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# Immunogenicity and Protection of a Recombinant Human Adenovirus Serotype 35-Based Malaria Vaccine against *Plasmodium yoelii* in Mice

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**Given the promise of recombinant adenovirus type 5 (rAd5) as a malaria vaccine carrier in preclinical models, we evaluated the potency of rAd35 coding for *Plasmodium yoelii* circumsporozoite protein (rAd35PyCS). We chose rAd35 since a survey with serum samples from African subjects demonstrated that human Ad35 has a much lower seroprevalence of 20% and a much lower geometric mean neutralizing antibody titer (GMT) of 48 compared to Ad5 (seroprevalence, 85%; GMT, 1,261) in countries with a high malaria incidence. We also demonstrated that immunization with rAd35PyCS induced a dose-dependent and potent, CS-specific $CD8^+$ cellular and humoral immune response and conferred significant inhibition (92 to 94%) of liver infection upon high-dose sporozoite challenge. Furthermore, we showed that in mice carrying neutralizing antibody activity against Ad5, mimicking a human situation, CS-specific T- and B-cell responses were significantly dampened after rAd5PyCS vaccination, resulting in loss of inhibition of liver infection upon sporozoite challenge. In contrast, rAd35 vaccine was as potent in naive mice as in Ad5-preimmunized mice. Finally, we showed that heterologous rAd35-rAd5 prime-boost regimens were more potent than rAd35-rAd35 because of induction of anti-Ad35 antibodies after rAd35 priming. The latter data provide a further rationale for developing rAd prime-boost regimens but indicate that priming and boosting Ad vectors must be immunologically distinct and also should be distinct from Ad5. Collectively, the data presented warrant further development of rAd35-based vaccines against human malaria.**

In spite of tremendous efforts to control the malaria epidemic, current prophylaxis and drug treatments are proving insufficient. Therefore, the development of a safe and effective malaria vaccine receives a high priority with the realization that repeated immunizations with radiation-attenuated (10, 17, 19, 27, 31) or genetically modified, replication-deficient *Plasmodium falciparum* sporozoites (30) can confer excellent protection. Unfortunately, widespread use of vaccines based on attenuated sporozoites is not feasible due to manufacturing hurdles. The most effective malaria vaccine preventing severe disease to date, is RTS,S adjuvanted with AS02A (18). This fusion protein has shown approximately 30 to 40% protection in human field trials in Africa (1, 7, 9, 21). Although the RTS,S vaccine elicits strong antibody responses, only weak memory T-cell responses are induced and the immune response is short-lived (41).

Several recombinant viral vectors have shown promise as malaria vaccine carriers because of their intrinsic capacity to evoke strong T-cell responses (24, 25, 35, 43), one of the most promising being replication-deficient (E1-deleted) recombinant adenovirus type 5 (rAd5) (15, 34). This vaccine carrier is promising not only because vaccination yields strong, insert-specific T- and B-cell responses in diverse preclinical models, but also since the vaccine manufacturing technology is highly functional; i.e., millions of vaccine doses can be produced on appropriate cell lines (12, 16), a prerequisite for the development of malaria vaccines. However, clinical trials with rAd5 HIVgag vaccines and healthy volunteers demonstrated that the presence of high levels of anti-Ad5 neutralizing antibodies dramatically reduced the number of responders to the gag antigen (39). Since anti-Ad5 preexisting immunity is highly prevalent and widespread within human communities worldwide, anti-Ad5 neutralizing antibodies potentially present a major hurdle for the further development of rAd5 vaccines (20, 44). In contrast to Ad5, we have reported that human Ad35 represents a virus with low seroprevalence (44). Also, we have demonstrated that a rAd35SIVgag vaccine induces potent T-cell responses in both naive mice and mice carrying anti-Ad5 neutralizing activity (3). However, using SIVgag antigen, we were unable to evaluate the protective efficacy afforded by rAd35 vaccines or to assess the impact of anti-Ad5 immunity on the protective efficacy of rAd5 and rAd35 vaccines in this prior study. Using a *Plasmodium yoelii* malaria mouse model, we demonstrated that the protective efficacy afforded by rAd5 vaccines is markedly reduced by the presence of anti-Ad5 immunity. In contrast, rAd35 malaria vaccines protected mice both with and without anti-Ad5 immunity against a high-dose sporozoite challenge. Furthermore, we demonstrated that heterologous rAd35-rAd5 prime-boost regimens were much more potent compared to homologous rAd35-rAd35 regimens, further supporting the development of Ad prime-boost regimens but indicating that the priming and boosting vectors should be

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distinct. Based on these immunogenicity and protection data on mice, further development of the rAd35 vector as a novel human malaria vaccine candidate is warranted.

## MATERIALS AND METHODS

**rAds and *P. yoelii* parasites.** Generation of replication-deficient Ad vectors on PER.C6/55K cells was performed as described previously (44). The circumsporozoite (CS) protein insert in both rAd5PyCS and rAd35PyCS consisted of amino acids 1 to 356 of the CS protein of *P. yoelii* (34) placed under the control of a cytomegalovirus promoter. The recombinant vectors were purified by cesium chloride density centrifugation, and vaccine preparations were stored at −80°C until further use. The virus titer, expressed as the number of virus particles (vp) per milliliter, was determined by high-performance liquid chromatography. Quality control testing of virus batches included identity PCR, absence of mycoplasma, remaining cesium chloride content, bioburden, and vp/plaque-forming unit ratio determination. Sporozoites of *P. yoelii* strain 17X NL were isolated from salivary glands of *Anopheles stephensi* mosquitoes 2 weeks after a blood meal on *P. yoelii*-infected mice.

**Mice, immunizations, and sporozoite challenge.** Six- to eight-week-old female BALB/c (H-2K$^d$) mice purchased from the National Institutes of Health (Bethesda, MD) or Taconic (Germantown, NY) were vaccinated intramuscularly (i.m.) in the quadriceps by a single administration of $10^6$ to $10^{10}$ vp of rAd35 or rAd5 vector expressing *P. yoelii* CS protein (rAdPyCS). Two weeks after vector administration, serum and spleen cells were isolated to determine vector- and CS-specific immune responses. An additional group of immunized mice was challenged with live *P. yoelii* sporozoites. Challenge was performed by intravenous injection of $10^4$ viable *P. yoelii* sporozoites. Parasite burden in the liver was determined 42 h after the challenge by measuring parasite-specific 18S rRNA by a quantitative real-time reverse transcription-PCR method as described previously (8). Protection, defined as percent inhibition of liver infection, was calculated as follows: $(1 - \mathrm{rRNA}_{\mathrm{immunized}}/\mathrm{rRNA}_{\mathrm{naive}}) \times 100$, where $\mathrm{rRNA}_{\mathrm{immunized}}$ represents the number of copies of parasite 18S rRNA in the livers of immunized challenged mice and $\mathrm{rRNA}_{\mathrm{naive}}$ represents the number of copies of parasite 18S rRNA in the livers of naive challenged mice.

For Ad5 preexisting immunity studies, mice were preimmunized i.m. with an Ad5 vector without transgene (Ad5Empty) (dose, $10^{10}$ vp). After 8 weeks, mice were injected with $10^9$ vp of either rAd5PyCS or rAd35PyCS. Two weeks after vector administration, serum and spleen cells were isolated to determine anti-vector and anti-CS immune responses. An additional group of immunized mice was challenged with live *P. yoelii* sporozoites, and livers were extracted 42 h later to determine protection.

For prime-boost experiments, mice were primed i.m. with $10^9$ vp of rAd5PyCS and rAd35PyCS, respectively, and boosted after 8 weeks either with homologous or heterologous vector ($10^9$ vp). Eight weeks after the boost vaccination, immune responses against the vector and CS antigen were determined. An additional group of immunized mice was challenged with live *P. yoelii* sporozoites, and protection was determined.

**Determination of CS-specific cellular and humoral immune response.** The numbers of CS-specific, gamma interferon-secreting CD8$^+$ cells in the spleens of immunized mice were determined with an ELISPOT assay as previously described (28). The CD8 immunodominant *P. yoelii* CS peptide SYVPSAEQI (H-2K$^d$ restricted) was used for stimulation. Tetramer analysis was performed with tetrameric H-2K$^d$ complexes containing the immunodominant *P. yoelii* CS epitope SYVPSAEQI (kindly provided by E. V. Ravkov and J. D. Altman, National Institutes of Health Tetramer Core Facility, Atlanta, GA). Mouse blood was collected in RPMI 1640 medium containing 40 U/ml heparin, and red blood cells were lysed with lysis buffer (BioWhittaker, Verviers, Belgium). White blood cells were stained with phycoerythrin-labeled K$^d$/CS tetramer and fluorescein isothiocyanate-labeled anti-CD8α monoclonal antibody (Ly-2; Pharmingen, San Diego, CA), and the frequency of CD8$^+$ Tet$^+$ cells was determined by flow cytometric analysis with a FACSCalibur (BD Biosciences, Mountain View, CA). Blood cells from naive mice were used as a negative control. The CS-specific humoral response was determined by enzyme-linked immunosorbent assay (ELISA). Briefly, Maxisorp ELISA plates (Nunc) were coated with 2 μg/ml of CS-specific peptide $(\mathrm{QGPGAP})_3$ in phosphate-buffered saline (PBS). Plates where washed and blocked with 200 μl PBS–0.05% Tween–10% fetal bovine serum for 1 h at 37°C. After washing of the plates, 100 μl serially twofold-diluted sera in PBS–0.05% Tween–5% fetal bovine serum was added to the wells and the plates were incubated for 1 h at room temperature. The plates were washed and incubated with 100 μl/well peroxidase-labeled goat anti-mouse immunoglobulin G (heavy and light chains, human absorbed; Kaplan, Gaithersburg, MD). Finally, the plates were washed and 100 μl per well 2,2′-azinobis(3-ethylbenzthiazoline-sulfonic acid) (ABTS; Kaplan, Gaithersburg, MD) substrate was added to each well and the plates were incubated for 15 min at room temperature. The optical density of developed staining was measured at 405 nm.

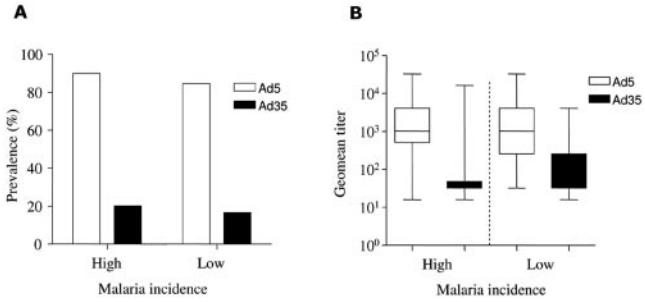


FIG. 1. Prevalence of Ad vector neutralizing antibodies in Africa. Sera collected from healthy adults from Africa were analyzed for neutralizing activity against Ad35 or Ad5. A total of 153 serum samples from 20 countries where malaria is endemic and 53 serum samples from 9 other African countries were available for screening (A). Corresponding GMTs are depicted in panel B.

**Determination of Ad vector-neutralizing antibodies.** The Ad antibody neutralization assay was performed with 96-well flat-bottom microtiter plates as previously described (40). Briefly, a twofold dilution of sera was prepared, starting from a serum dilution of 1/32. To this mixture, $5 \times 10^6$ vp of rAd containing the luciferase reporter gene (rAd5Luc or rAd35Luc) in a volume of 50 μl was added, followed by the addition of 100 μl medium containing $10^4$ A549 cells (multiplicity of infection of 500). Luciferase reporter gene expression in cells was assessed with a luciferase substrate and a Trilux luminescence detector (according to the manufacturer's instructions). Human serum samples were tested by the same protocol. The sera were obtained from anonymous patients of the Academic Medical Center outpatient clinic. The samples were used according to the Research Code of the Academic Medical Center in Amsterdam.

**Statistical analysis.** Data are presented as geometric means or medians. Statistical analyses were performed with SPSS version 12.0.1 (SPSS Software, Inc., 2004). Immune responses (logarithmically transformed) among groups of mice were assessed by independent-sample *t* tests for two groups of animals. For comparison of Ad5 and Ad35 dose-response experiments, analysis of variance was used where the vector and dose were entered into the model as covariables. In all cases, *P* values of less than 0.05 were considered statistically significant.

## RESULTS

**Prevalence of Ad-neutralizing antibodies in sera from African subjects.** Sera collected from healthy blood donors from Africa were analyzed for neutralizing activity against Ad5 and Ad35 as described previously (40). A total of 153 serum samples from 20 countries where malaria is endemic and 53 serum samples from African countries with a low malaria incidence were tested (http://www.mara.org.za/maps.htm). Results obtained demonstrate that neutralizing activity against Ad5 could be detected in more that 85% of the sera while the prevalence of anti-Ad35 immunity was less than 20%, irrespective of the origin, i.e., countries where malaria is endemic (high) or marginal/malaria epidemic-prone (low malaria incidence) countries (Fig. 1A). Also, geometric mean titers (GMTs) against Ad35 proved to be approximately 20-fold lower compared to Ad5 in sera tested for Ad5- and Ad35-neutralizing activities (Fig. 1B). These data thus show that seroprevalence for Ad35 is significantly lower than that for Ad5 in regions where malaria is endemic and thus further support the use of rAd35 vector as a vaccine delivery vehicle in developing parts of the world.

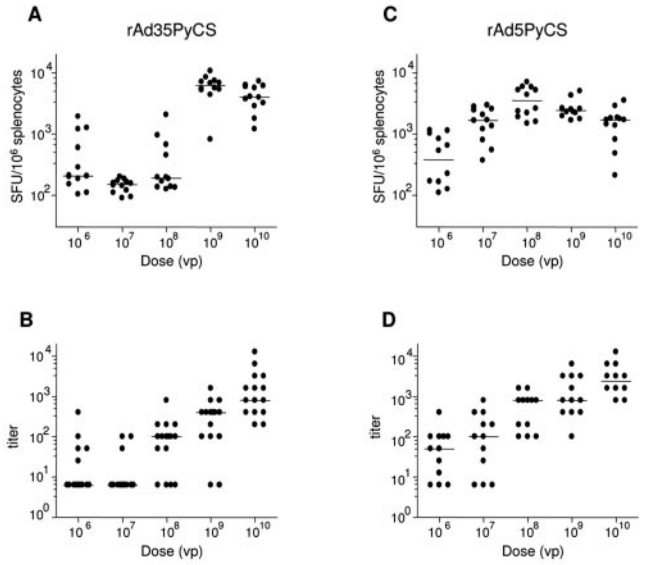


FIG. 2. Dose dependence of Ad35PyCS- or rAd5PyCS-induced immune responses and protection upon challenge. Groups of naive BALB/c mice (four per group) were immunized i.m. with $10^6$ to $10^{10}$ vp of rAd35PyCS (left panel) or rAd5PyCS (right panel). After 2 weeks, CS-specific cellular and humoral immune responses were determined by ELISPOT (A, C) (data from two independent experiments) and ELISA (B, D) (data from four independent experiments). Bars represent median values.

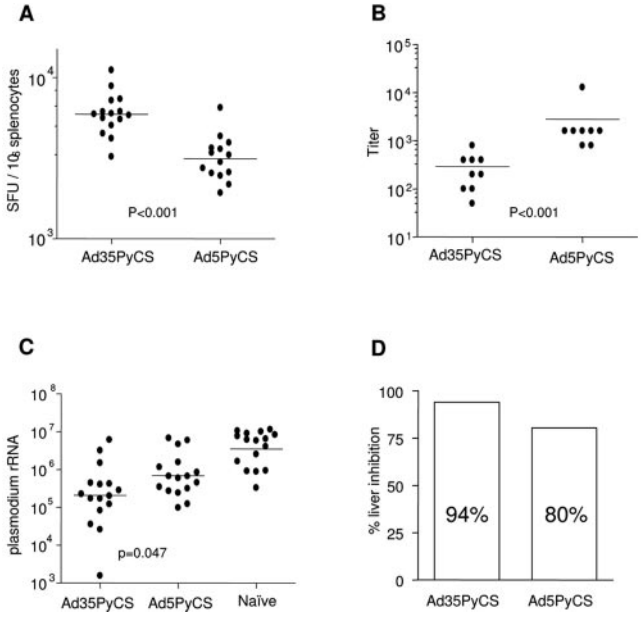


FIG. 3. Comparison between rAd35PyCS- and rAd5PyCS-induced immune responses and protection. BALB/c mice were immunized with $10^9$ vp of rAd5PyCS or rAd35PyCS. Splenocytes were isolated 2 weeks later, and the number of gamma interferon-secreting, CS-specific $CD8^+$ T cells was determined in an ELISPOT assay (A). CS-specific humoral responses were assessed in an ELISA (B). The parasite burdens in the livers of immunized and naive mice, as determined by real-time PCR, are depicted in panel C. The protection against a sporozoite challenge (i.e., inhibition of liver infection) that was achieved by immunization is shown in panel D. Data from three independent experiments are shown. Bars represent average values. SFU, spot-forming units.

**rAd35PyCS versus rAd5PyCS vaccine potency in mice.** Naive BALB/c mice were immunized with rAd5PyCS and rAd35PyCS vaccines at immunization doses ranging from $10^6$ to $10^{10}$ vp. Results obtained show that immunization with rAd35PyCS resulted in clear, dose-dependent, CS-specific T-cell responses (Fig. 2A) and antibody responses (Fig. 2B). However, in contrast to mice immunized with rAd5, demonstrating maximal CS-specific T-cell responses (approximately 2,000 spot-forming units/$10^6$ splenocytes) at a vaccine dose of $10^8$ vp (Fig. 2C), maximum T-cell responses with rAd35 vaccine (approximately 4,000 spot-forming units/$10^6$ splenocytes) were obtained at a dose of $10^9$ vp. For both the rAd35 and rAd5 vaccines, no upper threshold was observed in the CS-specific antibody level although antibody responses obtained after Ad5 vaccination proved consistently higher compared to those achieved with rAd35 vaccine (Fig. 2D). The observed difference in the dose dependence of the immune response between rAd35PyCS and rAd5PyCS proved significant for both the T-cell response ($P < 0.001$) and the antibody response ($P = 0.003$). Collectively, the results demonstrate that the vector dose dependence of the immune response obtained after immunization with rAd35 is distinct from that obtained with rAd5 in naive BALB/c mice, but both the Ad5PyCS and rAd35PyCS vaccines are capable of eliciting strong T-cell and antibody responses against the CS antigen.

**Inhibition of parasite liver infection by rAd35PyCS and rAd5PyCS vaccines.** Naive BALB/c mice were immunized with the rAd35PyCS and rAd5PyCS vaccines at the optimal dose for either vector ($10^9$ vp). The rAd35PyCS-induced CS-specific T-cell response was significantly higher ($P < 0.001$) than the rAd5PyCS-induced response at this dose (Fig. 3A). However, induction of anti-CS antibodies proved significantly less efficient ($P < 0.001$) after immunization with rAd35PyCS compared to rAd5PyCS (Fig. 3B). Determination of the parasite in the liver, 2 days after a high sporozoite challenge, demonstrated that both the rAd5 and rAd35 vaccines significantly reduced the liver burden (Fig. 3C). The difference in the inhibition levels obtained after immunization with rAd5 (80%) and rAd35 (96%) proved significant ($P = 0.047$). Thus, both the rAd5 and rAd35 vaccines prove capable of inducing potent T- and B-cell responses that are functional, i.e., reduced parasite infection. Furthermore, the increased inhibition seen after immunization with rAd35 compared to rAd5, related to the observed B- and T-cell responses, suggests that T cells play a key role in the control of the *P. yoelii* parasite.

**Potency of rAd5PyCS and rAd35PyCS vaccines in the presence of anti-Ad5 immunity.** BALB/c mice were preimmunized with a replication-incompetent rAd5 vector not carrying any transgene by a single i.m. immunization with $10^{10}$ vp 8 weeks prior to vaccination with $10^9$ vp of either rAd5PyCS or rAd35PyCS. This immunization resulted in a high anti-Ad5 neutralizing antibody titer (512 to 1,024) without generating antibodies that can cross-react with rAd35 (data not shown). As shown in Fig. 4A, T-cell responses decreased more than fourfold in Ad5-preimmunized mice compared to naive mice upon vaccination with rAd5, in contrast to rAd35 vaccination,

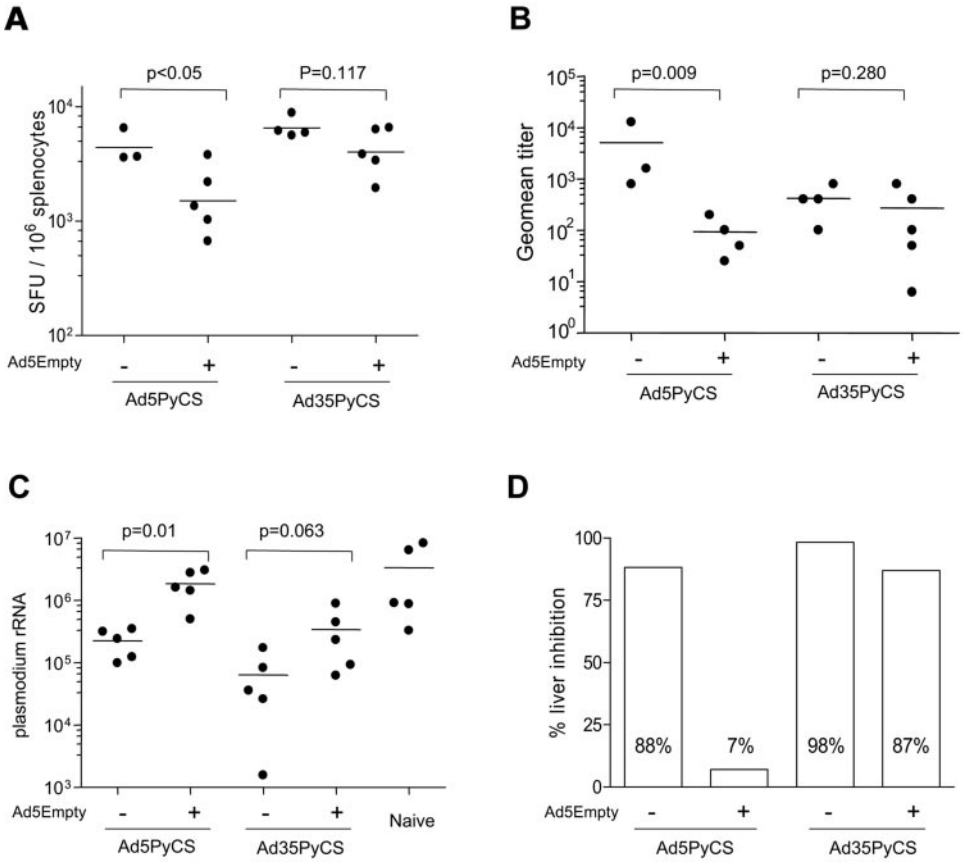


FIG. 4. rAd35PyCS immunogenicity and protection in the presence of anti-Ad5 immunity. BALB/c mice were preimmunized with $10^{10}$ vp of rAd5empty 8 weeks before immunization. Groups of naive mice or mice with anti-Ad5 immunity (three to five per group) were immunized with $10^9$ vp of rAd35PyCS or rAd5PyCS. CS-specific responses were analyzed 2 weeks after the immunization by ELISPOT (A) and ELISA (B). Parasite burdens in the livers of sporozoite-challenged mice are depicted in panel C, while protection (inhibition of liver infection) is shown in panel D. Bars represent geometric values. SFU, spot-forming units.

for which only a marginal and nonsignificant ($P = 0.117$) decrease in the T-cell level could be detected. Also, CS-specific antibody levels dropped to 5% in preimmunized mice compared to naive mice with rAd5, with no apparent decrease when they were immunized with rAd35 (Fig. 4B). The detrimental effects of anti-Ad5 immunity on levels of CS-specific T- and B-cell responses induced by rAd5 vaccine translated into a complete loss of inhibition of sporozoite liver infection, whereas rAd35 vaccine in such a host still significantly protected animals against liver infection (Fig. 4C and D). These results thus show that in a host carrying anti-Ad5 neutralizing antibodies, rAd5 vaccine potency is seriously affected, resulting in loss of protection, in contrast to that of the rAd35-based vaccine.

**Prime-boost immunization regimens.** BALB/c mice were primed at week 0 with $10^9$ vp rAd35PyCS and boosted at week 8 with either rAd5PyCS (heterologous) or rAd35PyCS (homologous) vector. Also, mice were primed with rAd5PyCS and subsequently boosted with rAd35PyCS to identify possible differences in the priming or boosting efficacy of the rAd35 vaccine. The kinetics of the CS-specific T-cell response was monitored by $K^d$/CS tetramer staining of peripheral blood mononuclear cells isolated at different times after boost vaccination. As shown in Fig. 5A, the rAd35 prime followed by rAd5 boost vaccination regimen in particular induced a rapid (day 10) and high-level (>20%) $CD8^+$ T-cell response. The increase in the frequency of CS-specific T cells continued until day 28 postboost and then declined to ±15% tetramer-positive $CD8^+$ T cells 60 days after the boost. Although boosting with rAd35 upon rAd35 priming resulted in a clear induction of $CD8^+$ T cells, the homologous booster was clearly less effective than heterologous prime-boost regimens. This phenomenon is probably due to the presence of antivector antibodies, induced at the priming, that neutralize homologous vector at the boosting (neutralizing anti-Ad35 GMT after prime, 798). The heterologous regimens are not affected since the neutralizing antibodies against Ad5 and Ad35 are not cross-reactive, as determined by Ad neutralization assay. Mice primed with rAd5 vaccine developed titers against Ad5 (GMT, 412), while no titer of antibody against Ad35 could be detected (GMT, <16). Similarly, rAd35-primed mice exhibited no titer of antibody against Ad5 (GMT, <16). ELISPOT analyses performed with splenocytes isolated 60 days after a boost further demonstrated that heterologous prime-boost combinations elicit higher CS-specific T-cell levels compared to homologous prime-boost combinations (Fig. 5B). Similarly, CS-specific antibody responses proved less efficient after homologous prime-boost compared to heterologous regimens (Fig. 5C). Finally, as

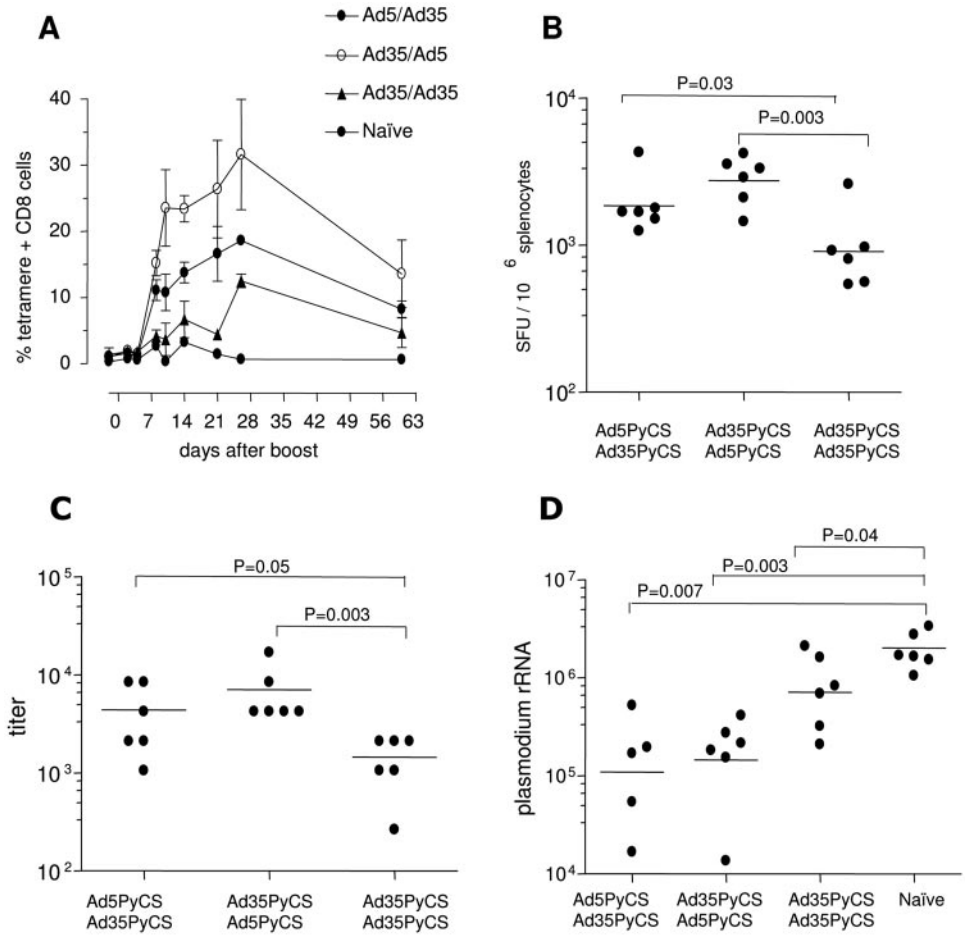


FIG. 5. Immunogenicity and protection in prime-boost immunization regimens. Groups of naive BALB/c mice (five to six per group) were primed with $10^9$ vp of rAd35PyCS or rAd5PyCS at week 0 and boosted with $10^9$ vp of homologous or heterologous vector at week 8. Mean percentages of $K^d$/CS tetramer-positive, $CD8^+$ T cells at various time points after boost immunization are shown (A). Bars represent standard deviations of the means. After 8 weeks, CS-specific cellular and humoral immune responses were determined by ELISPOT (B) and ELISA (C), respectively. Parasite burdens in the livers of sporozoite-challenged mice are shown in panel D. Percent inhibition of liver infection was calculated on the basis of rRNA data. In homologous prime-boost regimens, 62% inhibition was observed, compared to 92% and 94%, respectively, in heterologous prime-boost regimens. Bars represent geometric means. SFU, spot-forming units.

shown in Fig. 5D, inhibition of liver infection induced by the vaccine proved less efficient when using a rAd35-rAd35 vaccination regimen (62%), whereas both heterologous vaccine prime-boost combinations greatly inhibited liver infection (94 and 92%, respectively). Taken together, these results demonstrate that, in mice, anti-Ad35 antibodies induced after priming effectively dampen a rAd35 boost, resulting in a significant reduction of CS-specific T- and B-cell responses and a partial loss of inhibition of sporozoite liver infection. In contrast, heterologous rAd5-rAd35 prime-boost regimens elicit potent CS-specific immune responses and still confer protection 60 days after a booster immunization.

## DISCUSSION

Replication-deficient rAd vectors are attractive vaccine delivery vehicles because of their safety, immunogenicity, and easy manipulation and the availability of suitable cell lines for high-yield production (12, 16). The feasibility of a rAd5 malaria vaccine has been demonstrated since single immunization with a rAd5 vector coding for the CS protein induced protective immune responses in the accepted and established *P. yoelii* rodent malaria model (34). However, translation of these promising experimental findings to humans might prove difficult since anti-Ad5 immunity is widespread among human populations and high titered, particularly in Africa (32). Previously we have reported that among the 51 Ad strains isolated to date, B2 group vectors Ad11 and Ad35 display low seroprevalence with low titers worldwide (20, 22, 44). Here we extended these observations and show that in countries with a high malaria incidence, Ad35 prevalence and titers are low compared to those of Ad5, further supporting the use of Ad35-derived vaccine carriers in these regions. These serology studies were performed with serum samples from healthy adults, and given the low seroprevalence in adults it can be speculated that infants would also have low serum titers of antibody against Ad35. The latter is important since the incidence of clinical malaria peaks at 1 to 5 years of age in areas where malaria is endemic (6). However, further studies with serum samples

from infants are needed to determine whether, indeed, Ad35 has a low seroprevalence in infants and whether, due to maternal antibodies, Ad5 seroprevalence is high in this population, precluding further pediatric vaccine development on the basis of the use of the rAd5 vector.

Our vaccination results reported here demonstrate that rAd35PyCS elicits potent CS-specific cellular and humoral immune responses and significant inhibition of liver infection upon high-dose sporozoite challenge. In naive mice, the induced protective response proved comparable to responses obtained after vaccination with rAd5PyCS, although clearly the dose dependence was distinct between rAd5- and rAd35-based vaccines. Whereas T-cell responses were maximal at a $10^9$-vp dose and exhibited a steep dose-dependent curve for rAd35, the optimal dose of rAd5PyCS proved to be 1 log lower ($10^8$ vp), with a gradual dose dependence. Although the difference in rAd35 and rAd5 vaccine kinetics in mice is poorly understood, it has been established that Ad35 requires the high-affinity receptor protein CD46, which is lacking in mice (14, 38). In contrast, the receptor for Ad5, CAR (4, 11, 42), is ambiguously expressed in mice (42). One hypothesis is that immune responses measured in mice upon immunization with rAd35, especially at low immunization doses, underestimate the actual immunogenic potential of rAd35, due to suboptimal receptor usage. Further investigation with CD46-transgenic mice (46), which have proven of great value in measles virus research (5, 29), might reveal the role of the CD46 receptor as regards Ad35 vaccine potency in dosing.

Results obtained with rAd35, regarding the high levels of liver inhibition in combination with strong T-cell responses but weaker B-cell responses, suggest an important role for T cells in control of the *P. yoelii* parasite. These studies further support earlier data demonstrating, through either depletion of $CD8^+$ T cells in vivo (33, 35, 45) or adoptive transfer of CS-specific $CD8^+$-T-cell clones (36, 37), that T cells are indeed key in preventing malaria in mice.

The advantage of rAd35 over rAd5 as a vaccine carrier was demonstrated in experiments performed with mice with or without anti-Ad5 neutralizing activity, wherein the immunogenicity of rAd35 vaccine was maintained while the potency of rAd5 vaccine was abrogated in mice with anti-Ad5 neutralizing activity. This finding confirms our earlier observations with rAd35SIVgag vaccine (3). Importantly, the rAd35PyCS vaccines protected mice both in the presence and in the absence of anti-Ad5 immunity against a high-dose sporozoite challenge in an established rodent challenge model. Since the level of anti-Ad5 neutralizing activity in the mice equaled the levels reported for humans (22), these data suggest that the potency of rAd5 vaccine can be significantly hampered in clinical trials (39), thus warranting Ad serology testing of patients enrolled in clinical trial studies.

One of the major hurdles encountered thus far with experimental pre-erythrocytic malaria vaccines in humans is a short duration of protection upon challenge. The most promising clinical results have been obtained with RTS,S/AS02A, an adjuvanted fusion protein of *P. falciparum* CS protein with hepatitis B surface antigen. This protein vaccine appeared safe and immunogenic but failed to elicit $CD8^+$-T-cell responses, and protection proved short-lived (41). A possible solution to induce $CD8^+$ T cells and to overcome short-lived immunity might be via heterologous prime-boost immunization regimens. In this respect, it would be interesting to test prime-boost combinations utilizing RTS,S and rAd vectors to achieve high levels of both neutralizing antibodies and T cells. Alternatively, results shown here demonstrate that heterologous prime-boost combinations of rAd35PyCS with rAd5PyCS elicit T-cell responses that proved extremely strong (15% tetramer-positive $CD8^+$ cells) even 60 days after boosting, indicating that a strong memory T-cell response was generated. Since it can be expected that any vaccine prime-boost combination containing rAd5 will be seriously inhibited, a novel Ad vector distinct from rAd35 and rAd5 is required. Such a vector could be generated from the pool of human Ads, provided that human serotypes with low seroprevalence are still available, or prime-boost combinations could be tested utilizing Ad vectors developed from nonhuman Ad serotypes such as bovine (2), canine (23), avian (26), or chimpanzee (13) vectors.

In summary, our results demonstrate that an rAd35-based CS malaria vaccine induces high CS-specific cellular and humoral immune responses and significantly inhibits liver infection upon a high-dose sporozoite challenge. Potency of rAd35 vaccine is maintained in the presence of anti-Ad5 immunity, in contrast to Ad5 vaccine. Also, the high efficacy of rAd35 in a prime-boost immunization schedule with a second rAd vector further supports the development of rAd-based prime-boost regimens and emphasizes the role for the rAd35 vaccine carrier as a component of a human malaria vaccine.

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