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Attenuated Vaccinia Virus-Circumsporozoite Protein Recombinants Confer Protection against Rodent Malaria

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NYVAC-based vaccinia virus recombinants expressing the circumsporozoite protein (CSP) were evaluated in the *Plasmodium berghei* rodent malaria model system. Immunization of mice with a NYVAC-based CSP recombinant elicited a high level of protection (60 to 100%). Protection did not correlate with CS repeat-specific antibody responses and was abrogated by in vivo $CD8^+$ T-cell depletion. Protection was not enhanced by modification of the subcellular localization of CSP. These results suggest the potential of poxvirus-based vectors for the development of vaccine candidates for human malaria.

The most effective form of experimental malaria vaccination to date consists of immunization with irradiated sporozoites, which has conferred protection in humans, monkeys, mice, and fowl (27). Both humoral (16, 32, 58) and cell-mediated (34) immune responses against the circumsporozoite protein (CSP), a major component of the sporozoite surface coat (27), contribute to protection against sporozoites in rodents. Vaccination studies with rodents and recombinant CS protein (12) or synthetic peptides derived from CSP coupled to carrier molecules (12, 20, 44) demonstrated protection, but not to the level achieved with irradiated sporozoites. Clinical trials with humans and recombinant or synthetic *Plasmodium falciparum* CSP-based candidate vaccines were disappointing, with less than 20% protective efficacy reported (2, 15).

The use of live vectors, particularly vaccinia virus, expressing immunologically relevant antigens from infectious agents as potential vaccines is encouraged by numerous studies. Vaccinia virus recombinants can stimulate both humoral (6, 8, 10, 17, 28, 29, 43, 46, 52) and cellular (7, 8, 46, 56, 57) immune responses and protect against an experimental or natural challenge (3, 8, 10, 14, 25, 46, 52). In a few cases, vaccinia virus recombinants have failed to provide adequate protection against challenge (11, 26, 33, 59), perhaps because of the quality or temporal regulation of foreign gene expression (11, 33, 59) or the immunizing potential of the particular vaccinia virus vector utilized (11, 26). Notably, no protection of rodents from sporozoite challenge was demonstrated after immunization with vaccinia virus-CSP recombinants derived from either a laboratory (23, 37) or a vaccine strain (40).

NYVAC is a highly attenuated strain of vaccinia virus derived from the Copenhagen vaccine strain by precise deletion of 18 open reading frames, some of which are associated with virulence and host range (45). The highly attenuated phenotype of NYVAC does not significantly diminish its immunizing potential as a vector in both experimental and target species (4, 9, 19, 45, 47). NYVAC-based recombinants expressing rabies virus, Japanese encephalitis virus, and malarial antigens (49) are currently undergoing human clinical evaluation. In this communication, we report the immunogenicity of NYVAC-based recombinants expressing the *P. berghei* CS protein and their ability to confer protective immunity against rodent malaria.

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MATERIALS AND METHODS

Generation of NYVAC(K1L)-CSP recombinants. NYVAC(K1L) recombinants were generated by in vivo recombination in RK_{13} (ATCC CCL37) cells with COPAK donor plasmids containing CSP gene expression cassettes and NYVAC rescuing virus (30). The COPAK donor plasmid consists of a multiple cloning region and the vaccinia virus K1L (13) open reading frame, which are flanked on the left and right by vaccinia virus open reading frames A24R and A27L (13), respectively. COPAK donor plasmids direct the insertion of foreign genes (and the K1L gene) into the ATI site of the NYVAC genome, between open reading frames A24R and A27L. COPAK donor plasmid pMLB-CS.2, which contains an expression cassette consisting of the *P. berghei* ANKA strain CSP gene (nucleotides 1 to 999) (21) under the control of the vaccinia virus early-late H6 promoter (31), was used to generate NYVAC(K1L)CSP by in vivo recombination. COPAK donor plasmids pMLB-CS.1 (H6/CSP-anchorless [nucleotides 1 to 951] expression cassette) and pMLB-CS.4 (H6/CSP-leaderless [nucleotides 1 to 3 and 64 to 999] expression cassette) were used to generate NYVAC(K1L)CSPΔ318-332 and NYVAC(K1L)CSPΔ2-21, respectively (see Fig. 1).

Expression analysis. Immunofluorescence, immunoprecipitation (48), and flow cytometric (49) analyses of Vero (ATCC CCL81) or HeLa (ATCC CCL2.2) cells infected with NYVAC(K1L)-CSP recombinants were performed as previously described, with mouse anti-*P. berghei* sporozoite serum or CS repeat-specific monoclonal antibody (MAb) PB3.28.1 (53).

Challenge studies. Groups of 10 BALB/c mice were immunized intraperitoneally with 10^7 PFU of NYVAC(K1L) (vP993) parental control virus, NYVAC (K1L)CSP (vP936), NYVAC(K1L)CSPΔ318-332 (vP957), or NYVAC(K1L) CSPΔ2-21 (vP1032) at week 0. Some groups were boosted with the same dose of the appropriate virus at week 3 or 4. Controls were inoculated intravenously (i.v.) with 10,000 irradiated *P. berghei* ANKA strain sporozoites or saline. One to two weeks after the second immunization, mice were challenged either by i.v. inoculation of 10,000 sporozoites or by the bites of infected mosquitoes. The mosquito bite challenge was performed as previously described (42) so that anesthetized mice received bites from at least five infected mosquitoes, which was sufficient to infect 100% of the control mice. Protection was scored as the absence of blood stage parasitemia as determined by microscopic examination of thin blood films obtained from individual mice on days 5 to 14 after challenge.

Enzyme-linked immunosorbent assays (ELISAs). CSP repeat-specific antibody titers were determined by ELISA as previously described (12). Sera from individual mice were evaluated before inoculation with NYVAC(K1L)CSP (prebleed) and again at the time of challenge with sporozoites (shown in Fig. 3).

Determination of memory cytolytic activity. The ability of NYVAC(K1L)CSP and NYVAC(K1L)CSPΔ2-21 to elicit memory CSP-specific cytolytic activity in mice was assessed essentially as previously described (9). Twenty-one days after BALB/c mice were immunized by intraperitoneal inoculation with 5×10^7 PFU

of control NYVAC, NYVAC(K1L)CSP, or NYVAC(K1L)CSPΔ2-21, spleen cells were harvested for in vitro stimulation and assessment of specific cytolytic activity. The spleen cells were incubated with infected [NYVAC, NYVAC (K1L)CSP, or NYVAC(K1L)CSPΔ2-21] or uninfected naive syngeneic stimulator spleen cells at a responder-to-stimulator ratio of 5:1. After 5 days, the cells were evaluated for cytotoxic activity by incubation with ^{51}Cr-labelled *H-2*^{d} P815 murine mastocytoma cells, P815 cells pulsed with 50 μg of $CS_{242–253}$ peptide (NDDSYIPSAEKI) (1) per ml, or P815 cells infected with vaccinia virus IHD-W in a 5-h ^{51}Cr release assay (5). Specific percent cytotoxicity was calculated as (experimental ^{51}Cr release − spontaneous ^{51}Cr release)/(maximum ^{51}Cr release − spontaneous ^{51}Cr release) × 100. Maximum release was determined by addition of 5% sodium dodecyl sulfate, and spontaneous release was determined by incubating target cells in the absence of effector cells. In none of the experiments presented did spontaneous release exceed 20% of the maximum ^{51}Cr release.

Determination of secondary cytolytic activity. The ability of NYVAC(K1L)CSP and NYVAC(K1L)CSPΔ2-21 to elicit secondary CSP-specific cytolytic activity in mice was assessed essentially as previously described (9). BALB/c mice were immunized with 5×10^7 PFU of the appropriate virus [NYVAC, NYVAC (K1L)CSP, or NYVAC(K1L)CSPΔ2-21] on days 0 and 28. Five days after the second inoculation, spleen cells from the immunized mice were directly assayed for cytotoxicity against P815 target cells, P815 cells pulsed overnight with 50 μg of $CS_{242–253}$ peptide (NDDSYIPSAEKI) (1) per ml, or P815 cells infected overnight with vaccinia virus IHD-W. Specific percent cytotoxicity was calculated as described above.

T-cell subset depletion. MAbs GK1.5 (rat anti-CD4 immunoglobulin G) and 2.43 (mouse anti-CD8 immunoglobulin G2a), used for depletion of T-cell subsets, were a gift from S. Hoffman. MAb UPC10 (immunoglobulin G2a) served as a control. Mice were inoculated intraperitoneally with 10^7 PFU of either NY VAC(K1L)CSP or NYVAC(K1L) on days 0 and 21. On days 25, 26, and 27, each of 13 mice in four groups received either 0.5 mg of MAb 2.43 or UPC10 or 1 mg of MAb GK1.5. On day 30, three mice from each group were sacrificed and the effectiveness of the depletion was determined by two-color flow cytometric analysis of splenic lymphocytes using phycoerythrin-conjugated anti-THY and fluorescein-conjugated anti-CD4 or anti-CD8 MAbs. The $CD4^+$ and $CD8^+$ T-cell populations were reduced by 97.7 and 99.9%, respectively, by the appropriate antibody treatment. Mice that received MAb UPC10 had normal levels of both T-cell subsets. The 10 mice remaining in each group were challenged with 10,000 *P. berghei* ANKA strain sporozoites by i.v. inoculation. On days 33, 36, and 39, the appropriate groups were again treated with the anti-CD4, anti-CD8, or control antibodies. Blood films from each mouse were read on days 7, 12, and 14 postchallenge. Mice were considered protected if no parasites were seen by day 14 postchallenge (day 44). None of the mice that were negative on day 14 postchallenge developed parasitemia during the following 3 weeks.

RESULTS

Generation and characterization of NYVAC(K1L)-CSP recombinants. A NYVAC-based recombinant, NYVAC(K1L) CSP (vP936), was generated which contains the full-length *P. berghei* CSP gene (encoding 332 amino acids) from the ANKA strain (21) (Fig. 1) plus the vaccinia virus K1L host range gene (31). Expression of CS protein in NYVAC(K1L)CSP-infected Vero cells was confirmed by indirect immunofluorescence and immunoprecipitation analyses, and cell surface expression in infected HeLa cells was demonstrated by flow cytometric analysis (data not presented). To study how immune responses and protective potency were affected by changing the intracellular targeting of CSP, additional recombinants containing modified forms of the CSP gene were constructed. The NYVAC(K1L) CSPΔ2-21 (vP1032) recombinant contains the CSP gene with the sequence encoding amino acids 2 to 21, the putative hydrophobic leader peptide, deleted (Fig. 1). Removal of the leader sequence prevented cell surface expression and caused CSPΔ2-21 to remain localized to the cytoplasm (data not presented), presumably by preventing its insertion through the endoplasmic reticulum membrane during translation. NYVAC (K1L)CSPΔ318-332 (vP957) contains the CSP gene with the sequence encoding amino acids 318 to 332, which includes the putative transmembrane anchor domain, deleted (Fig. 1). Removal of the anchor sequence prevented cell surface expression and resulted in secretion of CSPΔ318-332 from infected cells (data not presented). CSP and CSPΔ2-21 were not secreted from infected cells (data not presented). This panel of recombinants allowed the design of a series of experiments to evaluate the effects of modified CSP targeting on immune induction and protection from challenge.

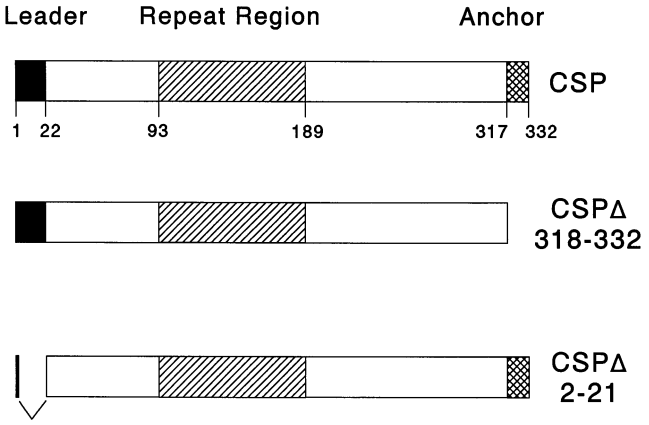

FIG. 1. CS proteins expressed by NYVAC(K1L) vaccinia virus recombinants. Schematic representations of the CS proteins expressed by NYVAC(K1L) CSP (CSP), NYVAC(K1L)CSPΔ318-332 (CSPΔ318-332), and NYVAC(K1L) CSPΔ2-21 (CSPΔ2-21) are shown. The regions of CSP containing the leader sequence, repeats, and transmembrane anchor domain are indicated by filled, striped, and hatched boxes, respectively. The amino acid positions defining these regions are indicated.

Protective efficacy of NYVAC(K1L)-CSP recombinants. The most direct means of evaluating the NYVAC(K1L)-CSP recombinants was to assess their ability to confer protection from infection by malaria parasites. A series of experiments were performed in which BALB/c mice, after one or two inoculations with the NYVAC(K1L)-CSP recombinants, were infected with sporozoites either by i.v. inoculation or by the bites of infected mosquitoes. Overall, 80% of the mice immunized with NYVAC(K1L)CSP were protected (Table 1 and Fig. 2). Immunization with NYVAC(K1L)CSPΔ318-332 or NYVAC(K1L) CSPΔ2-21 achieved 73 and 60% protection, respectively (Table 1 and Fig. 2). Thus, alteration of intracellular targeting such that CSP was either secreted or localized to the cytoplasm did not drastically affect the potency of the recombinant viruses. The sizes of the challenge groups were not sufficient to determine if the reduced protection obtained with NYVAC(K1L) CSPΔ2-21 was indeed significant. Interestingly, the levels of protection obtained after one or two inoculations of NYVAC (K1L)CSP were similar (85 versus 75%, respectively). Complete protection was achieved in two experimental sets, once with one inoculation and once with two inoculations of NYVAC (K1L)CSP (Table 1, experiments 1 and 5). Significantly, immunization with irradiated sporozoites always induced complete protection against challenge.

Humoral responses elicited by NYVAC(K1L)CSP. To understand the relationship between humoral responses to CSP and protection in mice inoculated with NYVAC(K1L)CSP, ELISA antibody responses to the CSP repeats were assessed just prior to challenge. Although CSP repeat-specific antibodies were detectable in many immunized mice, there was no correlation between these antibody levels and the ability of individual mice to block parasite development before the blood stage (Fig. 3). In fact, some mice were protected in the absence of detectable repeat-specific antibodies. Antibodies to the nonrepetitive regions of CSP were not measured. Thus, the ability of such antibodies to abate the number of developing parasites, if any, cannot be determined from these studies. However, in mice immunized with irradiated sporozoites, most, if not all, antisporozoite antibodies were directed against the CSP repeat epitope (35).

TABLE 1. Challenge studies with NYVAC(K1L) *P. berghei* CSP recombinants

Expt and immunogen[a]	No. of doses[b]	Challenge[c]	No. protected/ no. challenged	% Protected
1				
NYVAC(K1L)CSP	1	SP	10/10	100
NYVAC(K1L)CSP	2	SP	7/10	70
NYVAC(K1L)	2	SP	0/10	0
Irr Spz	1	SP	10/10	100
Saline	2	SP	0/10	0
2				
NYVAC(K1L)CSP	2	SP	6/10	60
NYVAC(K1L)CSP	2	MB	9/10	90
NYVAC(K1L)CSPΔ318-332	2	SP	7/10	70
NYVAC(K1L)CSPΔ318-332	2	MB	8/10	80
NYVAC(K1L)CSPΔ2-21	2	SP	5/10	50
NYVAC(K1L)CSPΔ2-21	2	MB	6/10	60
NYVAC(K1L)	2	SP	0/10	0
NYVAC(K1L)	2	MB	0/10	0
Irr Spz	1	SP	10/10	100
Irr Spz	1	MB	10/10	100
Saline	2	SP	0/10	0
Saline	2	MB	0/10	0
3				
NYVAC(K1L)CSP	2	MB	6/10	60
NYVAC(K1L)CSPΔ318-332	2	MB	7/10	70
NYVAC(K1L)CSPΔ2-21	2	MB	7/10	70
NYVAC(K1L)	2	MB	0/10	0
Irr Spz	1	SP	10/10	100
Saline	2	SP	0/10	0
4				
NYVAC(K1L)CSP	1	MB	7/10	70
NYVAC(K1L)	1	MB	1/10	10
Saline	2	SP	0/10	0
5				
NYVAC(K1L)CSP + UPC10[d]	2	SP	10/10	100
NYVAC(K1L)CSP + anti-CD4[d]	2	SP	9/10	90
NYVAC(K1L)CSP + anti-CD8[d]	2	SP	3/10	30
NYVAC(K1L) + UPC10	2	SP	2/10	20
Saline	2	SP	0/5	0

[a] NYVAC(K1L)CSP expresses the *P. berghei* CS protein, NYVAC(K1L)CSPΔ318-332 expresses secreted CSP, NYVAC(K1L)CSPΔ2-21 expresses intracytoplasmic CSP, NYVAC(K1L) is the parental control virus, and Irr Spz are irradiated sporozoites.

[b] For NYVAC(K1L) recombinants, one or two doses of 10^7 PFU were administered intraperitoneally. For irradiated sporozoites, one dose of 10,000 sporozoites was administered by i.v. inoculation.

[c] SP, i.v. inoculation of 10,000 live sporozoites; MB, bites of five infected mosquitoes.

[d] UPC10, control immunoglobulin G2a MAb; anti-CD4, MAb GK1.5; anti-CD8, MAb 2.43.

Cellular responses elicited by NYVAC(K1L)-CSP recombinants. Because protection elicited by the NYVAC(K1L)-CSP recombinants did not correlate with CSP repeat-specific antibody responses, the ability of these recombinants to elicit cellular immunity was studied. First, the induction of cytotoxic T lymphocytes (CTL) in mice immunized with NYVAC(K1L) CSP and NYVAC(K1L)CSPΔ2-21 was evaluated. The development of an intracytoplasmic CSP construct (CSPΔ2-21) was based in part on the demonstration that removal of the leader sequence from an extrinsic antigen expressed by vaccinia virus resulted in enhanced degradation and overcame the defective presentation of the antigen to CTL (51). It was of interest, therefore, to determine if enhancing the degradation of CSP would enhance the elicitation of CSP-specific CTL. All immunized mice developed vaccinia virus-specific lytic activity, indicating effective immunization and the presence of memory cell precursors which can be expanded by in vitro restimulation (Fig. 4A). Mice inoculated once with NYVAC(K1L)CSP developed CSP-specific lytic activity directed against a synthetic peptide corresponding to the $CS_{242–253}$ CTL epitope (1) (Fig. 4A). It appears, however, that NYVAC(K1L)CSPΔ2-21 failed to prime memory CTL after one immunization. The reason for this lack of priming by NYVAC(K1L)CSPΔ2-21 is not immediately obvious. However, both CSP recombinants were capable of restimulating strong cytolytic activity from NYVAC (K1L)CSP-primed mice (Fig. 4A).

The induction of cytolytic activity 5 days after a second immunization with the NYVAC(K1L)-CSP recombinants, but

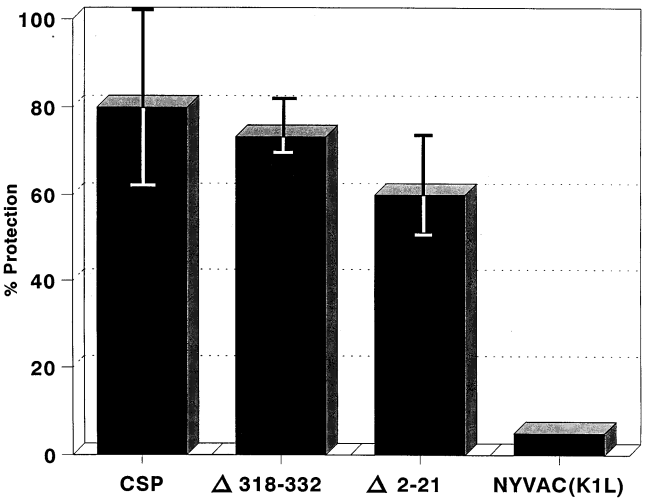

FIG. 2. Protection from sporozoite challenge by immunization with NYVAC (K1L) recombinants expressing *P. berghei* CSP. Groups of 10 mice were immunized with NYVAC(K1L)CSP (CSP), NYVAC(K1L)CSPΔ318-332 (Δ 318-332), NYVAC(K1L)CSPΔ2-21 (Δ 2-21), or NYVAC(K1L) prior to challenge. Cumulative data derived from Table 1 are shown.

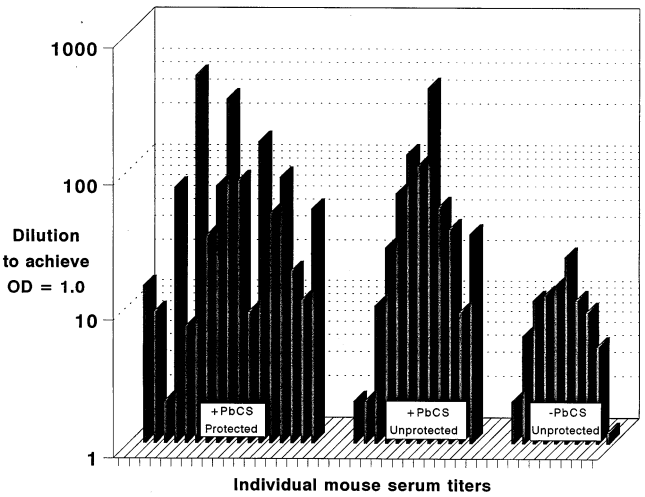

FIG. 3. Protection conferred by immunization with NYVAC(K1L)CSP is not associated with antibodies to the CSP repeats. CSP repeat-specific ELISA titers of individual mice immunized with NYVAC(K1L)CSP (+PbCSP) or NYVAC (K1L) (−PbCSP) were determined at the time of challenge. OD, optical density.

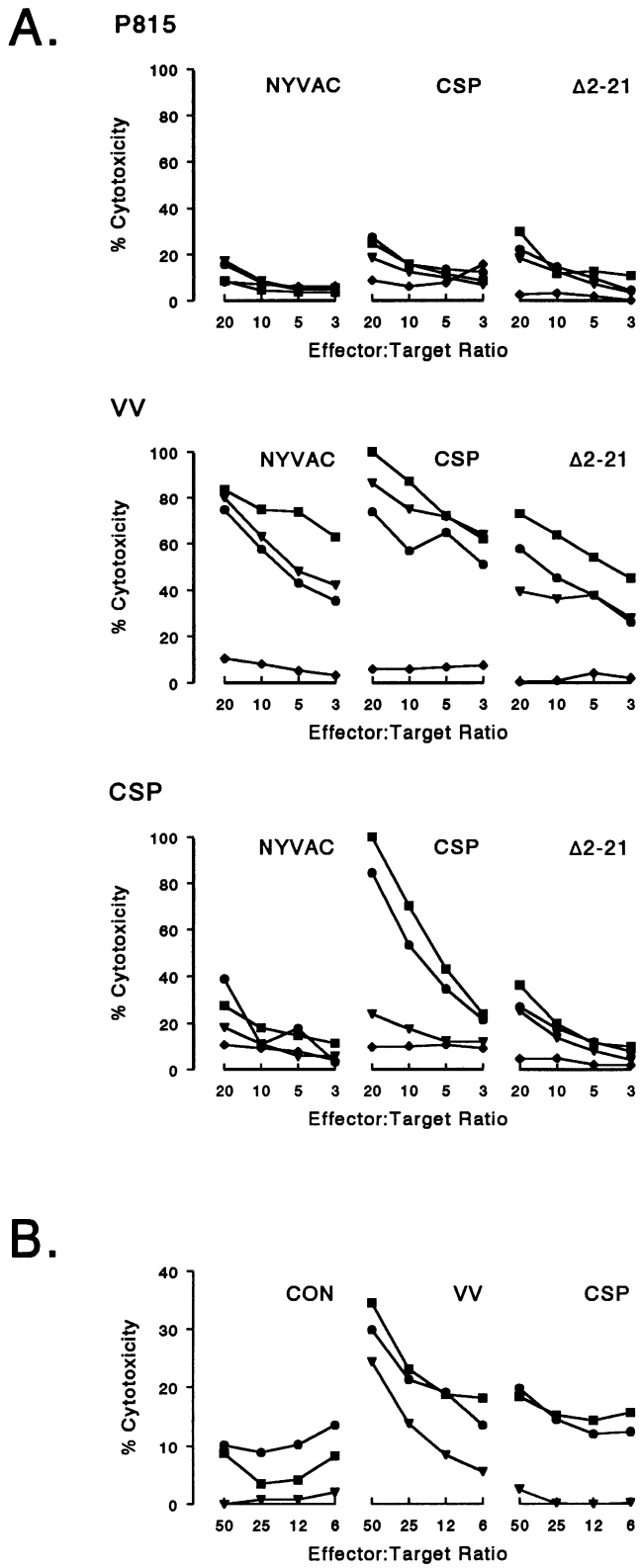

FIG. 4. NYVAC(K1L) vaccinia virus recombinants expressing *P. berghei* CS protein induce CSP-specific cytolytic responses in mice. (A) Memory CSP-specific cytolytic responses were determined in mice inoculated with NYVAC, NYVAC(K1L)CSP (CSP), or NYVAC(K1L)CSPΔ2-21 (Δ2-21). Twenty-one days after inoculation, the spleen cells of experimental mice were incubated with uninfected (◆) or NYVAC (▼)-, NYVAC(K1L)CSP (●)-, or NYVAC(K1L) CSPΔ2-21 (■)-infected syngeneic spleen cells. Five days later, the spleen cell cultures were assayed for CTL activity against unmodified (panel P815), vaccinia virus IHD-W-infected (panel VV), or $CS_{242–253}$ peptide-pulsed (panel CSP) P815 target cells. Error bars were eliminated for clarity, since standard errors were within 10% of the means. (B) Secondary CSP-specific cytolytic responses were determined as described in Materials and Methods. Twenty-eight days after the first inoculation, mice were reimmunized with NYVAC (▼), NYVAC(K1L) CSP (●), or NYVAC(K1L)CSPΔ2-21 (■). Five days later, unstimulated spleen cells were assayed for cytotoxicity against unmodified (CON), vaccinia virus IHD-W-infected (VV), and $CS_{242–253}$ peptide-pulsed (CSP) P815 target cells. Error bars were eliminated for clarity, since standard errors were within 10% of the means.

without in vitro restimulation, was also assessed. Despite the discrepancy between the abilities of the two recombinants to generate memory CTL precursors as assessed in the in vitro restimulation system, mice immunized with both recombinants responded to secondary immunization by developing nearly equivalent levels of cytolysis to both vaccinia virus and CSP targets (Fig. 4B).

Contribution of T-cell subsets to protection. To determine if the in vitro cytolytic activity was functional in vivo and associated with protection, in vivo depletion experiments were performed to rid mice of either $CD4^+$ or $CD8^+$ T cells prior to challenge. Treatment with an anti-CD8 MAb greatly reduced the protective effect of NYVAC(K1L)CSP vaccination, whereas an anti-CD4 MAb had little effect (Fig. 5), indicating that the protection was predominantly cellular in nature and mediated primarily by $CD8^+$ T cells. Presumably, the mechanism of protection was elimination of parasite-infected hepatocytes in the liver by $CD8^+$ T cells.

DISCUSSION

The results presented here show that immunization of mice with a NYVAC(K1L) vaccinia virus recombinant expressing CS protein elicits CSP-specific cellular immune responses that provide protection from sporozoite challenge. These results and others (1, 12, 23, 34, 36, 39, 41, 54) demonstrate the importance of cellular immunity to pre-erythrocytic antigens in controlling infection by sporozoites. Despite these studies, most CSP-based vaccine candidates evaluated in humans have not been designed to elicit strong cellular immune responses (50).

The basis of the failure of vaccinia virus WR or Wyeth strain recombinants expressing CSP to confer protection in other studies (23, 37, 40) is unclear but may suggest that the vaccinia virus strain chosen as a vector for malaria antigens and possibly other factors, such as the route of administration or the promoter selected for expression, can affect the subsequent development of protective responses. Further, WR strain recombinants expressing blood stage antigens of *P. falciparum* were not successful in protecting *Saimiri* monkeys from challenge with parasitized erythrocytes (33), although the gene constructs were not optimized for expression by vaccinia virus (48).

Previous studies have suggested that altering the localization of a normally secreted malaria antigen such that it was expressed on the cell surface resulted in enhanced immunogenicity when expressed by a vaccinia virus recombinant (22). The results presented here indicate that, at least for CSP, there is no apparent advantage to modifying the intracellular targeting of the protein. The levels of protection achieved with NYVAC(K1L)CSPΔ318-332 (secreted CSP) were not significantly different from those obtained with NYVAC(K1L)CSP (Fig. 2). Surprisingly, removal of the leader sequence from CSP appeared to adversely affect the priming of memory CTL precursors after one immunization as assessed following in vitro restimulation (Fig. 4A). However, equivalent cytolysis was observed after two immunizations with NYVAC(K1L)CSP and NYVAC

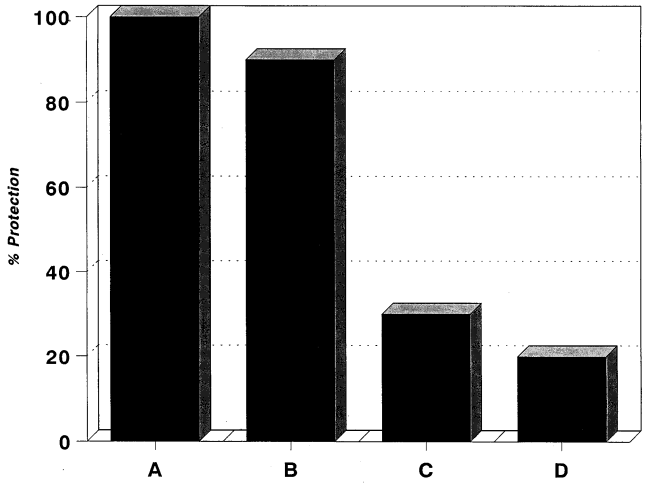

FIG. 5. Protection conferred by immunization with NYVAC(K1L)CSP is mediated by $CD8^+$ T cells. Mice were immunized with NYVAC(K1L)CSP (A, B, and C) or NYVAC(K1L) (D) and treated with a control MAb (A and D), an anti-CD4 MAb (B), or an anti-CD8 MAb (C) prior to sporozoite challenge.

(K1L)CSPΔ2-21 when assayed directly (Fig. 4B). Vaccinia virus-expressed leaderless CS protein may be less efficient at eliciting cellular immune responses than unmodified CSP, but this can apparently be overcome by multiple inoculations. Unfortunately, T-cell depletion studies with animals vaccinated with NYVAC(K1L)CSPΔ2-21 or NYVAC(K1L)CSPΔ318-332 were not performed, so the dependence of the protection conferred by these recombinants on particular T-cell subsets cannot be determined.

Comparative studies of various mouse strains have shown that BALB/c ($H\text{-}2^d$), the strain used here, is the most readily protected by immunization with irradiated sporozoites (24, 55). Although a genetic restriction of immune responses to sporozoites or recombinant CSP peptides has not been demonstrated, levels of response do vary among mouse strains (35). The NYVAC(K1L)-CSP recombinants have only been evaluated in BALB/c mice, so it is not known whether the protection elicited by these recombinants is strain dependent.

This is the first demonstration of significant protection in a rodent malaria model system elicited by a recombinant-vector approach that is currently acceptable for human clinical evaluation. Other studies have demonstrated that *Salmonella typhimurium* transformants expressing CSP (1, 36), *S. typhimurium*-expressed hybrid hepatitis B virus core particles containing a CSP repeat epitope (38), sequential administration of an influenza virus recombinant expressing a CSP CTL epitope followed by a WR vaccinia virus-CSP recombinant (23), and direct injection of DNA encoding CSP (41) can confer protection on mice.

The ability of irradiated-sporozoite inoculation to provide complete protection from challenge, coupled with the inability of CSP-based vaccine candidates to elicit such levels of protection, strongly implies that immunity against multiple pre-erythrocytic antigens is necessary to consistently achieve high levels of protection. Support for this concept has been obtained in the *P. yoelii* model, where it was demonstrated that a combination of two pre-erythrocytic antigens was more effective in conferring protection than either antigen alone (18). Although the NYVAC(K1L)-CSP recombinants did not consistently elicit complete protection, the results presented here do support the continued evaluation of the highly attenuated NYVAC vaccinia virus strain as a vector for vaccines against human malaria. NYVAC has a large capacity for exogenous DNA and so is an ideal vector for the expression of multiple components of the malaria parasite. Thus, a NYVAC recombinant expressing several *P. falciparum* antigens derived from multiple stages of the parasite life cycle could provide a safe, rational malaria vaccine candidate (49).

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