or seroconversion rate for a given subject, the antibody titer from sera drawn after the first immunization was compared with the antibody titer at day 0 for a significant increase (one-sided *t* test, $P < .05$). If there was not a statistically significant response after immunization 1, the sera after immunization 2 or 3 were analyzed by the same method. If there was no response after immunization 3, the subject was considered a nonresponder. Regression analysis was used to measure the relationship between the log of vaccinia neutralization titer at day 0 and the mean differences in postimmunization antimalaria antigen titers. Comparisons between times to positive malaria parasitemia were based on the Wilcoxon 2-sample test (normal approximation, continuity correction of 0.5).

Results

The study began in October 1994 and ended in September 1995. Fifty-nine volunteers were enrolled: 25 volunteers received at least two immunizations of low-dose NYVAC-Pf7, 24 volunteers received at least two immunizations of high-dose NYVAC-Pf7, and 10 control volunteers received at least two intramuscular injections of normal saline. Four volunteers in each vaccine group and 2 volunteers in the saline control group were unable to complete the study due to scheduling conflicts or unwillingness to continue and did not receive the third im-

Table 1. Adverse reactions to NYVAC-Pf7 within 72 h of vaccination.

	After dose 1		After dose 2		After dose 3	
	Low dose (n = 25)	High dose (n = 24)	Low dose (n = 25)	High dose (n = 24)	Low dose (n = 21)	High dose (n = 20)
Local reactions						
Erythema and/or induration (⩾5 mm)	2 (8)	1 (4.2)	2 (8)	4 (16.7)	0	3 (15)
Pain or tenderness	15 (60)	21 (87.5)	19 (76)	22 (91.7)	18 (85.7)	15 (75)
Axillary adenopathy	4 (16)	2 (8.3)	1 (4)	1 (4.2)	1 (4.8)	0
Limited abduction of arm	0	2 (8.3)	0	5 (20.8)	1 (4.8)	2 (10)
Systemic reactions						
Fever (>38.0°C)	0	1 (4.2)	0	0	0	0
''Feverish'' or chills	1 (4)	5 (20.8)	0	13 (54.2)	0	4 (20)
Malaise	2 (8)	3 (12.5)	0	10 (41.7)	1 (4.8)	3 (15)
Headache	0	5 (20.8)	0	9 (37.5)	0	4 (20)
Myalgia	1 (4)	1 (4.2)	2 (8)	7 (29.2)	0	3 (15)
Anorexia	0	4 (16.7)	0	5 (20.8)	0	0
Nausea	1 (4)	2 (8.3)	0	4 (16.7)	1 (4.8)	0

NOTE. Data are no. of subjects (%).

munization dose. Volunteers were recruited with the intention to include persons who had a history of smallpox vaccination and/or physical evidence of scarification as an indicator of prior immunity to vaccinia virus (vaccinia-immune) and to include subjects who had never received a smallpox vaccination (vaccinia-naive). The accuracy of the history and physical examination was validated by obtaining vaccinia neutralization antibody titers from preimmunization sera.

Safety and reactogenicity. All three immunizations of low- and high-dose NYVAC-Pf7 were well tolerated; the most common side effect was local tenderness at the site of injection (table 1). Transient ''flu-like'' symptoms, including pain, malaise, feverishness, chills, and headache, were reported in several volunteers who had received the high-dose NYVAC-Pf7 vaccine. All adverse reactions were classified as mild or moderate and occurred within 24 h of immunization. Most reactions had resolved by 72 h after immunization, and no reactions were reported after 7 days of follow-up. The frequency of adverse reactions peaked after the second dose of vaccine. There were no clinically relevant deviations in laboratory tests associated with the administration of either the low- or high-dose vaccine, and no skin ulcerations were noted at the site of inoculation. In addition, there was no relationship between vaccinia immune status at time of enrollment and vaccine reactogenicity after any of the three immunizations.

Antibody responses. All volunteers had vaccinia serology testing as determined by the sera dilution that gives 50% reduction in plaque-forming units. As expected, volunteers with prior exposure to vaccinia as children (vaccinia-immune) boosted their antibody responses after the first immunization with NYVAC-Pf7 (figure 1). The difference in magnitude of vaccinia neutralization titers between the groups receiving the low or high dose of NYVAC-Pf7 was statistically significant after the first immunization. The vaccinia serologic conversion rate following two NYVAC-Pf7 immunizations in volunteers without a history of prior smallpox vaccination (vaccinia-naive) was low, confirming the highly attenuated nature of the vaccinia virus construct.

Antibody responses to full-length recombinant malaria antigens (Pfs25, AMA1) or to immunodominant epitopes from recombinant fragments (LSA1, MSP1, SERA) or synthetic peptides (CS, SSP2) were analyzed by ELISA, indirect IFA, and in some cases immunoprecipitation of metabolically labeled malaria-infected erythrocytes. The magnitude of antibody response (antibody titer) was assessed in an ELISA by the rate of change in optical density units per unit of time (kinetic rate),

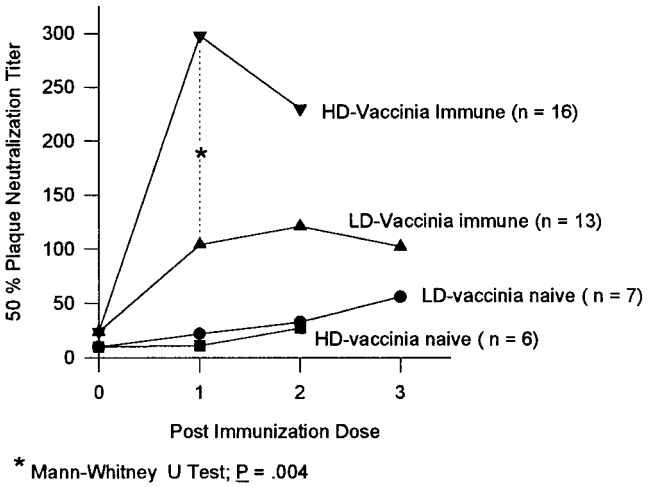

Figure 1. Presence of vaccinia virus–neutralizing antibodies in individual sera was determined by plaque neutralization assay and indicated as 50% end-point titer. Statistical differences between low (LD) and high dose (HD) vaccine and vaccinia-naive and -immune were made by Mann-Whitney U Test.

and the serologic conversion rate (time to seroconversion) was measured by comparison of antibody titer after each immunization dose with baseline (preimmunization) values. Serologic responses to 6 of 7 malaria antigens were elicited after immunization with low-dose and high-dose NYVAC-Pf7 (table 2). The highest level of antibody responses to any malaria antigen was elicited against the sexual-stage parasite antigen, Pfs25. Despite the high level of anti-Pfs25 antibody responses, no transmission blocking activity was found as measured by membrane feeding studies. We were unable to detect an antibody response to the SERA antigen in any subject, although pooled hyperimmune sera from individuals from malaria-endemic areas recognized both SERA recombinant proteins [9] used in the ELISA. Control subjects who received saline alone did not seroconvert to any malaria antigen.

The NYVAC-Pf7 high-dose vaccine was more immunogenic than the low-dose vaccine in that a greater proportion of volunteers seroconverted to CS, LSA1, MSP1, and AMA1 (table 3). There was no statistically significant difference in the magnitude of the antibody response between the low- and high-dose vaccine recipients, regardless of vaccinia immune status. However, in subgroup analysis, there was a statistically significant difference in the magnitude of antibody responses when comparisons were based on prior vaccinia exposure due to antecedent smallpox vaccination. For example, antibody titers to the CS repeat peptide and to sporozoites by IFA were highest in volunteers who had never had a history of smallpox vaccination (vaccinia-naive) (figure 2). Furthermore, regression analysis revealed a significant inverse correlation between antimalarial antibody titers (CS antigen, $P = .0001$; Pfs25, $P = .004$; SSP2, $P = .004$) after three NYVAC-Pf7 immunizations and antivaccinia neutralization titers at the time of study entry. Antecedent vaccinia virus exposure during childhood did not prevent the subsequent ability of volunteers to respond to new antigens with multiple immunizations. In fact, significant boosting in antibody titers and increased seroconversion rates in volunteers occurred after both the second and third immunization doses (figure 2).

Table 2. Cumulative seroconversion after third immunization to NYVAC-Pf7 malaria antigens.

		Vaccine dose		Vaccinia immune status	
Antigen	Total ($n = 39$)	Low ($n = 21$)	High ($n = 18$)	Naive ($n = 12$)	Immune ($n = 27$)
Pfs25	36 (92.4)	18 (85.7)	18 (100)	12 (100)	24 (88.9)
CSP	29 (74.4)	12 (57.1)	17 (94.4)	11 (91.7)	18 (66.7)
LSA1	20 (51.3)	9 (42.9)	11 (61.1)	8 (66.7)	12 (44.4)
SSP2	24 (61.5)	14 (66.7)	10 (55.6)	10 (83.3)	14 (51.8)
MSP1	20 (51.3)	5 (23.8)	15 (83.3)	7 (58.3)	13 (48.1)
AMA1	24 (61.5)	10 (47.6)	14 (77.8)	9 (75)	15 (55.6)
SERA	0	0	0	0	0

NOTE. Data are no. of subjects.

Table 3. Cell-mediated immune responses to NYVAC-Pf7 malaria antigens.

	CTL responses		Proliferative responses	
Antigen	Low dose ($n = 23$)	High dose ($n = 15$)	Low dose ($n = 21$)	High dose ($n = 17$)
CSP	1 (4.3)	2 (13.3)	15 (71.4)	10 (58.8)
SSP2	4 (17.4)	5 (33.3)	ND	ND
LSA-1	2 (8.7)	0	15 (71.4)	10 (58.8)
PRBC	ND	ND	11 (52.4)	9 (52.9)

NOTE. Data are no. positive of those tested (%). CTL, cytotoxic lymphocytes; ND, not done.

Antibody responses by ELISA to blood-stage antigens MSP1 and AMA1 (figure 3) were low or not detected (SERA). Positive IFA responses to malaria-infected erythrocytes were observed in only 2 of 38 volunteers (1 from each dosage group). The antibody levels to MSP1 and AMA1, as measured by ELISA after immunization with NYVAC-Pf7, were low compared with antibody levels measured in human volunteers after a single infection of the 3D7 clone of *P. falciparum* (unpublished observations). Nevertheless, immunization with NYVAC-Pf7 primed the immune system for increased seroreactivity of blood-stage antigens MSP1 and AMA1, as evidenced by increased antibody titers in sera drawn 2 weeks after *P. falciparum* infection and drug treatment compared with sera from nonimmunized malaria-infected and drug-cured volunteers (figure 3). This antibody response to blood-stage antigens after malaria challenge and drug cure was dependent on both the dose and vaccinia immune status. To determine whether the antibody response to MSP1 elicited by NYVAC-Pf7 and detected by ELISA was directed against native malaria protein, detergent extracts of [^{35}S]methionine-labeled parasites grown in culture were immunoprecipitated with preimmune sera or sera drawn 2 weeks after administration of the high-dose vaccine. Immunization with NYVAC-Pf7 elicited antibodies within 2 weeks of the first immunization that recognized native malaria MSP1 protein (figure 4).

Immunization with high-dose NYVAC-Pf7 resulted in a greater proportion of volunteers seroconverting to $\geqslant 3$ malaria antigens compared with volunteers immunized with low-dose NYVAC-Pf7 (figure 5). Greater than 30% (6/18) of volunteers immunized with the high-dose vaccine seroconverted to 6 of 7 antigens while 0 of 21 volunteers immunized with the low-dose vaccine seroconverted to 6 of 7 antigens (figure 6B). Two volunteers (low-dose group) did not seroconvert to any of the 7 antigens included in NYVAC-Pf7. In a similar manner, $>70\%$ of vaccinia-naive subjects seroconverted to at least 5 malaria antigens compared with 28% of vaccinia-immune volunteers (figure 6C).

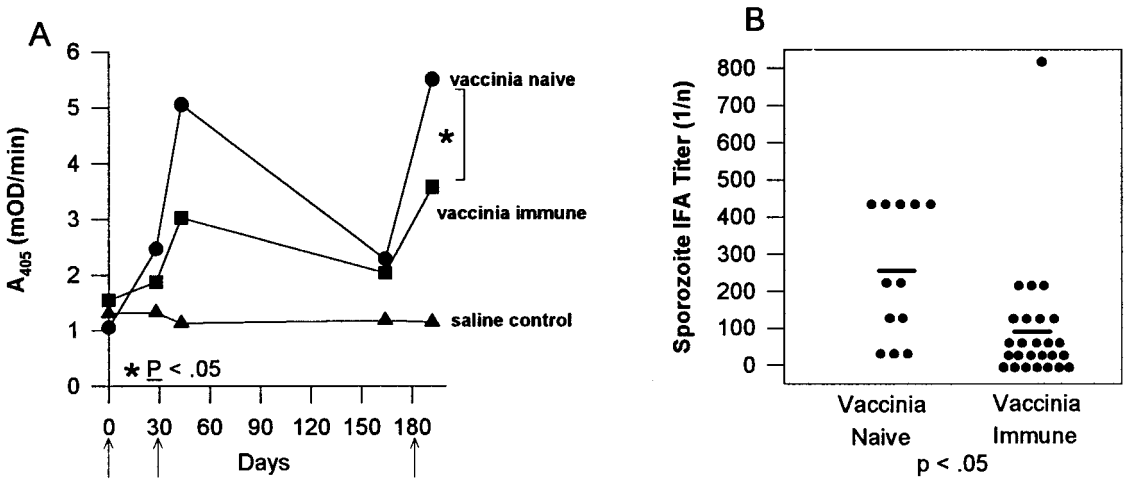

Figure 2. Geometric mean antibody responses to CS repeat peptide by ELISA (**A**) and to sporozoites by IFA (**B**). Arrows (**A**) indicate day of immunization. Horizontal bar (**B**) indicates mean sporozoite IFA titer. Statistical differences between vaccinia-naive and -immune groups were determined by Mann-Whitney U test.

Cell-mediated immune responses. Cell-mediated immune responses to NYVAC-Pf7 vaccination were evaluated by in vitro CTL assays and lymphoproliferative responses to antigen-based peptide sequences. Several approaches were used to evaluate CTL activity in volunteers. Autologous EBV-transformed B cells from subjects infected with vaccinia constructs (WR strain) encoding individual CS, SSP2, or LSA1 antigen were used as targets, and most of these assays were performed in the presence of the ^{51}Cr-unlabeled ''cold targets'' (EBV-transformed B cell lines infected with parental vaccinia virus) to eliminate or lower lysis induced by vaccinia virus alone. In other cases, target cells were transfected with plasmid DNA encoding SSP2. The background lysis observed in these assays prevented meaningful interpretation of specific cytolytic activity attributed exclusively to malaria-specific CTL compared with lytic activity attributed to immune response induced by infection with vaccinia. Therefore, representative CTL assays were designed using HLA-matched volunteers and malaria antigen peptides with known HLA-specific motifs. On the basis of these results, CTL activity was detected in volunteers receiving either the low- or high-dose vaccine, but no activity was observed in PBMC collected

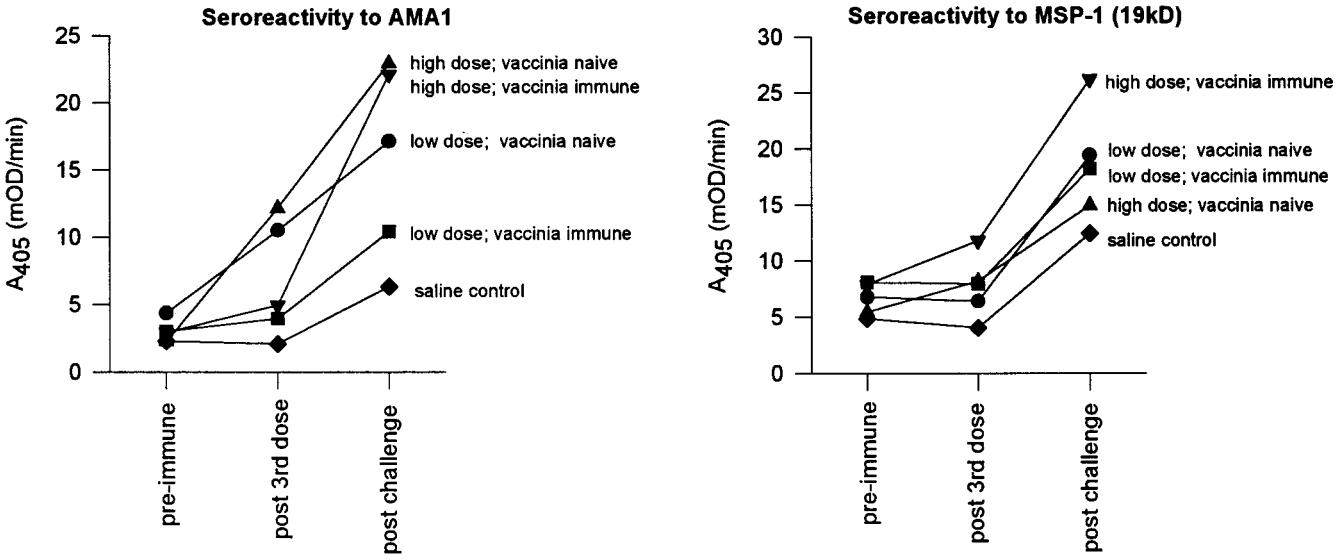

Figure 3. Geometric mean antibody responses measured by kinetic rate to AMA1 and MSP1.

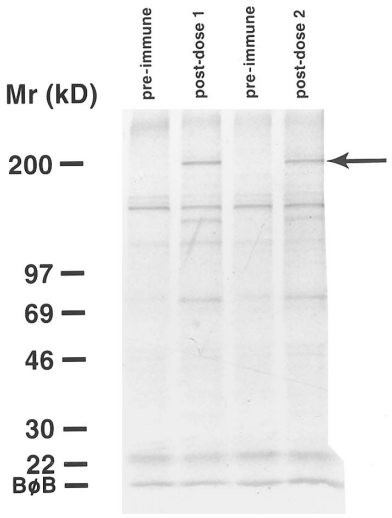

Figure 4. Immunoprecipitation of [^{35}S]methionine-labeled 3D7 *P. falciparum* parasite extracts with preimmune sera and sera drawn after first and second immunizations. Arrow indicates position of native MSP1 recognized by postimmunization sera. Schizont-stage parasite equivalents (5×10^7) extracted with 1% TX-100 were immunoprecipitated with 10 μL sera and protein A–Sepharose for 1 h. Immunoprecipitates were separated by SDS-PAGE, and autoradiographs were developed after 72 h.

prior to the first immunization dose (table 3). In addition, no differences were observed with respect to vaccinia immune status. All individuals with demonstrated CTL activity responded primarily after the second immunization dose, and little CTL activity was observed after the third dose. CTL activity was not observed to >1 antigen in any given volunteer, reflecting the restricted genetic responsiveness of host and known CTL epitopes of CS, SSP2, and LSA1 (table 4).

Another measure of cellular immune reactivity was established by evaluating lymphoproliferative responses to pre–erythrocytic-stage peptides or parasitized erythrocytes. We have previously demonstrated that recognition of antigens normally associated with erythrocytic stages occurs in lymphocyte cultures from volunteers immunized with irradiated sporozoites but not in lymphocyte cultures from sporozoite-naive volunteers [12]. In the present study, we investigated whether volunteers immunized with NYVAC-Pf7 would recognize antigens associated with parasitized red blood cells. This assay provided additional meaningful information on the induction of immune responses to the asexual stage antigens. Since IFA responses to blood stage antigens were observed in only 2 volunteers, we assessed whether the NYVAC-Pf7 vaccine primed the cellular immune system to recognize blood stage antigens. Indeed, ~50% of subjects responded to blood-stage malaria antigens according to proliferation assays, confirming that the antigens in the vaccine were being expressed and recognized by the immune system, and that the primed PBMC recognized native antigen (table 3).

Vaccine efficacy. The challenge phase of the vaccine trial was conducted in two time periods separated by ~11 weeks. Volunteers consenting to malaria challenge (19 low-dose, 16 high-dose, 8 control) were challenged with 5 mosquitoes infected with the *P. falciparum* 3D7 clone for 5 min. One volunteer (low-dose, vaccinia immune) was completely protected against malaria challenge. All unimmunized control volunteers became parasitemic on days 9–12. There was a significant delay in prepatent period (time to detectable parasitemia on Giemsa-stained thick blood smear) among volunteers receiving both the low-dose and high-dose NYVAC-Pf7 vaccine (figure 6). There was no difference in prepatent period with respect to vaccinia immune status, and there was no relationship between delay in prepatent period and antibody titer, CTL activity, or lymphoproliferative responses.

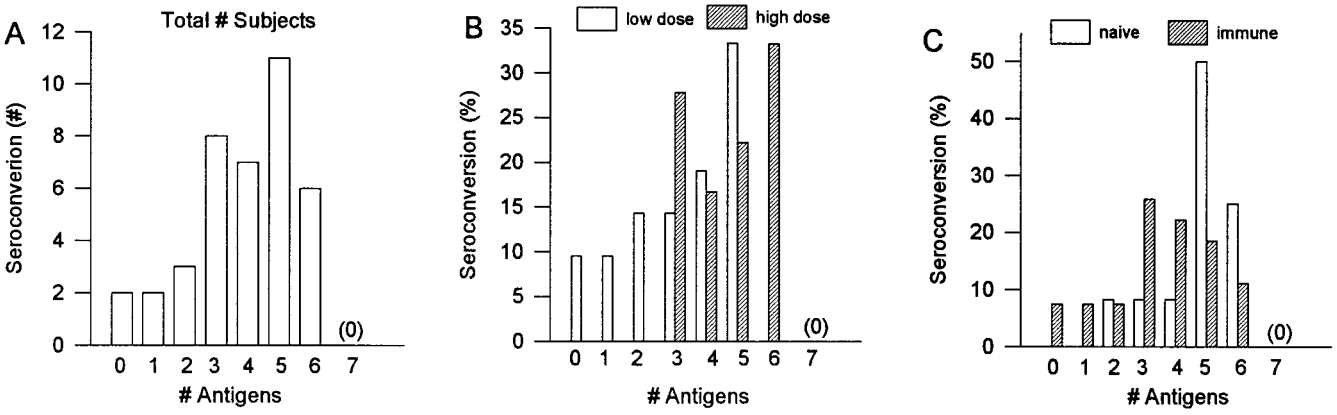

Figure 5. Seroconversion to malaria antigens included in NYVAC-Pf7 vaccine. **A**, No. of volunteers who seroconverted to individual antigen components. **B**, Proportion of volunteers (%) in low- and high-dose groups who seroconverted to no. of antigens (no seroconversion to all 7 antigens) included in NYVAC-Pf7 vaccine. **C**, Proportion (%) of volunteers in vaccinia-naive and -immune groups who seroconverted to 0–7 antigens.

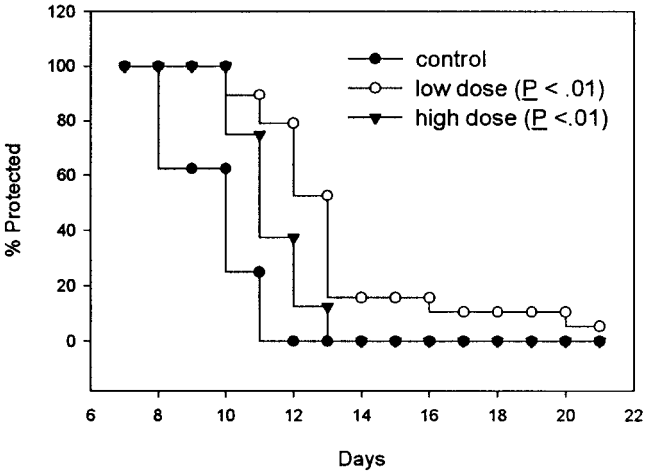

Figure 6. Time to parasitemia (days) in volunteers challenged with 5 *P. falciparum*–infected mosquito bites. Low-dose (n = 19) and high-dose (n = 16) groups were compared with control volunteers (n = 8) by Mann-Whitney U test. Only volunteer in low-dose group who was protected from malaria was not included in statistical analysis.

Discussion

Attenuated virally vectored vaccines for parasitic diseases are attractive alternatives to more traditional approaches for vaccine development. The interest in live-virus vaccinia vectors as vehicles for antigen delivery is based on the need to generate diverse cellular [13–17] as well as humoral [14, 18–23] immune responses that elicit protective immunity on infection. In particular, the prevention of infection with the malaria parasite, *P. falciparum*, poses daunting challenges to vaccinologists. The complexities associated with developing antimalarial vaccines include the diverse antigenic repertoire associated with the organism's many life-cycle changes, antigenic variation and diversity of wild-type isolates, intracellular development (hepatocyte and erythrocyte), and restricted host genetic responsiveness. In this context, NYVAC-Pf7 offers the first multistage, multicomponent vaccine against any infectious disease to test the concept that attenuated live-virus vectors are safe, immunogenic, and efficacious.

NYVAC-Pf7 proved to be a well-tolerated vaccine, and the side effects after immunization were consistent with proven safe vaccines. The level of attenuation and safety as reflected by host range specificity and lack of virulence in immunodeficient animals has been reported [6]. In accordance with previous studies attesting to its attenuation, NYVAC-Pf7 elicited low levels of vaccinia-neutralizing antibodies in subjects with no history of poxvirus exposure. However, the vaccine is a potent booster of vaccinia neutralization titers in volunteers previously exposed to vaccinia.

Since it has not been previously known whether repeated immunization of humans with live attenuated pox-based vectors would dampen the immune response to new antigens, these studies clearly indicate that repeated immunization with live virus vectors can elicit not only new immune responses but also boost existing antibody levels against foreign antigens, despite high vaccinia-neutralizing antibodies. Nevertheless, antivaccinia immunity did have a significant effect on the magnitude of the antibody response in volunteers previously exposed to vaccinia virus.

Humoral and cell-mediated immune responses against all stages of the malaria parasite were elicited in volunteers in both dose groups and without regard to prior vaccinia immune status. Since the evaluation of antibody-mediated immunity contributed by each antigen was limited by the lack of full-length recombinant protein for all of the antigens included in the vaccine, the serologic responses detected to the vaccine components are in fact an underestimation of the true antibody response. The lack of potent antibody responses in volunteers as detected in both ELISA and IFA probably reflects the highly attenuated nature of the virus vector used for vaccine construction and immunization. This interpretation is supported by the slow-to-develop as well as absolute low antivaccinia titers in vaccinia-naive vaccinated subjects.

Although detectable immune responses were elicited by NYVAC-Pf7 immunization, the level of immunity generated by this vaccine does not confer protection against malaria infection despite a delay in the prepatent period. We speculate that genetic immunization with malaria-encoded plasmids will elicit similar low-level antibody responses in humans and that boosting with appropriately adjuvanted recombinant proteins will be required for protective immunity. In contrast to immunization with NYVAC-Pf7, immunization with the CS protein alone, which we recently demonstrated, is expressed on hepatitis B surface antigen particles and, administered with a cocktail of three potent adjuvants, elicits a high degree of protective immunity [4] with significantly higher antibody levels to CS protein by both ELISA and IFA compared with sera from subjects immunized with NYVAC-Pf7 (data not shown).

NYVAC-Pf7 was designed principally to elicit cell-mediated immune responses directed toward the intrahepatic stages of the malaria parasite, and in this context, it was successful. The

Table 4. Proportions of volunteers with cytotoxic lymphocytes (CTL) or lymphoproliferative responses to CS, SSP2, or LSA1 antigen.

	CTL responses		Proliferative responses	
	Low dose (n = 23)	High dose (n = 15)	Low dose (n = 21)	High dose (n = 17)
Nonresponders	17 (73.9)	7 (46.7)	3 (14.3)	1 (5.9)
Response to 1 antigen	6 (26.1)	8 (53.3)	4 (19.0)	6 (35.3)
Response to 2 antigens	0	0	7 (33.3)	7 (41.2)
Response to 3 antigens	0	0	7 (33.3)	3 (17.6)

NOTE. Data are no. positive of those tested (%).

hepatic stage is the target for radiation-attenuated sporozoite-induced protection in animals and human volunteers exposed to experimental malaria challenge [24, 25]. The relevance of the detection of CTL in peripheral circulation with regard to malaria immunity is uncertain. Indeed, in the murine model of *Plasmodium berghei* malaria, immunization with NYVAC-CS conferred protection in >80% of mice, and this protection was afforded by CD8 cells [26]. In this context, it is significant that circulating CTL activity was detected in volunteers not protected from malaria challenge and no CTL activity was detected in the lone volunteer protected against the infection. Although these results might appear to suggest that CTL responses are less protective in humans, the CTL levels induced here were below the protective levels required in murine models. Furthermore, our evaluation of the induced CTL response was necessarily incomplete, as only selected peptides of particular HLA types were used and only the peripheral blood compartment could be sampled. Presumably, the only relevant tissue location for CTL activity in malaria infection is within the confines of the hepatic lymphoid compartment, a site not accessible for study.

The evaluation of this vaccine under the experimental conditions described here are limited by the ethical constraints in permitting volunteers infected with malaria to generate a potential anti–blood-stage immune response in the presence of continuous parasite multiplication. All volunteers were treated for malaria infection at first signs of parasitemia. Under conditions of natural infection, clinical immunity against disease only develops after parasites enter the peripheral circulation upon completing the intrahepatic stage of development. Therefore, any immune responses generated against the blood-stage components in this vaccine were unevaluable. However, it can reasonably be expected that because of the low level of immunogenicity of the blood-stage antigens in the volunteers, neither significant antiparasite nor antidisease immunity would have been generated. However, volunteers primed with NYVAC-Pf7 had significantly increased antibody responses after a primary malaria challenge infection to both MSP1 and AMA1, 2 antigens known to provide protective immunity in primate models of malaria [10, 27]. One can speculate that anti–blood-stage vaccines that prime the immune system to particular malaria antigens may ameliorate the clinical symptoms after an initial and subsequent malaria infections in nonimmune persons vulnerable to the most devastating forms of the disease. This hypothesis can only be tested in field trials under conditions of intense malarial transmission.

How can immunogenicity be enhanced and protection afforded against clinical infection? There is a growing consensus that immune responses primed by live virus vectors can be dramatically boosted by subsequent immunization with subunit recombinant vaccines [17]. Indeed, we have observed that the immunization of both rhesus monkeys and human volunteers primed with NYVAC-Pf7 and boosted with recombinant Pfs25 antigen demonstrated a significant rise in antibody titers (unpublished results). Currently, we are investigating the prime-boost strategy in primate models of *P. falciparum* malaria using attenuated live virus vectors (NYVAC and ALVAC) followed by boosting with multiple recombinant proteins or naked DNA vectors to enhance immunogenicity. In summary, NYVAC-Pf7 proved to be safe, well tolerated, and immunogenic for multiple antigens. Future formulations will require additional measures to boost immunogenicity prior to advanced development in human vaccine trials.

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