INFECTION AND IMMUNITY, Sept. 1996, p. 3833–3844 Vol. 64, No. 9
0019-9567/96/$04.00+0

NYVAC-Pf7: a Poxvirus-Vectored, Multiantigen, Multistage Vaccine Candidate for *Plasmodium falciparum* Malaria

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Received 16 January 1996/Returned for modification 27 February 1996/Accepted 5 June 1996

The highly attenuated NYVAC vaccinia virus strain has been utilized to develop a multiantigen, multistage vaccine candidate for malaria, a disease that remains a serious global health problem and for which no highly effective vaccine exists. Genes encoding seven *Plasmodium falciparum* antigens derived from the sporozoite (circumsporozoite protein and sporozoite surface protein 2), liver (liver stage antigen 1), blood (merozoite surface protein 1, serine repeat antigen, and apical membrane antigen 1), and sexual (25-kDa sexual-stage antigen) stages of the parasite life cycle were inserted into a single NYVAC genome to generate NYVAC-Pf7. Each of the seven antigens was expressed in NYVAC-Pf7-infected culture cells, and the genotypic and phenotypic stability of the recombinant virus was demonstrated. When inoculated into rhesus monkeys, NYVAC-Pf7 was safe and well tolerated. Antibodies that recognize sporozoites, liver, blood, and sexual stages of *P. falciparum* were elicited. Specific antibody responses against four of the *P. falciparum* antigens (circumsporozoite protein, sporozoite surface protein 2, merozoite surface protein 1, and 25-kDa sexual-stage antigen) were characterized. The results demonstrate that NYVAC-Pf7 is an appropriate candidate vaccine for further evaluation in human clinical trials.

Malaria remains a major cause of disease and death in much of the world. More than 2 billion people populate malarious areas, and as many as 500 million contract clinical cases of malaria each year (76). About half of malaria cases are caused by infection with *Plasmodium falciparum*, which claims approximately 2.5 million lives each year, mostly of children under the age of five (76). The appearance and rapid spread of drug-resistant parasites and insecticide-resistant mosquitoes have seriously impaired the effectiveness of these common tools for malaria control. The need for an effective vaccine is thus urgent and of prime importance to global public health.

More than a decade of intensive malaria vaccine research (24, 53) has identified several major obstacles to the development of an effective vaccine, including the developmental regulation of antigen expression during parasite replication, nonresponsiveness of individuals to particular parasite antigens or epitopes, and variability of antigens among different parasite isolates. Subunit vaccines based on single malarial antigens may fail to protect an individual because of any one of these factors. One means of circumventing these barriers is the inclusion in a vaccine formulation of multiple antigens from different stages of the parasite life cycle. In this way, the inability to mount a fully effective immune response to a particular antigenic component of the vaccine or to antigens of a given stage of the life cycle may be compensated by effective responses to other antigens or life cycle stages, resulting in protective immunity. Immunization with combinations of antigens or epitopes derived from single stages of the parasite life cycle is more effective in rodent and monkey models of malaria infection than is immunization with individual antigens or epitopes (20, 40, 57). Despite such multicomponent strategies, the problem of overcoming nonresponsiveness to vaccine components derived from a single life cycle stage remains a difficult one. This is clearly illustrated by field studies with the SPf66 multicomponent *P. falciparum* blood stage vaccine, in which 26 to 45% of inoculated individuals do not seroconvert (52, 83). A multiantigen, multi-life cycle stage approach, by which immune responses are elicited against multiple antigens of several of the major stages of the parasite life cycle (including the infectious sporozoite and the liver, blood, and sexual stages), may provide a more effective vaccination strategy.

An attractive approach for the delivery of multiple antigens in a vaccine formulation is the use of vaccinia virus vectors (60), in part because of their large capacity to incorporate and express exogenous DNA. Vaccinia virus recombinants expressing antigens from pathogenic agents can stimulate both humoral (8, 13, 15, 34, 55, 56, 75, 79, 85) and cellular (12, 13, 79, 88, 91) immunity and confer protection against both experimental and natural challenge (4, 13, 26, 49, 79). Notably, however, vaccinia virus recombinants derived from laboratory or vaccine strains that express single malarial antigens have failed

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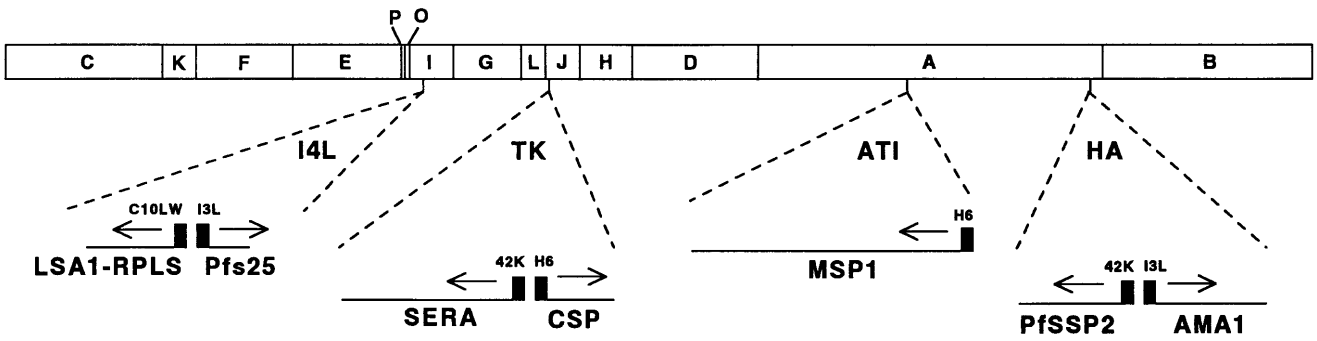

FIG. 1. Genomic organization of NYVAC-Pf7 (vP1209). The sites of insertion of the *P. falciparum* genes are indicated on a schematic representation of the *Hin*dIII map of the NYVAC genome. Solid boxes represent poxvirus promoter elements, and arrows indicate the orientation of transcription. RPLS, repeatless.

to confer protection in rodents (46, 68, 71) and monkeys (64). The failure of these recombinants may relate to the choice of poxvirus vector or the suboptimal quality of foreign-gene expression (64, 82).

NYVAC is a highly attenuated strain of vaccinia virus derived from the Copenhagen vaccine by the precise deletion of 18 open reading frames, some of which are associated with virulence or host range (78). The attenuation of NYVAC does

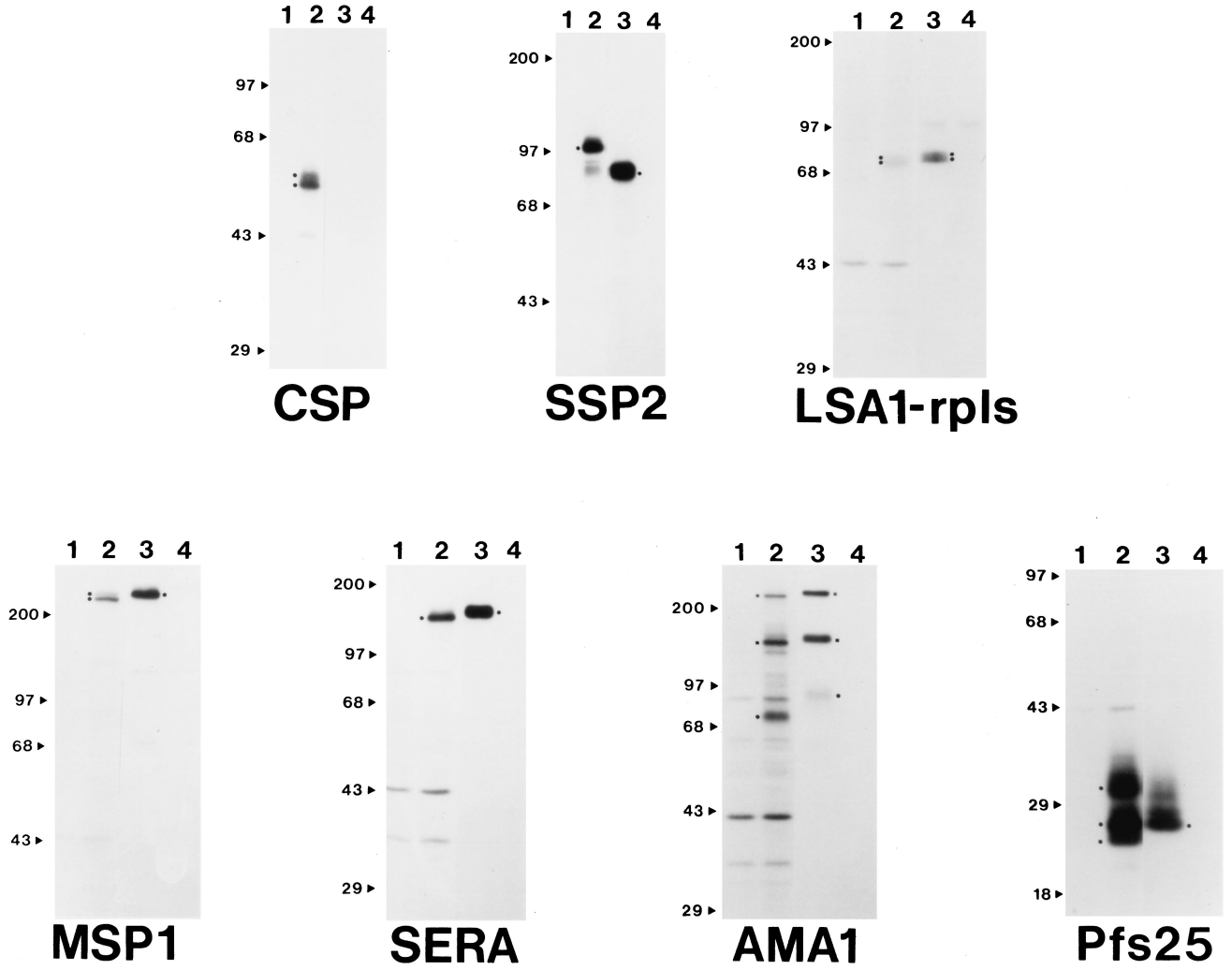

FIG. 2. Expression of *P. falciparum* antigens by NYVAC-Pf7. Lysates of cell pellets and culture supernatants from infected, [^{35}S]methionine-labeled HeLa cells were analyzed by IP at 18 h postinfection. The specific antibodies used were MAb Pf2A10 (for CSP), mouse anti-PfSSP2 (for PfSSP2), rabbit anti-LSA1 (for LSA1), rabbit anti-gp195 (for MSP1), rabbit anti-p126 (for SERA), human anti-malarial pool (AMA1), and MAb 4B7 (for Pfs25). Lanes: 1, NYVAC cell pellet; 2, NYVAC-Pf7 cell pellet; 3, NYVAC-Pf7 culture supernatant; 4, NYVAC culture supernatant. Molecular mass markers are indicated in kilodaltons. *P. falciparum* antigens are indicated by dots. IP of MSP1 and SERA, as well as AMA1 (top to bottom, respectively), by the pooled human antimalarial serum is evident. rpls, repeatless.

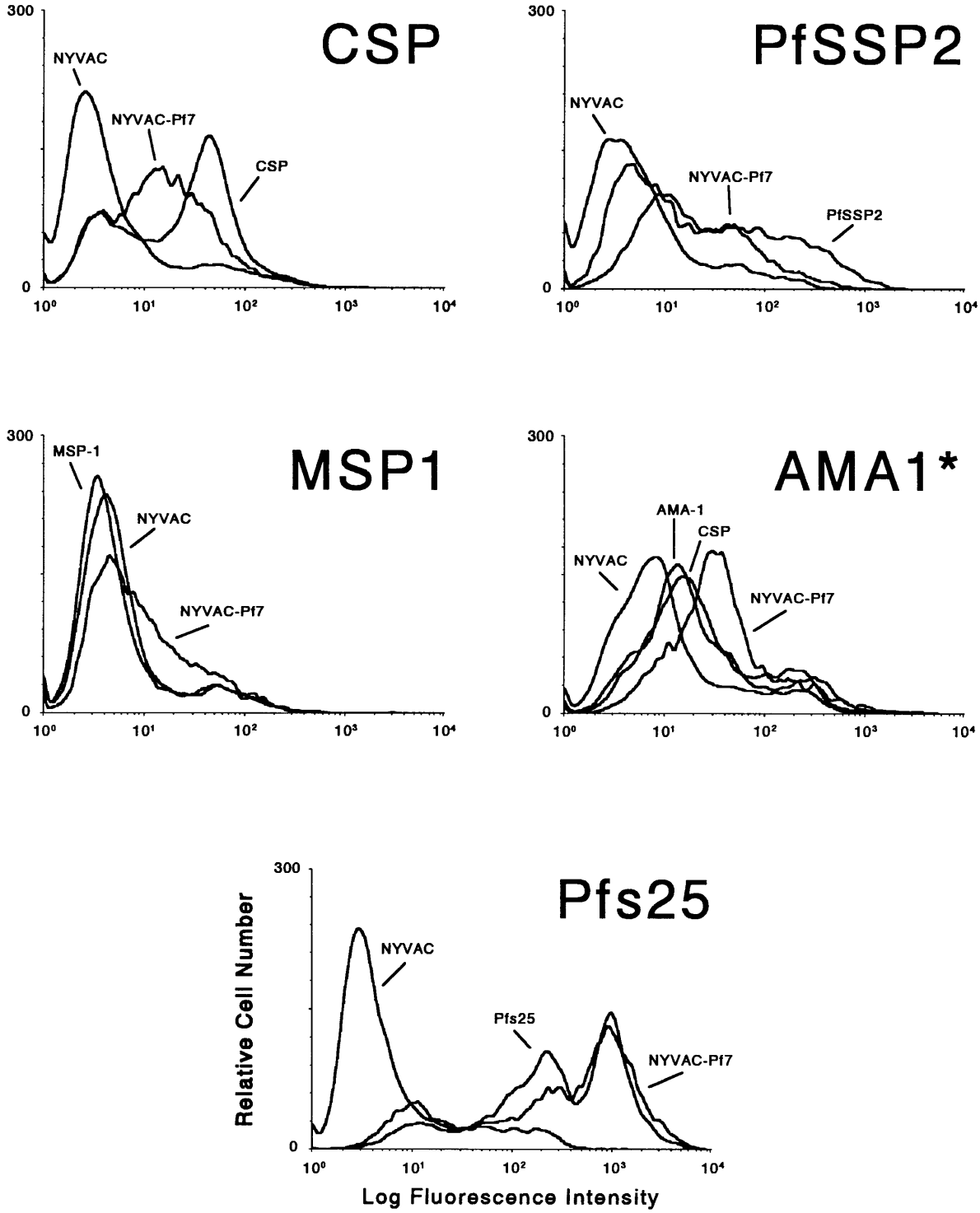

FIG. 3. Cell surface expression of *P. falciparum* antigens by NYVAC-Pf7. HeLa cells infected with NYVAC-Pf7 were evaluated for surface expression of five malarial antigens with specific antibodies. Controls included HeLa cells infected with NYVAC parental virus or NYVAC recombinants singly expressing CSP, PfSSP2, MSP1, AMA1, or Pfs25. The designation in the upper right corner of each histogram refers to the specificity of the antibody used for analysis. The asterisk by the AMA1 histogram indicates that a polyclonal reagent was used to detect expression of this antigen (see text for details). All other specific reagents were monoclonal or monospecific.

not diminish its immunizing potential as a vector in experimental or target species (5, 14, 43, 78, 80). In contrast to the failure of other vaccinia virus-malaria recombinants (46, 64, 68, 71), the potential of NYVAC-based recombinants to confer protection from experimental challenge with malaria parasites has been demonstrated (45). NYVAC thus provides a suitable foundation for the development of a multiantigen, multistage vaccine for human malaria.

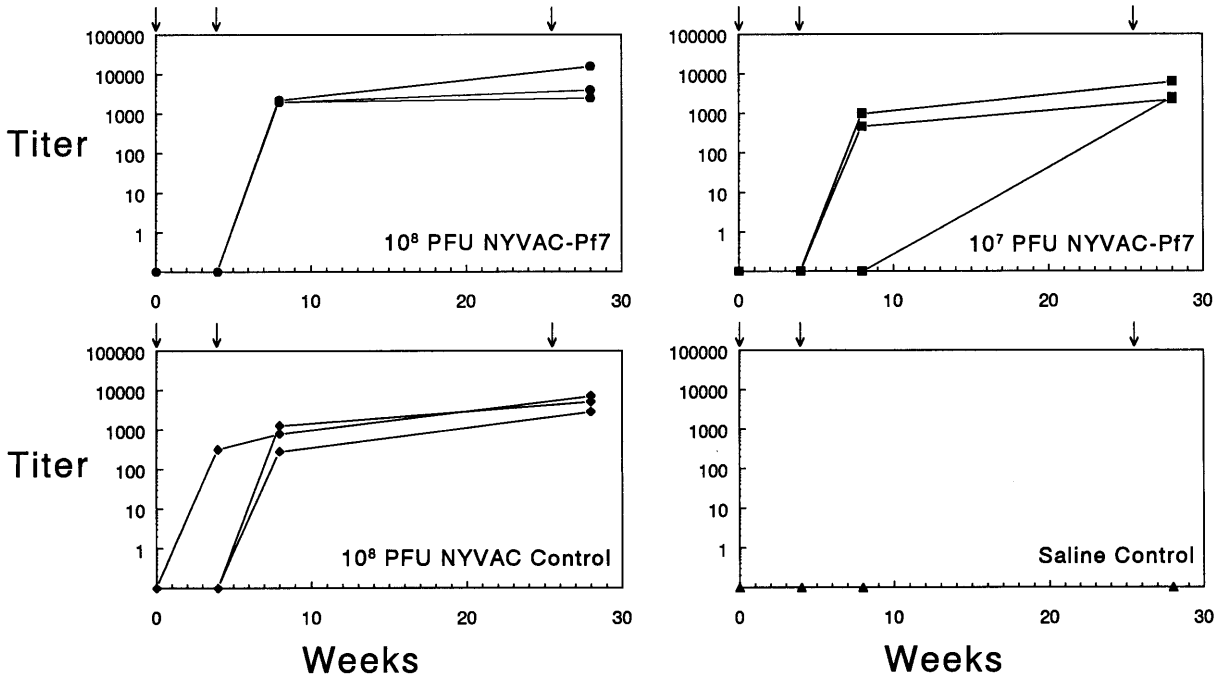

FIG. 4. NYVAC-Pf7 elicits vaccinia virus-neutralizing antibodies in rhesus monkeys. Serum samples from individual monkeys obtained at weeks 0, 4, 8, and 28 were evaluated for the presence of vaccinia virus-neutralizing antibodies by a plaque microneutralization assay, and 50% end points were calculated. The immunogen and dose for each group are indicated within the graphs. The timing of immunizations is indicated by arrows above each graph.

There are a number of *P. falciparum* proteins with potential as vaccinating antigens (23, 62). They include two antigens that are expressed on the surface of sporozoites, circumsporozoite protein (CSP) and sporozoite surface protein 2 (PfSSP2); one expressed only within infected liver cells, liver stage antigen 1 (LSA1); three expressed during various stages of schizogony in the blood and liver, merozoite surface protein 1 (MSP1), serine repeat antigen (SERA), and apical membrane antigen 1 (AMA1); and one expressed during the sexual stage on the surface of zygotes and ookinetes, the 25-kDa sexual-stage antigen (Pfs25). In some studies, these antigens have been shown to provide a level of protection in experimental models of malaria infection. CSP and SSP2 elicit protective immune responses, particularly cellular responses mediated by $CD8^+$ T cells (1, 45, 46, 57, 67, 70, 72), in rodents. Although CSP-based vaccine candidates evaluated in human clinical trials have provided disappointing results (2, 30), the tested vaccines have in general been designed to elicit humoral rather than cellular responses. MSP1 (20, 27, 61, 74), SERA (17, 61), and recombinant derivatives of these antigens (21, 28, 29, 32, 35, 36, 37, 42, 44) confer partial to full protection from *P. falciparum* blood stage challenge in monkeys. The *Plasmodium knowlesi* and *Plasmodium fragile* analogs of AMA1 confer partial protection from blood stage challenge in monkeys (11, 16).

The potential importance of LSA1 as a vaccine antigen was indicated by recent studies suggesting that resistance to severe malaria in West Africans who carry the HLA-B53 major histocompatibility complex class I allele may be associated with the development of LSA1-specific cytotoxic-T-lymphocyte (CTL) responses (31). Mice immunized with a peptide derived from *P. falciparum* LSA1 were partially protected from *Plasmodium berghei* sporozoite challenge (33).

Pfs25 is unique among the above-noted antigens in that the immune responses it elicits will most likely be of no direct benefit to the vaccinated individual. Rather, Pfs25 can elicit antibodies which block the transmission of the parasite from the vertebrate host to mosquitoes, as shown in mice and monkeys with recombinant Pfs25 immunogens (3, 38, 81a).

A NYVAC recombinant expressing a constellation of seven *P. falciparum* antigens could provide a vaccine candidate with the potential to elicit immunity directed against multiple stages in the malarial life cycle.

MATERIALS AND METHODS

***P. falciparum* genes.** The CSP gene (derived from the NF54/3D7 clone) utilized here differs from the published NF54 sequence (7) in that 10 repeat units were deleted and a base change at position 1199 results in an amino acid change from S to F. The PfSSP2 gene (NF54/3D7 clone) has been described previously (66). The repeat region of the LSA1 gene was found to be unstable (81b). Therefore, a repeatless form of the LSA1 gene (NF54 strain) was engineered which consists of nucleotides 1 to 458 of the full-length LSA1 gene (92) linked to nucleotides 1630 to 1909 by the sequence GCGCGCATCTAAAGAAACG (amino acids 1 to 458 linked to 1630 to 1909 by the sequence RASKET). The MSP1 gene (Uganda-Palo Alto strain) has been previously described (10) and corrected (50). The SERA gene (FCR3 strain) has been previously described (82). The AMA1 gene (NF54/3D7 clone) differs from the published sequence of the FC27 strain's AMA1 gene at 10 predicted amino acid positions (81). A five-amino-acid insertion of sequence RRIKS was generated between amino acids 377 and 378 during the isolation of this gene for subcloning. The Pfs25 gene (NF54/3D7 clone) has been described previously (39). Coding sequences were modified to remove occurrences of vaccinia early transcription termination signals (T_5NT) by PCR mutagenesis as follows: one early transcription termination signal was eliminated from the CSP gene by altering nucleotides 37 to 43 from TTTTTAT to TTCC TAT, two were eliminated from the MSP1 gene by altering nucleotides 16 to 40 from TTTTTATGTTCATTTCTTTTTTTTA to TTTCTATGTTCATTTCTTT TCTTTA, and one was removed from the AMA1 gene by altering nucleotides 600 to 606 from TTTTTAT to TTTCTAT.

Generation of NYVAC-Pf7. To generate NYVAC-Pf7 (vP1209), expression cassettes consisting of the poxvirus promoter-*P. falciparum* gene combinations were subcloned into NYVAC donor plasmids, which were then used to insert the expression cassettes into defined sites in the NYVAC genome by in vivo recombination (58). The promoters we have utilized are the vaccinia virus early-late H6 promoter (59), the Pi or C10LW early promoter from vaccinia virus strain WR

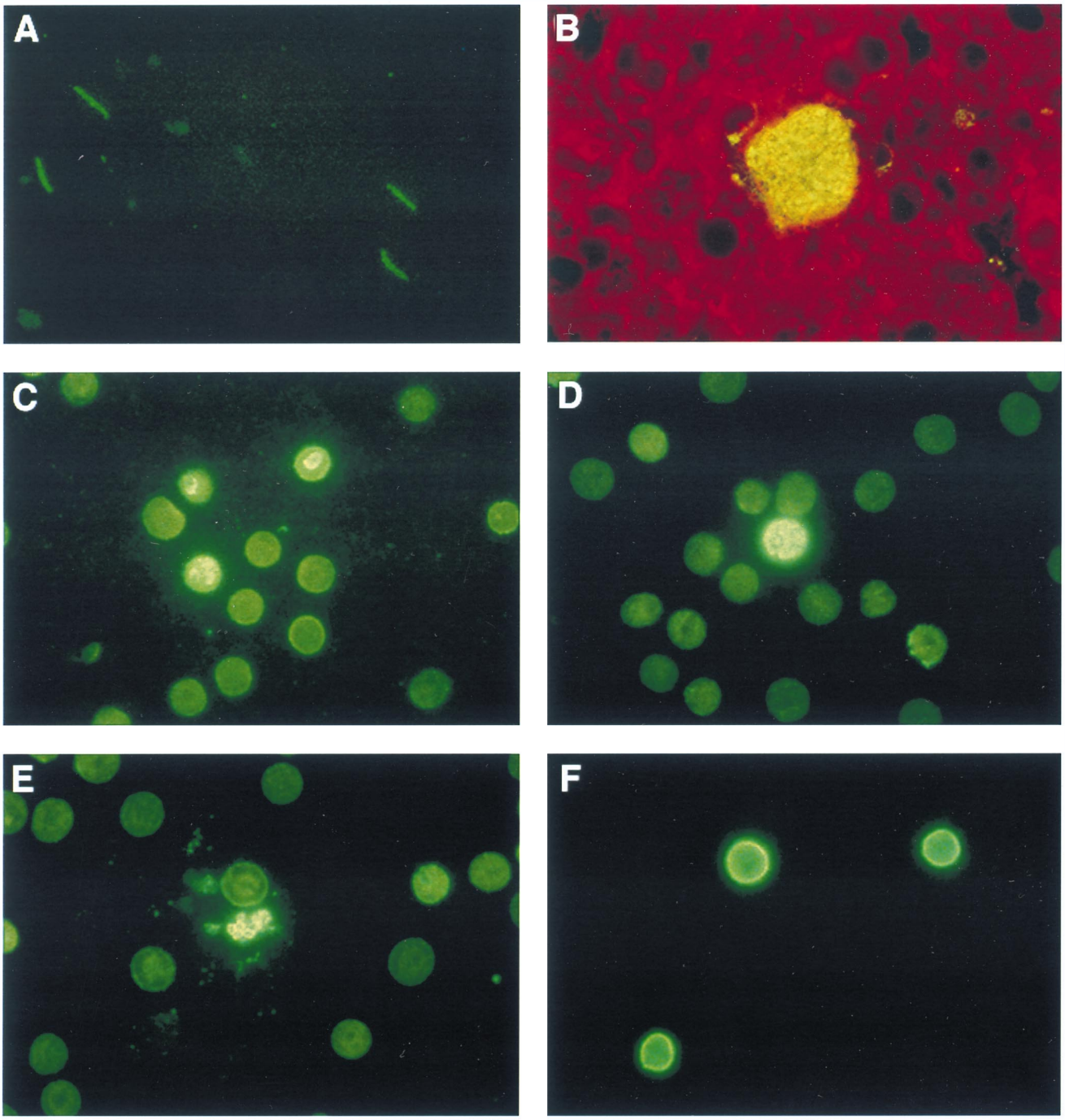

FIG. 5. NYVAC-Pf7 elicits antibody responses against multiple stages of the malarial life cycle. Week 28 serum from monkey E39 was evaluated by IFA against sporozoites at a dilution of 1:50 (A), infected chimpanzee liver sections at a dilution of 1:250 (B), infected erythrocytes at a dilution of 1:400 (C, D, and E), and live zygotes at a dilution of 1:100 (F). There was no reactivity against these stages with similarly diluted serum from a NYVAC-immunized control monkey.

(86), the vaccinia virus I3L early-intermediate promoter (60, 69, 84), and the entomopoxvirus 42K early promoter (5′-TCAAAATTGAAAATATATAATTA CAATATAAA-3′) (21a) (Fig. 1). The insertion sites for the expression cassettes were the I4L, TK, ATI, and HA loci, which were generated by the deletion of open reading frames I4L, J2R, A26L, and A56R, respectively, during the generation of NYVAC (22, 78).

Serological reagents for expression analysis. CSP was detected with the repeat-specific monoclonal antibody (MAb) Pf2A10 (6). PfSSP2 was detected with mouse anti-PfSSP2 serum (66) or MAb 88:10:161 (31a). Repeatless LSA1 was detected with serum from a rabbit inoculated with a peptide corresponding to a predicted LSA1 epitope (DNEILQIVDELSEDITKYFMKL) (92). MSP1 was detected with rabbit anti-gp195 (MSP1) serum or with MAb CE2.1 (9) or MAb 3D3 (47). SERA was detected with rabbit anti-p126 (SERA) serum or MAb 23D5 (17, 18). AMA1 was detected with a pool of immunoglobulins from African donors with high antimalaria titers (this serum detects poxvirus-expressed AMA1 and CSP by flow cytometric analysis and AMA1, MSP1, and SERA by immunoprecipitation analysis [Fig. 2 and 3] [81c]) or MAb 28G2dc1 (51). Pfs25 was detected with MAb 4B7 (3).

Immunoprecipitation analysis. Analysis of the expression of *P. falciparum* antigens in NYVAC-Pf7-infected HeLa cells was performed as previously described (82).

Flow cytometric analysis. HeLa cells were infected with NYVAC-Pf7,

TABLE 1. Expression of *P. falciparum* antigens by NYVAC-Pf7

Antigen	Expression on surface[a]	Molecular mass of antigen (kDa)[b]	
		In cell	Secreted
CSP	+	59, 55	NA[c]
PfSSP2	+	107	91
LSA1 (repeatless)	−	73, 70	75, 72
MSP1	+/−	230, 220	230
SERA	−	135	137
AMA1	+	83	90
Pfs25	+	33, 27, 25	27

[a] Determined by flow cytometric analysis (Fig. 3). +, expressed on cell surface; −, not expressed on cell surface.
[b] Determined by IP analysis (Fig. 2).
[c] NA, not applicable.

NYVAC, or appropriate control recombinants at a multiplicity of 5 PFU/cell for 16 h. Unfixed infected cells were then stained by standard indirect methods with appropriate serological reagents. In all, 10,000 live stained cells were evaluated for surface fluorescence with a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, Calif.). Fluorescence was measured with logarithmic amplification after gating on forward-angle light scatter versus 90° light scatter to exclude dead cells and debris. Antibodies used for evaluation were MAb Pf2A10 (for CSP), mouse anti-PfSSP2 (for PfSSP2), rabbit anti-gp195 (for MSP1), the pooled human antimalarial serum (for AMA1), and MAb 4B7 (for Pfs25). The control recombinants are indicated in Fig. 3.

Plaque immunoassays. Plaque immunoassays to detect internal expression of malarial antigens by NYVAC-Pf7 were performed as previously described (48) with MAb Pf2A10 (for CSP), MAb 88:10:161 (for PfSSP2), MAb 3D3 (for MSP1), MAb 23D5 (for SERA), MAb 28G2dc1 (for AMA1), or MAb 4B7 (for Pfs25). Controls included the NYVAC parent, NYVAC-CSP, NYVAC-PfSSP2, NYVAC-MSP1, NYVAC-SERA, NYVAC-AMA1, and NYVAC-Pfs25.

Immunization of monkeys with NYVAC-Pf7. Rhesus monkeys (20 to 30 lb [ca. 900 to 1,400 g]) were selected on the basis of nonmalarial history, physical examination, clinical laboratory results, and negative antibody titers to simian retrovirus, CSP, and MSP1 by enzyme-linked immunosorbent assay (ELISA). All monkeys were monitored monthly for 5 months before immunizations to establish baseline data. Physical examinations included the determination of weight, temperature, pulse rate, respiration, auscultation, and palpation before the immunizations and continued weekly or biweekly until week 56 of the study. Blood chemistry profiles (urea nitrogen, albumin, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, potassium, sodium, calcium, CO_2, Cl^-, creatine kinase, creatinine, γ-glutamyltransferase, glucose, lactate dehydrogenase, phosphorus, total globulin, total protein, total bilirubin, cholesterol, and triglycerides) and a blood hematology screen (hematocrit, hemoglobin, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, mean corpuscular volume, platelets, erythrocytes, and leukocytes) were performed at these times by personnel of the clinical laboratory of the Division of Pathology, Walter Reed Army Institute of Research.

Determination of vaccinia virus-neutralizing antibodies. The presence of vaccinia virus-neutralizing antibodies in individual serum specimens of immunized monkeys was determined by plaque neutralization assaying (54) performed in 96-well microtiter plates. Calculation of 50% end points (Fig. 4) was by the method of Reed and Muench (65).

IFAs. Sporozoite (87), liver stage (63), and blood stage (41) immunofluorescence assay (IFA) titers were determined as described previously. For zygote IFAs, titers were determined in the same manner as that used for blood stages, with fixed 3-h gametes. The assay depicted in Fig. 5F was performed with live 7-h zygotes. In all experiments, IFA titers were considered reliable only if they reflected at least a fourfold increase over prebleed titers from the same animal. Concurrent specimens from control monkeys inoculated with NYVAC or saline consistently had low or zero IFA titers.

ELISAs. ELISAs to detect antibodies to CSP, PfSSP2, or Pfs25 in monkey serum were performed as previously described (3, 89).

Immunoblot analysis. Evaluation of antibodies to MSP1 in monkey sera by immunoblot analysis was performed as previously described (9).

Nucleotide sequence accession number. The GenBank nucleotide sequence accession number for the AMA1 gene (NF54/3D7 clone) is U65407.

RESULTS

Construction and characterization of NYVAC-Pf7. NYVAC-Pf7 (vP1209) was derived by sequentially inserting genomic or cDNA copies of the genes encoding CSP, PfSSP2, a repeatless form of LSA1, MSP1, SERA, AMA1, and Pfs25 at four defined sites in the NYVAC genome (Fig. 1). In constructing the expression cassettes for the insertion of these genes into NYVAC, each gene was engineered so that it was placed under the control of a separate poxvirus promoter element such that no extraneous DNA sequences separated the promoter and the foreign gene coding sequences. In addition, vaccinia virus early transcriptional termination sequences (90) within the *P. falciparum* genes were removed by modifying the nucleotide sequences to eliminate the termination signals without changing the encoded amino acids. Such modifications can enhance the immunogenicity of extrinsic antigens expressed by vaccinia virus (19). Further modifications included the insertion of early transcription termination sequences after the translational stop codons and the addition of a series of stop codons in all reading frames to prevent the generation of spurious translational products. The genomic organization of NYVAC-Pf7 was confirmed by restriction enzyme analysis of genomic DNA (data not presented).

The genetic stability of NYVAC-Pf7 was investigated by performing DNA sequence analysis on subcloned restriction fragments from genomic DNA which contained the *P. falciparum* expression cassettes and NYVAC flanking arms. The NYVAC-Pf7 DNA was from a clinical lot prepared in accordance with good manufacturing practice guidelines. Comparison of the sequence of the malarial genes from this clinical material with the sequence obtained at the plasmid level prior to insertion indicated that no point mutations had been introduced during the generation of the recombinant or during the manufacture of the clinical vaccine lot (data not presented). Thus, the NYVAC-Pf7 vaccine is genotypically stable.

The expression of the seven malarial genes in culture cells infected with NYVAC-Pf7 was evaluated with sera specific for each of the antigens. By immunoprecipitation (IP) analysis of radiolabelled lysates of infected HeLa cells, expression of each of the seven antigens was demonstrated (Fig. 2, Table 1). This

TABLE 2. Determination of phenotypic homogeneity of NYVAC-Pf7 by plaque-based immunoassay

Infecting virus[a]	*P. falciparum* antigen expressed											
	CSP		PfSSP2		MSP1		SERA		AMA1		Pfs25	
	No. of plaques	% Positive	No. of plaques	% Positive	No. of plaques	% Positive	No. of plaques	% Positive	No. of plaques	% Positive	No. of plaques	% Positive
NYVAC	71	0	94	0	66	0	76	0	30	0	71	0
NYVAC-Pf7	522	100	607	100	635	100	442	100	561	100	674	100
NYVAC-Pf7 P5 (0.1)	601	100	238	100	620	100	222	100	ND[b]	ND	231	100
NYVAC-Pf7 P5 (0.01)	658	100	197	100	652	100	199	100	ND	ND	227	100

[a] P5, inoculum from fifth passage in chicken embryo fibroblast cells. The numbers in parentheses indicate the multiplicities of infection in PFU per cell.
[b] ND, not determined.

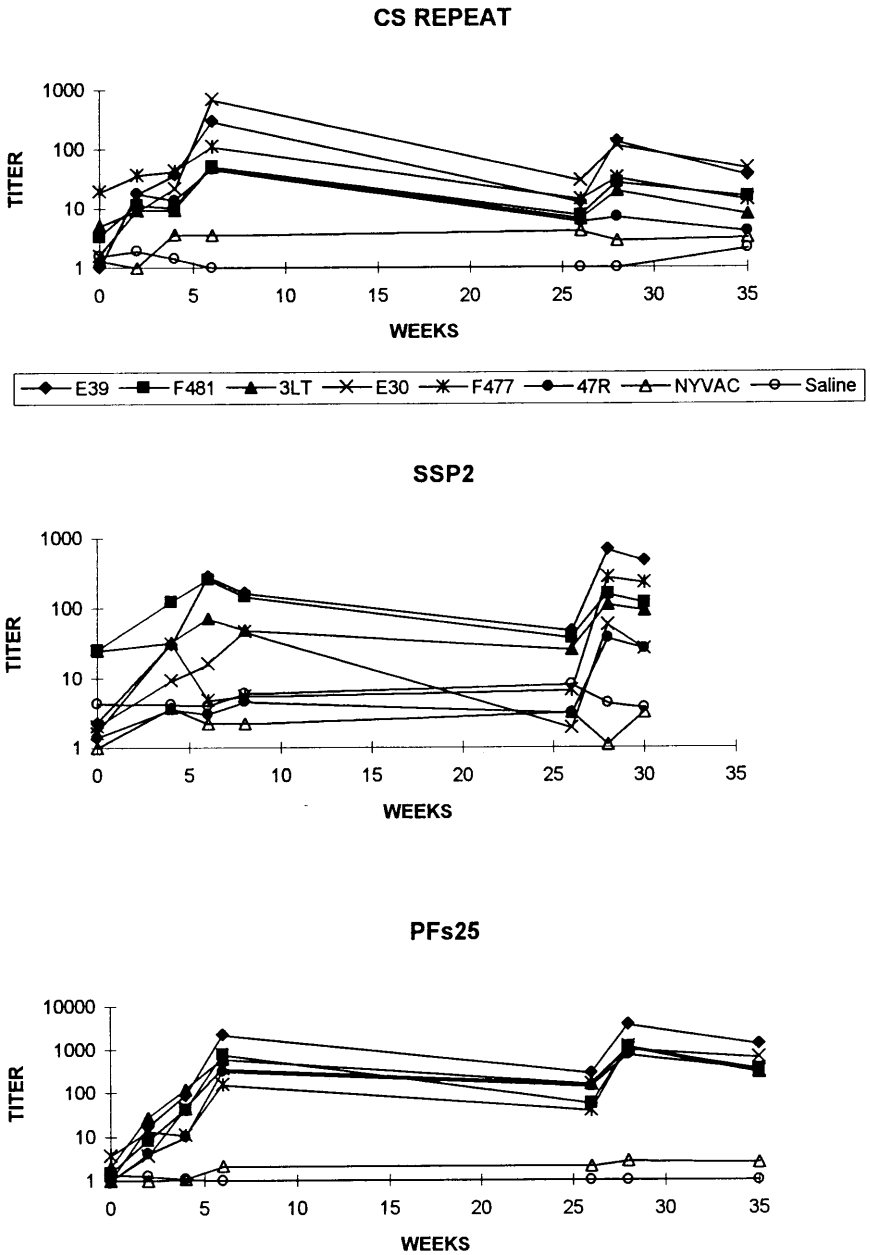

FIG. 6. NYVAC-Pf7 elicits CSP-, PfSSP2-, and Pfs25-specific antibodies in rhesus monkeys. Serum samples from individual monkeys inoculated with 10^8 (E39, F481, and 3LT) or 10^7 (E30, F477, and 47R) PFU of NYVAC-Pf7 and pooled sera from monkeys inoculated with NYVAC parental virus or saline were evaluated for antibodies to the CS repeats ($NANP_{10}$), PfSSP2 (*Escherichia coli* expressed; partially purified), or Pfs25 by ELISA. All animals were inoculated at weeks 0, 4, and 26. Titers are expressed as the serum dilutions required to achieve an optical density at 420 nm of 1.0.

analysis indicated that repeatless LSA1 and SERA were secreted from infected cells, which is consistent with the localization of these proteins to the parasitophorous vacuole of the developing parasite (33, 82). The other malarial antigens, except CSP, were also released from infected cells. Multiple peptidic forms of each of the malarial antigens were detected (Fig. 2, Table 1). Such forms may represent biosynthetic intermediates in the synthesis of these antigens or differential posttranslational modifications, such as glycosylation, which have been observed previously with malarial antigens expressed by vaccinia virus (82). Flow cytometric analysis indicated that several of the malarial antigens are localized on the surface of infected cells (Fig. 3). In particular, surface presentation of CSP, PfSSP2, and Pfs25 was readily demonstrated. MSP1 was also present on the cell surface, albeit at low levels. AMA1 expression was evaluated with a pooled human antimalarial serum that recognizes both AMA1 and CSP by flow cytometric analysis, but not the other antigens under study (data not presented). The reactivity of this serum with NYVAC-Pf7-infected HeLa cells, compared with its reactivity with cells infected with NYVAC-AMA1 and NYVAC-CSP single recombinants, suggests that AMA1 is expressed on the cell surface (Fig. 3). SERA and repeatless LSA1 are not localized on the surface of infected cells (data not presented). Thus, five of the seven *P. falciparum* antigens expressed by NYVAC-Pf7 are localized on the surface of infected culture cells.

TABLE 3. IFA determination of stage-specific antibody responses elicited by NYVAC-Pf7

Monkey	Immunogen	IFA titer against[a]:			
		Sporozoites	Liver stage	Blood stage	Zygotes
E39	10^8 PFU of NYVAC-Pf7	200	200	800	4,000
F481	10^8 PFU of NYVAC-Pf7	200	<25	800	4,000
3LT	10^8 PFU of NYVAC-Pf7	100	25	800	4,000
E30	10^7 PFU of NYVAC-Pf7	200	ND[b]	200	4,000
F477	10^7 PFU of NYVAC-Pf7	100	ND	3,200	4,000
47R	10^7 PFU of NYVAC-Pf7	<10	ND	400	4,000
F485	10^8 PFU of NYVAC	<10	ND	<50	<1,000
H31	10^8 PFU of NYVAC	<10	ND	<50	<1,000
OK6	10^8 PFU of NYVAC	<10	ND	<50	<1,000
F483	Saline	<10	ND	<50	<1,000
H293	Saline	<10	ND	<50	<1,000
G617	Saline	<10	ND	<50	<1,000

[a] Endpoint IFA titers of week 28 sera from individual monkeys are indicated.
[b] ND, not determined.

The expression of the *P. falciparum* components of NYVAC-Pf7 was evaluated at the population level by use of a plaque-based immunoassay with specific serological reagents. One hundred percent of the evaluated NYVAC-Pf7 plaques expressed CSP, PfSSP2, MSP1, SERA, AMA1, and Pfs25 (Table 2). Repeatless LSA1 expression could not be evaluated because the LSA1-specific serological reagent was not functional in this assay. The expression of these antigens by NYVAC-Pf7 was further evaluated after five blind passages at two multiplicities of infection in chick embryo fibroblast cells. After passage, 100% of the evaluated plaques retained expression of the five tested *P. falciparum* antigens (Table 2) and there was no alteration in the sizes of the seven expressed antigens by IP analysis (data not presented). These results clearly demonstrate the phenotypic stability of NYVAC-Pf7.

Safety and immunogenicity in nonhuman primates. The safety and immunogenicity of preclinical batches and clinical vaccine batches of NYVAC-Pf7 prepared in accordance with good manufacturing practice guidelines were evaluated in mice, rabbits, and rhesus monkeys (*Macaca mulatta*). The safety of the vaccine and induction of immune responses in mice and rabbits were documented (data not presented). In the monkey study, groups were composed of three adult males who received either 10^8 PFU of NYVAC-Pf7, 10^7 PFU of NYVAC-Pf7, 10^8 PFU of NYVAC parental control virus, or saline by the intramuscular (i.m.) route at weeks 0, 4, and 26. The primary goal of this study was to monitor, by clinical and laboratory examination, any local reactogenicity or systemic toxicities of the major organ systems in the monkeys after vaccination. There was no febrile reaction to the immunizations, no weight loss, and no evidence of physical discomfort during the course of the study. There was no induration, erythema, or ulceration observed at the injection site after any inoculation. In all monkeys, hematologic parameters and serum chemistry results after each immunization were consistent with preimmunization values. There was no evidence of adverse reactions to immunization in any animal, indicating that three inoculations with the two doses of NYVAC-Pf7 by the i.m. route was safe and nontoxic in rhesus monkeys.

The immunogenicity of NYVAC-Pf7 was studied in these monkeys by evaluating antibody responses to both malarial parasites and the NYVAC vector. Vaccinia virus-neutralizing antibodies were elicited in each of the monkeys immunized with NYVAC-Pf7, as well as in those receiving the NYVAC parental virus, after two or three inoculations (Fig. 4).

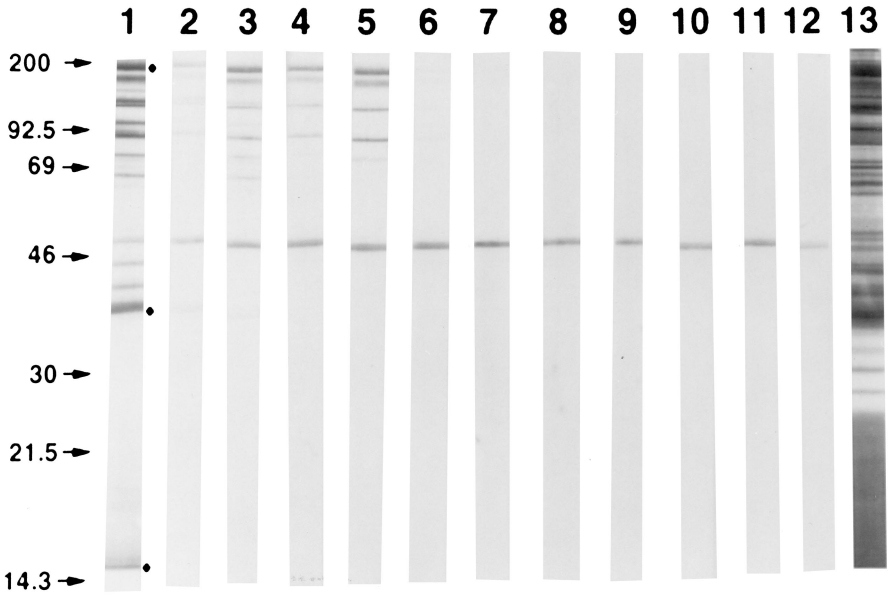

FIG. 7. NYVAC-Pf7 elicits MSP1-specific antibodies in rhesus monkeys. Week 28 serum specimens from individual monkeys (1:200 dilution) inoculated with NYVAC-Pf7, NYVAC parental virus, or saline were evaluated by immunoblotting with MSP1 affinity purified from Uganda-Palo Alto strain parasites. The diamonds between lanes 1 and 2 identify the 200-kDa MSP1 precursor and the 42- and 19-kDa C-terminal processing fragments. Lanes 1, 2, and 3, monkeys E39, F481, and 3LT, respectively (10^8 PFU of NYVAC-Pf7); lanes 4, 5, and 6, monkeys E30, F477, and 47R, respectively (10^7 PFU of NYVAC-Pf7); lanes 7, 8, and 9, monkeys F485, H31, and OK6, respectively (10^8 PFU of NYVAC); lanes 10, 11, and 12, monkeys F483, H293, and G617, respectively (saline); lane 13, rabbit anti-MSP1 serum (1:1,000 dilution). Molecular mass markers are indicated in kilodaltons. Although difficult to visualize in this reproduction, detection of the 19-kDa C-terminal fragment by serum samples from monkeys F481 and 3LT (lanes 2 and 3, respectively) was evident on the original immunoblot.

Because NYVAC-Pf7 was designed as a multiantigen, multistage vaccine, the determination of immune responses in the immunized monkeys reactive with the various stages of the parasite life cycle was a key element in the evaluation of this vaccination strategy. Thus, serum samples from immunized monkeys were evaluated by IFA of sporozoites, infected chimpanzee liver sections (liver stage), infected erythrocytes (blood stage), and zygotes (sexual stage). IFA titers against sporozoites were weak but were reproducibly detected in five of six monkeys inoculated with NYVAC-Pf7 (Table 3). All monkeys immunized with NYVAC-Pf7 developed IFA titers against blood stage parasites and zygotes (Table 3). Serum specimens from two of three monkeys inoculated with 10^8 PFU of NYVAC-Pf7 detected liver schizonts in tissue sections of liver from a chimpanzee infected 6 days previously with *P. falciparum* sporozoites. Figure 5 provides examples of the reactivity patterns obtained against the various life cycle stages with week 28 serum from monkey E39, who was inoculated with 10^8 PFU of NYVAC-Pf7. The serum from this monkey contained antibodies which recognized the surfaces of sporozoites (Fig. 5A), infected liver hepatocytes (Fig. 5B), blood stage ring forms and trophozoites (Fig. 5C), schizonts (Fig. 5D), and released merozoites (Fig. 5E), as well as fertilized zygotes (Fig. 5F), which are found only in the mosquito gut.

It was of further interest to determine the contribution of the individual malarial antigens to the multistage antibody responses observed in immunized monkeys. The availability of recombinant parasite proteins and epitopes has allowed the development of reliable ELISAs for the evaluation of antibody responses to the CSP repeat epitope (89), PfSSP2, and Pfs25 (3). By use of these assays, the elicitation of antibodies to these antigens in sera from immunized monkeys was investigated (Fig. 6). Monkeys inoculated with NYVAC-Pf7 developed modest antibody responses to the CSP repeat epitope which peaked after the second inoculation. The third inoculation resulted in a minor boost in titer. This pattern of response is typical of responses to the CSP repeats elicited by vaccination (2, 30). Similar responses were observed to PfSSP2. Vigorous responses to Pfs25, which were boosted by subsequent inoculations, were evident in all NYVAC-Pf7-immunized animals (Fig. 6). Immunoblot analysis with purified parasite-derived MSP1 revealed that after three inoculations, all monkeys immunized with NYVAC-Pf7 developed antibodies recognizing the 195-kDa MSP1 precursor protein (Fig. 7). Each of the monkeys immunized with 10^8 PFU of the vaccine also developed antibodies to the p42 and p19 C-terminal MSP1 fragments (Fig. 7). In general, a dose-response effect was observed for each evaluated antigen.

DISCUSSION

The results presented here describe the generation, characterization, and some preclinical evaluation of NYVAC-Pf7, the first multiantigen, multistage vaccine candidate for *P. falciparum* malaria. Evaluation of the immunogenicity of NYVAC-Pf7 has thus far focused on antibody responses in immunized rhesus monkeys. The serological analyses presented here indicate that NYVAC-Pf7 does indeed elicit immune responses against multiple stages of *P. falciparum*. The ability of antibodies against both preerythrocytic and blood stage antigens to contribute to protection has been suggested (23, 63). A dissection of the contribution of the particular malarial antigens to these multistage responses was begun, and as demonstrated here, all monkeys generated antibodies to CSP, PfSSP2, MSP1, and Pfs25. Reliable assays that allow the evaluation of responses to repeatless LSA1, SERA, and AMA1 in these animals are not yet available. Work continues on the development of appropriate assays for these antigens.

One rationale for developing a poxvirus-based vaccine was to maximize the elicitation of cellular immunity, particularly CTL responses (45). The inclusion of at least five antigens expressed during the liver stage of parasite development (25, 66, 77), and probably a sixth (AMA1), provides NYVAC-Pf7 with the potential to direct a broad-based CTL response against infected liver cells. $CD8^+$ T-cell responses elicited by preerythrocytic antigens can confer some protection in rodents (1, 45, 46, 57, 67, 70, 72). Furthermore, LSA1-specific CTLs may play a role in resistance to severe *P. falciparum* malaria in West Africans (31). These studies support the notion that the elicitation of appropriate cellular responses may be an important characteristic of a successful malaria vaccine. Cellular responses elicited by NYVAC-Pf7 are currently under study.

Demonstrating the functional nature of immune responses to human malaria parasites is difficult. Although in vitro assays of immune function do exist, none correlate with protection. Furthermore, there are no available animal model systems that can accurately predict the protective potential of a malaria vaccine in humans. Although blood stage infection with *P. falciparum* is supported in *Aotus* monkeys and has provided a useful model for evaluation of blood stage malaria vaccine antigens, vaccination results are more reliable in some karyotypes than in others (73). The multistage nature of NYVAC-Pf7, consisting of components derived from preerythrocytic as well as blood and sexual stages, makes it difficult, if not impossible, to fully evaluate this vaccine candidate in such models in which only blood stage challenge is feasible. Fortunately, the availability of cloned, chloroquine-sensitive *P. falciparum* strains makes possible the administration of controlled challenge infections with sporozoites to evaluate the efficacy of promising candidate malaria vaccines in humans (2, 30).

The demonstrated safety of NYVAC-Pf7 in rhesus monkeys and the capacity to elicit humoral immune responses against multiple stages of the *P. falciparum* developmental cycle justify further evaluation in human clinical trials. Only in such studies can the functional nature of the immune responses elicited by NYVAC-Pf7, as measured by protective efficacy, be reliably assessed. These studies are now under way.

ACKNOWLEDGMENTS

We thank J. Tartaglia for critical review of the manuscript; H. Martinez for synthesis of oligonucleotides; J. Winslow, S. Davis, S. Mercer, S. Jones, D. Pavlovic, C. Bongermino, H. Martinez, K. Catlin, and D. Conte for expert technical assistance; M. Harmon and D. Schaefer for production of GMP-grade NYVAC-Pf7; M. Topper (Clinical Laboratory, Division of Pathology, WRAIR) for performing blood chemistry and hematology profiles on rhesus monkeys; J. Williams and D. Scheumann for ELISA procedures; J. Sacci for performing liver stage IFAs; B. Wizel for development of PfSSP2 assays; J. Lyon for MAb 3D3; R. Nussenzweig for MAb Pf2A10; P. Delplace for rabbit anti-p126 and MAb 23D5; M. Hommel for pooled human antimalarial sera; and A. Thomas for MAb 28G2dc1.

This investigation received serological reagents produced with financial support from the UNDP/WORLD BANK/WHO Special Programme for Research and Training in Tropical Diseases (TDR) and support from the United States Agency for International Development cooperative agreement HRN-6001-A00-2020-00.

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Editor: D. H. Howard