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Immunology

Priming with recombinant influenza virus followed by administration of recombinant vaccinia virus induces CD8$^+$ T-cell-mediated protective immunity against malaria

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ABSTRACT **Live vectors expressing foreign antigens have been used to induce immunity against several pathogens. However, for the virulent rodent malaria parasite *Plasmodium yoelii*, the use of recombinant vaccinia virus, pseudorabies virus, or *Salmonella*, expressing the circumsporozoite protein of this parasite, failed to induce protection. We generated a recombinant influenza virus expressing an epitope from the circumsporozoite protein of *P. yoelii* known to be recognized by CD8$^+$ T cells and demonstrated that this vector induced class I major histocompatibility complex-restricted cytotoxic T cells against this foreign epitope. Immunization of mice with this recombinant influenza virus, followed by a recombinant vaccinia virus expressing the entire circumsporozoite protein, induced protective immunity against sporozoite-induced malaria. The sequence of immunization appears to be crucial, since a primer injection with recombinant vaccinia virus, followed by a booster injection with recombinant influenza virus, failed to induce protection. The protection induced by immunization with these recombinant viruses is mostly mediated by CD8$^+$ T cells, as treatment of mice with anti-CD8 monoclonal antibody abolishes the anti-malarial immunity. The use of different live vectors for primer and booster injections has a synergistic effect on the immune response and might represent an effective general strategy for eliciting protective immune responses to key antigens of microbial pathogens.**

Recombinant live viruses expressing foreign antigens are attractive delivery systems, designed to elicit protective immunity against microbial pathogens. A number of experimental findings provide strong evidence that recombinant viruses, such as attenuated vaccinia virus expressing microbial antigens, can induce protective immunity against viral and bacterial infections (1–7). However, the use of recombinant vaccinia virus expressing distinct plasmodial antigens had, to our knowledge, failed to generate a significant degree of protection against the various developmental stages of malaria parasites (8, 9).

The need for additional live carriers that would induce efficient protective immunity against certain infectious agents led to the engineering of influenza viruses expressing microbial epitopes. This negative-strand RNA virus had, until recently, not been amenable to genetic manipulation, because of the lack of an appropriate rescue system. Recently, a general procedure was described (10, 11) by which the genome of influenza virus could be altered, so that the expression of a cDNA-derived RNA became feasible. The construction of such a recombinant influenza virus containing a plasmodial antigen has provided the opportunity to define its immunogenicity and compare it with that of a recombinant vaccinia virus.

The rodent malaria parasite *Plasmodium yoelii* was selected for these studies, since a number of its protective immune mechanisms and the corresponding antigens have been relatively well characterized. Several studies have demonstrated that CD8$^+$ T cells play a major role in immunity against preerythrocytic stages of rodent malaria (12–14). In the *P. yoelii* system, CD8$^+$ T cells recognize the major histocompatibility complex (MHC) class I-restricted epitope SYVPSAEQI present in the circumsporozoite (CS) protein of this parasite (15–17). Adoptive transfer, to naive mice, of CD8$^+$ T-cell clones specific for this epitope inhibits the development of the liver stages of the parasite and confers extensive protection against sporozoite-induced malaria (17, 18).

In the present report, we describe studies in which we immunized mice with a recombinant vaccinia virus expressing the entire *P. yoelii* CS protein and a recombinant influenza virus expressing the cytotoxic epitope of this protein. We determined the immunogenicity of these live vectors, administered individually or sequentially, and characterized the protective anti-malaria immune mechanisms they induce.

MATERIALS AND METHODS

Construction of a Recombinant Influenza Virus Expressing the Cytotoxic CS Epitope of *P. yoelii*. A unique *Bst*EII restriction site was engineered into a full-length cDNA of WSN virus hemagglutinin (HA), immediately upstream of the antigenic site E (PT3/WSN-HAm) (19). The recombinant ME virus HA [where ME is the malarial cytotoxic T-lymphocyte (CTL) epitope] was constructed by replacing the *Bst*EII–*Hin*dIII fragment of the WSN HA with a PCR product, in which the nucleotide sequence encoding the 8 aa between the Cys and Trp residues was replaced by the sequence encoding the plasmodial peptide NEDSYVPSAEQI. Transfection of *in vitro*-synthesized RNA into Madin–Darby bovine kidney cells and rescue of infectious recombinant influenza virus were done as described (19). The nucleotide sequence encoding NEDSYVPSAEQI has been confirmed by direct sequencing of purified viral RNA. This recombinant influenza virus was designated influenza ME.

Construction of a Recombinant Vaccinia Virus Expressing the Entire *P. yoelii* CS Protein (PYCS). A *Xba* I fragment of

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Abbreviations: HA, hemagglutinin; CS, circumsporozoite; CTL, cytotoxic T lymphocyte; pfu, plaque-forming unit; ME, malaria CTL epitope; wt, wild type; MHC, major histocompatibility complex; mAb, monoclonal antibody.

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1.75 kb, containing the gene of the *P. yoelii* CS protein, was isolated from the plasmid pBS-PY1993. This DNA fragment was blunt-ended by treatment with a large fragment of *Escherichia coli* DNA polymerase (Klenow), purified by agarose gel electrophoresis, and cloned into the *Sma* I site of the vaccinia virus insertion vector pSC11 (20). As a result of this cloning strategy, we isolated a plasmid, pJR88, containing the *P. yoelii* CS gene under control of the vaccinia virus early-late promoter p7.5, the β-galactosidase gene *lacZ* under the control of the vaccinia virus late promoter p11, and flanking regions from the vaccinia virus thymidine kinase gene. To generate vaccinia PYCS recombinants, the insertion vector pJR88 was introduced by homologous recombination into the thymidine kinase locus of the vaccinia virus genome. The recombinant virus was selected by β-galactosidase expression (20) and purified (21).

Cytolytic Assay. P815 target cells (5×10^5 cells) were incubated with 5×10^6 plaque-forming units (pfu) of influenza ME or wild-type (wt) WSN virus for 1 h at 37°C. After adsorption, the target cells were resuspended in fetal calf serum and labeled for 2 h with 200 μCi of ^{51}Cr (ICN; 1 Ci = 37 GBq). For the chromium release assay, these target cells were washed, distributed at 2.5×10^3 cells per well, and incubated with the CTL clone YA26, at various effector/target cell ratios. Clone YA26 was generated and maintained as described (17, 18). This CTL clone recognizes the epitope represented by the peptide SYVPSAEQI, in association with MHC class I H-2k^d. After 5 h at 37°C, the supernatants were collected with a semiautomatic harvester (Skatron, Sterling, VA). The percentage of specific lysis was calculated as follows: (experimental − spontaneous release)/(total − spontaneous release) × 100.

The spleen cells of influenza ME and wt virus-immunized mice were obtained 14 days after aerosol exposure. These spleen cells (4×10^7 cells per 10 ml) were restimulated *in vitro* in the presence of 2.5×10^6 P815 cells pulsed with 1 μM NEDSYVPSAEQI plus 1% of the EL-4 supernatant. After 6 days in culture, these cells were washed and incubated at various ratios with 2.5×10^3 ^{51}Cr-labeled P815 cells per well, in the presence or absence of 0.2 μM NEDSYVPSAEQI.

Parasites and Animals. *P. yoelii* (17X NL strain) was maintained as described in ref. 17. Four- to 8-week-old female BALB/c mice, used for the immunological studies, were purchased from The Jackson Laboratory and from Charles River Breeding Laboratories. Parasite injection was done i.v. into the tail vein and parasitemia was determined by microscopic examination of Giemsa-stained thin blood smears, collected daily from the 3rd to the 14th day after challenge.

Immunizations and Challenge. BALB/c mice were immunized, by aerosol, with 500 pfu of influenza ME or wt virus. When indicated, a second dose containing equivalent amounts of virus was administered by the same route.

Mice immunized with recombinant vaccinia PYCS or wt vaccinia virus received 5×10^7 pfu, injected i.p. When a second dose of these viruses was administered, 10^8 pfu was used. In mice that received two immunizing doses, consisting of different combinations of viruses, the second dose was administered 3 weeks after the first dose.

All immunized mice were challenged 13 days after the last immunizing dose. In those experiments in which protection was assessed by blood-stage parasitemia, mice were challenged by intravenous injection of 100 sporozoites. When protection was assessed by measuring plasmodial rRNA, mice were challenged i.v. with 5×10^5 *P. yoelii* sporozoites.

Quantification of *P. yoelii* rRNA in the Liver of Infected Mice. Quantification of *P. yoelii* rRNA was performed as described (22). Briefly, total RNA was isolated from the livers of mice sacrificed 42 h after they had been injected i.v. with 5×10^5 sporozoites of *P. yoelii*. RNA was prepared by the method of Chomczynski and Sacchi (23). One-tenth of the whole liver RNA was precipitated with isopropanol. The RNA pellet was dissolved in 10 mM Tris·HCl, pH 8.0/1 mM EDTA and denatured at 65°C in 20× SSC (1× SSC = 150 mM NaCl/15 mM sodium citrate) plus 37% (vol/vol) formaldehyde, 1:1 (vol/vol). This preparation was diluted 1:45 and 0.2-ml samples were blotted onto nylon membranes. The RNA was fixed to the filters by UV cross-linking. Hybridization was performed overnight in 5× SSPE (1× SSPE = 180 mM NaCl/10 mM sodium phosphate/1 mM EDTA, pH 7.7)/1% SDS/500 μg of heparin at 42°C, containing three ^{32}P-labeled oligonucleotide probes (10^6 cpm/ml; specific activity, $>2 \times 10^8$ cpm/μg). The probes are described elsewhere (22). For comparison purposes, a standard curve was prepared using serial dilutions of purified RNA from *P. yoelii*-infected erythrocytes and blotted under the same conditions.

***In Vivo* Depletion of CD4$^+$ or CD8$^+$ T Cells.** The hybridomas producing rat IgG anti-CD4 (GK1.5) or anti-CD8 (2.43) were purchased from the American Type Culture Collection. Ascites were produced in BALB/c nude mice, and the concentration of rat immunoglobulin was estimated by RIA using mouse-adsorbed anti-rat IgG heavy and light chains (Kirkegaard and Perry Laboratories, Gaithersburg, MD). For 3 consecutive days, each mouse received daily doses of 1 mg of anti-CD4 or anti-CD8 monoclonal antibody (mAb). These mice were challenged with *P. yoelii* sporozoites 2 days after receiving the last mAb dose. The efficacy of the depletion was estimated by two-color flow cytometry analysis of peripheral blood lymphocytes, using phycoerythrin-conjugated anti-THY-1 and fluorescein-conjugated anti-CD4 or anti-CD8 mAbs (all purchased from Accurate Chemicals, Westbury, NY). We found that the amount of CD4$^+$ T cells was reduced by 98% in mice treated with anti-CD4 mAb. The treatment with anti-CD8 mAb eliminated 95% of the CD8$^+$ T cells.

RESULTS

Construction of Recombinant Influenza and Vaccinia Viruses and Characterization of Their Immunogenic Properties. A peptide containing the cytotoxic epitope of the *P. yoelii* CS protein was expressed in antigenic site E of the HA protein of the influenza virus A/WSN/33. This recombinant influenza virus (ME) was generated by inserting the nucleotide sequence for the peptide NEDSYVPSAEQI into the influenza HA gene (Fig. 1*A*). Earlier studies had shown that the epitope SYVPSAEQI is recognized by MHC class I H-2K^d-restricted CD8$^+$ CTLs (15–17).

We also generated a recombinant vaccinia virus expressing the entire *P. yoelii* CS protein, by insertion of the *P. yoelii* CS gene into the thymidine kinase locus of the viral genome (Fig. 1*B*) (25).

We established that target cells infected by influenza ME virus express, process, and present the cytotoxic CS epitope of *P. yoelii*. As shown in Fig. 2*A*, target cells infected *in vitro* with influenza ME virus were lysed by the CD8$^+$ T-cell clone YA26, which recognizes the SYVPSAEQI epitope (17, 18). This CTL clone did not lyse target cells infected with the wt influenza virus or uninfected cells.

More importantly, we determined that H-2K^d mice immunized with influenza ME virus, unlike those immunized with the wt virus, generated CTLs that specifically lysed target cells coated with the synthetic peptide containing the cytotoxic plasmodial epitope (Fig. 2*B*). These findings demonstrate that a foreign epitope expressed in an influenza virus can be processed and presented in the context of class I MHC and can also induce specific cytotoxic T cells *in vivo*.

We obtained similar *in vitro* results with recombinant vaccinia constructs (data not shown), known to efficiently

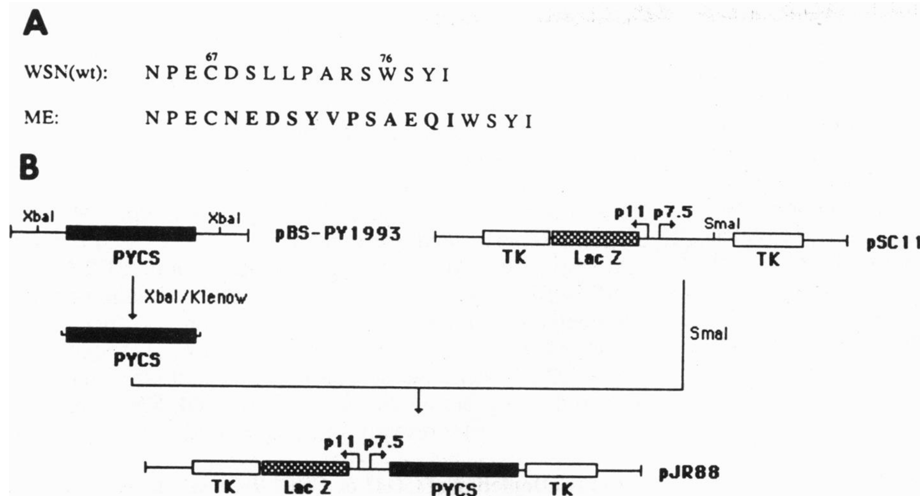

FIG. 1. Recombinant influenza virus (influenza ME) and recombinant vaccinia virus (vaccinia PYCS). (*A*) Amino acid sequences of the original antigenic site E of influenza A/WSN/33 (wt) virus HA and of the "substituted site" in the HA of the recombinant influenza ME virus. Boldface type corresponds to the amino acid sequence containing the cytotoxic epitope of the *P. yoelii* CS protein. Numbering in the WSN virus is according to ref. 24. (*B*) Introduction of the *P. yoelii* CS protein gene in vaccinia virus DNA.

induce $CD8^+$ T cells in various systems, including malaria (26–28).

Induction of Protective Immunity Against Malaria by Immunization with Recombinant Influenza and Vaccinia Viruses. To determine whether these recombinant viruses could induce protective immunity against preerythrocytic forms (namely, sporozoites and liver stages of *P. yoelii*), BALB/c mice immunized with one or two doses of these viruses were challenged with viable sporozoites. Parasite development was monitored by measuring plasmodial rRNA in the liver of the sporozoite-injected mice (22).

Immunization of mice with one or even two doses of the influenza ME virus did not appear to affect the intrahepatocytic development of the parasites. In fact, the levels of plasmodial rRNA in the liver of mice immunized with influenza ME virus were similar to control mice immunized with wt virus (Fig. 3*A*). In mice immunized with a single dose of the vaccinia PYCS virus, we observed a partial decrease (50–55%) of the parasite load in the liver (data not shown). A second immunizing dose of this vaccinia recombinant failed to enhance the level of anti-parasite immunity (Fig. 3*B*). Control mice immunized with wt vaccinia had a parasite load similar to that found in naive mice.

To determine whether the use of these two different live vectors, in a single immunization protocol, would enhance the protective immune response, mice were inoculated first with influenza ME virus and 3 weeks later with vaccinia PYCS virus. These immunized mice were challenged 13 days later with *P. yoelii* sporozoites. We found that this immunization resulted in a 96.3% decrease of plasmodial rRNA in the liver, revealing a remarkable synergistic effect of the vaccination with two distinct live vectors (Fig. 3*C*). Surprisingly, protective immunity failed to be induced when the two recombinant viruses were administered in the reverse order—namely, a primer injection with the vaccinia PYCS followed 3 weeks later by immunization with influenza ME virus (Fig. 3*D*).

The findings based on measuring rRNA in the liver of immunized and challenged mice were fully corroborated by experiments in which we observed protection against the development of blood infection in mice immunized with influenza ME and given booster injection with vaccinia PYCS virus. When these immunized mice were challenged with *P. yoelii* sporozoites, 60% were protected against this highly infective parasite, never developing patent parasitemia. Furthermore, those immunized mice that became infected (40%) did so only after a prolonged prepatent period, reflecting a

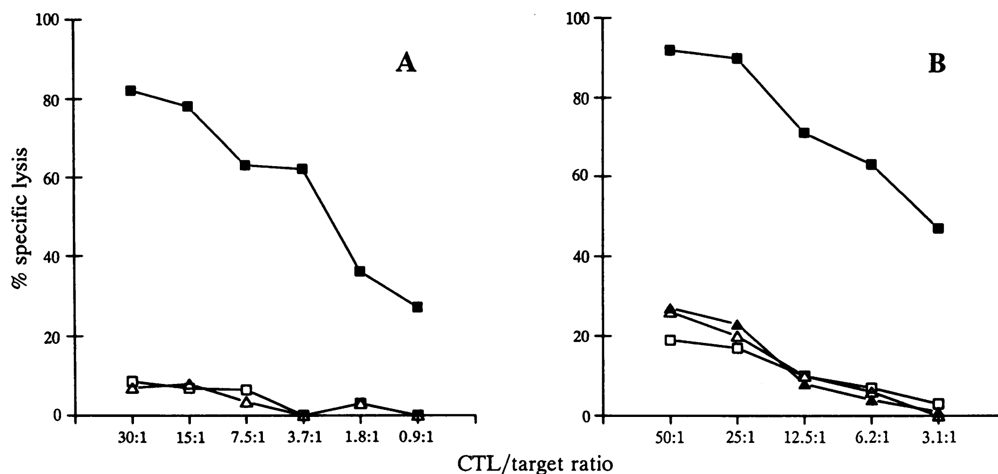

FIG. 2. Influenza ME-infected target cells process and present the cytotoxic CS epitope. (*A*) Chromium-labeled P815 target cells infected with influenza ME virus (■), control wt WSN virus (□), or uninfected P815 cells (△) were incubated with $CD8^+$ CTL clone YA26, at various effector (CTL)/target cell ratios. (*B*) BALB/c mice were immunized with 500 pfu of influenza ME or control wt WSN virus, by aerosol; 14 days later their spleen cells were collected, restimulated *in vitro* in the presence of irradiated P815 cells pulse-labeled with 1 μM NEDSYVPSAEQI. After 6 days in culture, these cells were assayed for the presence of peptide-specific cytolytic T cells as described (15). Spleen cells of mice immunized with influenza ME virus displayed considerable lysis of P815 target cells in the presence (■) but not in the absence (□) of NEDSYVPSAEQI. Spleen cells of mice immunized with wt WSN virus incubated with P815 target cells in the presence (▲) or absence (△) of peptide showed no specific lytic activity.

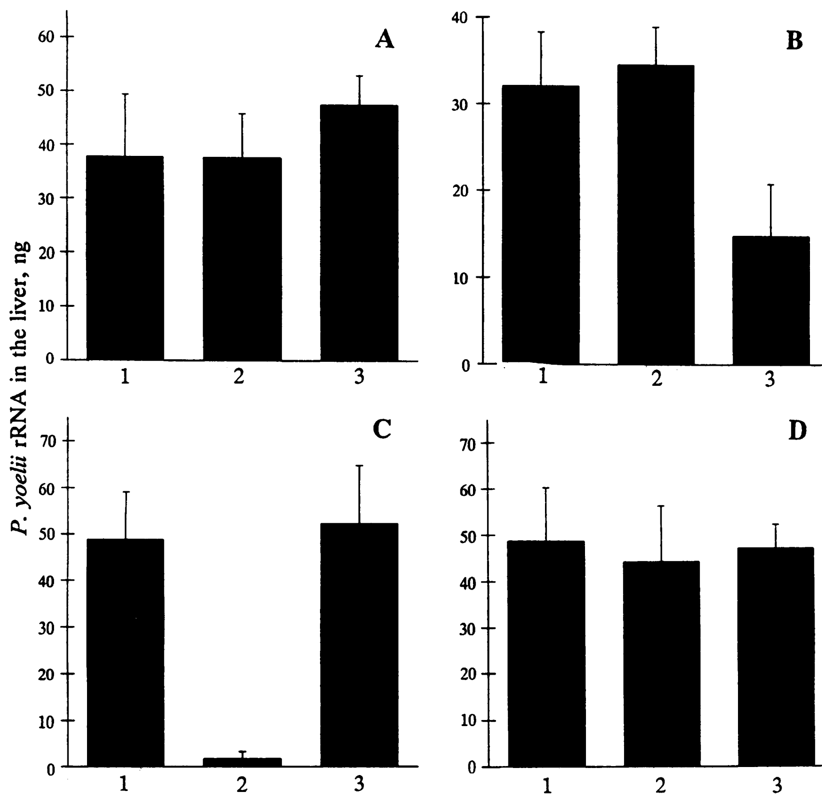

FIG. 3. Immunization with recombinant influenza ME followed by vaccinia PYCS viruses inhibits the development of liver stages of *P. yoelii*. BALB/c mice received two immunizing doses of different viruses. The second immunizing dose was administered 3 weeks after the first immunization. All mice were challenged 13 days after the second immunizing dose. Immunized and control mice were challenged i.v. with 5×10^5 *P. yoelii*. The amount (ng) of plasmodial rRNA of each group of mice obtained from the livers of five mice is expressed as the mean ± SEM. (*A*) Mice immunized twice by aerosol with 500 pfu of influenza ME or control wt WSN virus. Bars: 1, none; 2, wt virus; 3, influenza ME. (*B*) Mice immunized with two doses of vaccinia PYCS or control wt virus. The first dose and the second dose consisted of 5×10^7 and 10^8 pfu, respectively, administered i.p. Bars: 1, none; 2, wt virus; 3, PYCS. (*C*) Mice immunized by aerosol with 500 pfu of influenza ME and subsequently given a booster injection i.p. with 5×10^7 pfu of vaccinia PYCS or control wt vaccinia virus. Bars: 1, none; 2, influenza ME plus vaccinia PYCS; 3, influenza ME plus wt vaccinia virus. (*D*) Mice immunized i.p. with 5×10^7 pfu of vaccinia PYCS virus or wt vaccinia virus, and subsequently given a second immunizing dose, by aerosol, with 500 pfu of influenza ME virus. Bars: 1, none; 2, vaccinia PYCS plus influenza ME; 3, wt vaccinia plus influenza ME.

decrease of their preerythrocytic parasite load (Table 1). In contrast, all the nonimmunized mice and the mice immunized with the wt viruses developed patent blood infections.

Protective Anti-Malaria Immune Mechanisms Induced by Immunization with Recombinant Viruses. Mice immunized with these two recombinant viruses were injected with anti-CD8 or anti-CD4 mAb to identify the mechanisms mediating the protective immunity induced by priming with influenza ME virus, followed by a booster injection with vaccinia PYCS virus. As shown in Fig. 4, treatment with anti-CD8 mAb almost completely ablated the inhibition of liver stage development induced by immunization, whereas no effect was observed upon treatment with anti-CD4 mAb. These findings clearly indicate that protective immunity induced by these recombinant viruses is mostly mediated by $CD8^+$ T cells.

DISCUSSION

The present findings demonstrate that protective immunity against the highly virulent rodent malaria parasite *P. yoelii* can be induced by inoculation of recombinant live viruses expressing the CS protein or portions of it. This protective immunity is greatly enhanced when two distinct live vectors are used for primer and booster injections.

Earlier studies had used a recombinant *Salmonella* expressing the entire CS protein to generate protective immunity against the rodent malaria parasite *P. berghei* (29). However, protection against *P. yoelii*, a much more infective plasmodial species, failed to be induced by immunization with recombinant vaccinia, pseudorabies virus, or *Salmonella* (8, 30). In fact, protection against *P. yoelii* has so far only been obtained upon immunization with large numbers of transfected tumor cells expressing certain sporozoite antigens (31).

We have currently determined that a certain degree of protective immunity against this parasite can be induced by a single immunization with vaccinia PYCS virus but that a second dose of the same recombinant virus fails to enhance this immunity. These results are, in all likelihood, due to a vigorous primary immune response that rapidly neutralizes the second dose of the virus. In contrast, the immunization with vaccinia PYCS, after priming the mice with the influenza ME virus, enhances the effectiveness of the anti-parasite immunity, apparently by expanding the influenza virus-induced $CD8^+$ T cells.

The sequence in which these vectors are used for immunization appears to be crucial. Protective immunity can only be induced by a primer injection with influenza ME virus, followed by a booster injection with vaccinia PYCS virus, but not when the reverse protocol is followed. Since vaccinia,

Table 1. Immunization with recombinant influenza virus followed by recombinant vaccinia virus confers protection against sporozoite-induced *P. yoelii* malaria

Immunization dose		No. mice protected/	%	Prepatent period,
First	Second	no. challenged	protection	days
Influenza ME	Vaccinia PYCS	9/15	60	5.2*
Influenza wt	Vaccinia wt	0/10	0	4.2
None	None	0/10	0	4.2

Protocol of immunization was as described in Fig. 3*C*. Challenge dose consisted of 100 *P. yoelii* sporozoites per mouse. This dose represents at least five times the minimum dose required to infect 100% of normal BALB/c mice. The protected animals remained negative, but all the control mice developed patent parasitemia.

*Prepatent period in this group refers to the six animals that were not protected.

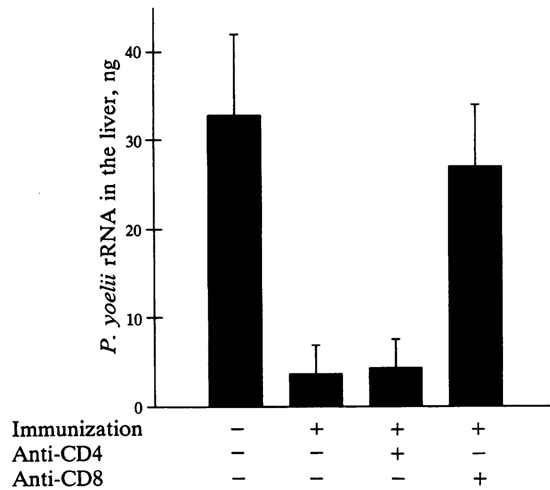

FIG. 4. Protective immunity induced by immunization with recombinant influenza and vaccinia viruses is mediated by CD8+ cells. Except for the controls, all mice were immunized (+) with influenza ME and subsequently given a booster injection with vaccinia PYCS virus, as described in Fig. 3*C*. Control mice were not immunized (−). As indicated, two groups of immunized mice were treated for 3 consecutive days with anti-CD4 or anti-CD8 mAb. Another group of immunized mice was left untreated. Immunized and control mice were challenged with *P. yoelii* sporozoites 2 days after the last antibody dose. The amount of plasmodial rRNA obtained from the livers of four mice is expressed as the mean ± SEM.

unlike influenza virus, is known to infect liver cells (32), a possible explanation for the observed difference of results is that a booster injection with vaccinia might increase the migration of CTLs to this target organ, including those induced by influenza ME virus. When the sequence of immunization is reversed (i.e., immunization with vaccinia followed by influenza), CTLs might be recruited away from the liver to the lung. If corroborated experimentally, this would suggest that localization of the CTLs in the liver, before sporozoite infection, is essential to achieve an efficient protective response against the intrahepatocytic stages of malaria parasites.

We have shown that the protection induced by immunization with these recombinant viruses is mostly mediated by $CD8^+$ T cells. It should, therefore, be possible to further enhance this protection by eliciting high titers of anti-sporozoite antibodies and $CD4^+$ T cells, known to have strong anti-parasite effects (33, 34).

Finally, it is worth emphasizing that the present results demonstrate that recombinant influenza viruses provide an efficient approach to the induction of CTLs directed against intracellular microorganisms. This finding and work demonstrating that immunization with recombinant influenza virus induces antibodies against foreign epitopes (19) indicate that recombinant influenza viruses should be considered as candidates for the development of vaccines against infectious intracellular pathogens, including malaria parasites. Our finding that immunity against malaria can be greatly enhanced by the successive use of two distinct live vectors may lead to additional strategies for the development of vaccines against microbial pathogens.

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