

Persistence of Protective Immunity to Malaria Induced by DNA Priming and Poxvirus Boosting: Characterization of Effector and Memory CD8⁺-T-Cell Populations

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The persistence of immunity to malaria induced in mice by a heterologous DNA priming and poxvirus boosting regimen was characterized. Mice were immunized by priming with DNA vaccine plasmids encoding the *Plasmodium yoelii* circumsporozoite protein (PyCSP) and murine granulocyte-macrophage colony-stimulating factor and boosting with recombinant vaccinia encoding PyCSP. BALB/c mice immunized with either high-dose (100 µg of p PyCSP plus 30 µg of pGM-CSF) or low-dose (1 µg of p PyCSP plus 1 µg of pGM-CSF DNA) priming were protected against challenge with 50 *P. yoelii* sporozoites. Protection 2 weeks after immunization was 70 to 100%, persisted at this level for at least 20 weeks, and declined to 30 to 40% by 28 weeks. Eight of eight mice protected at 20 weeks were still protected when rechallenged at 40 weeks. The antigen (Ag)-specific effector CD8⁺-T-cell population present 2 weeks after boosting had ex vivo Ag-specific cytolytic activity, expressed both gamma interferon (IFN-γ) and tumor necrosis factor alpha, and constituted 12 to 20% of splenic CD8⁺ T cells. In contrast, the memory CD8⁺-Ag-specific-cell population at 28 weeks lacked cytolytic activity and constituted only 6% of splenic CD8⁺ T cells, but at the single-cell level it produced significantly higher levels of IFN-γ than the effectors. High levels of Ag- or parasite-specific antibodies present 2 weeks after boosting had declined three- to sevenfold by 28 weeks. Low-dose priming was similarly immunogenic and as protective as high-dose priming against a 50-, but not a 250-, sporozoite challenge. These results demonstrate that a heterologous priming and boosting vaccination can provide lasting protection against malaria in this model system.

Immunity to malaria is often short lived. Some aspects of the clinical immunity which develops following life-long exposure to malaria are lost upon leaving an area where malaria is endemic, the sterile protection induced by immunization with radiation-attenuated sporozoites has not been shown to last more than 9 months, and the protection afforded by a recent candidate malaria vaccine, RTS,S (17), is short lived (16). It is important therefore to develop model malaria vaccines that provide long-term protection and to understand better the immune mechanisms involved in long-term protection against malaria.

Immunization of BALB/c mice with irradiated *Plasmodium yoelii* sporozoites confers sterile protection against high-dose challenge with infectious *P. yoelii* sporozoites (4, 9, 11, 20). This protection is dependent on CD8⁺ T cells and gamma interferon (IFN-γ) (4, 10, 20). Immunization with DNA plasmids encoding the *P. yoelii* circumsporozoite protein (PyCSP)

partially protects BALB/c mice against challenge with 50 to 100 *P. yoelii* sporozoites (6, 12, 13, 14, 19). Like the protection induced by immunization with irradiated sporozoites, the DNA-vaccine-induced protection is dependent on CD8⁺ T cells and IFN-γ (5, 12). This modest protective efficacy can be improved by adding a plasmid encoding murine granulocyte-macrophage colony-stimulating factor (GM-CSF) to the PyCSP plasmid (19) or by boosting with a recombinant vaccinia encoding PyCSP (13). Recently, Sedegah et al. demonstrated that combining these two strategies by priming with the combination of DNA plasmids encoding PyCSP and murine GM-CSF and boosting with recombinant PyCSP poxvirus induced a substantial increase in protection, and in antibody and T-cell responses, compared with immunizing with DNA alone (14). In addition, we found protection could be induced even if the priming dose of DNA was reduced 100-fold. In these studies, protection correlated most closely with the results of ex vivo cytotoxic T-lymphocyte (CTL) and IFN-γ enzyme-linked immunospot (ELISPOT) assays, consistent with CD8⁺-T-cell- and IFN-γ-dependent protection.

Here we further characterize the immunity induced by the DNA-GM-CSF viral boost regimen. We studied the persistence of the protection, its robustness in the face of challenge with increased numbers of infectious sporozoites, and the

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change in antibody and T-cell responses and the phenotype of responding T cells over time.

MATERIALS AND METHODS

Mice. Four- to 6-week-old female BALB/cByJ (*H-2^d*) mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and used in all experiments. The experiments reported herein were conducted according to the principles set forth in *Guide for the Care and Use of Laboratory Animals* (9a).

Parasites. *P. yoelii* (17XNL nonlethal strain, clone 1.1) parasites were maintained by alternating passage of the parasites in *Anopheles stephensi* mosquitoes and CD-1 mice. Sporozoites for challenge studies were obtained by hand dissection of infected mosquito glands in M199 medium containing 5% normal mouse serum 14 days after the mosquitoes had taken an infectious blood meal. Isolated sporozoites were diluted to a final concentration of 250 to 1,250 infectious sporozoites per ml.

Parasite challenge. Parasite challenge was accomplished by injecting 50 or 250 sporozoites into the tail vein, in a volume of 200 μ l, at 2, 4, 8, 12, 16, 20, 28, or 40 weeks postimmunization. Giemsa-stained blood films were prepared on days 5 to 14 postchallenge and examined microscopically for the presence of parasites, with up to 50 oil-immersion fields examined. Protection was defined as the complete absence of blood-stage parasitemia at all time points.

Immunogens. The plasmid DNA constructs encoding PyCSP in the VR1020 backbone (VR2516), designated p PyCSP herein, and the murine GM-CSF plasmid (VR1701), designated pGM-CSF herein, have been described previously (13, 19). Plasmid DNA was purified by using cesium chloride-ethidium bromide density gradient centrifugation. Plasmids were stored at -20°C and diluted in phosphate-buffered saline (PBS) (pH 7.2) to the appropriate concentration for injection. The recombinant vaccinia virus construct encoding PyCSP, designated rv PyCSP herein, and control virus have been described previously (13).

Immunizations. For high-dose (DG-V) immunization, 100 μ g of p PyCSP was mixed with 30 μ g of pGM-CSF; for low-dose (dg-V) immunization, 1 μ g of p PyCSP was mixed with 1 μ g of pGM-CSF. Negative-control mice were injected with unmodified plasmid DNA (VR1020). In all cases, mice received the total dose in two 50- μ l intramuscular injections with a 0.3-ml insulin syringe with a 29.5-gauge needle; one injection in each tibial anterior muscle. Mice received a boost intraperitoneally of 2×10^7 rv PyCSP or of control virus (vP993) containing no foreign gene delivered in a total volume of 0.2 ml of PBS with a 1-ml insulin syringe with a 26.5-gauge needle 3 weeks after priming with DNA.

Antibody assays. *P. yoelii* sporozoite-specific antibodies in prechallenge sera were assayed by immunofluorescent staining of air-dried *P. yoelii* sporozoites as described previously (3, 13). PyCSP-specific antibodies were assayed by standard enzyme-linked immunosorbent assay with a full-length recombinant PyCSP produced in yeast or linear synthetic peptide containing four copies of the major PyCSP repeat, (QGGPAP), as solid-phase antigens by using previously described methods(3).

CTL assays. Standard restimulated CTL assays were carried out as described previously (14) with a 16-mer peptide derived from the PyCSP [PyCSP(280-296), SYVPSAEQILEFVKQI] for restimulation and a 9-mer peptide [PyCSP(280-288), SYVPSAEQI] for target sensitization. The percent lysis was determined with the following formula: (experimental release – medium control release) / (maximum release – medium control release) \times 100. The percent specific lysis was calculated by subtracting the percent lysis of the control targets from the percent lysis of the specific peptide. Alternatively, freshly isolated immune spleen cells were used as effectors in ^{51}Cr release assays without the standard 6- to 7-day in vitro restimulation period. In brief, for effectors, various ratios (effector-to-target cell ratio = 300:1, 150:1, 75:1, and 37.5:1) of freshly isolated spleen cells suspended in complete medium containing 2% rat T-cell stim (Collaborative Biomedical Products Inc., Bedford, Mass.) were added to 96-well round bottom plates in a volume of 100 μ l per well in triplicate. For targets, P815 (*H-2^d*) cells (American Type Culture Collection, Rockville, Md.) were labeled overnight with 0.1 mCi of ^{51}Cr (Dupont-New England Nuclear Inc., Boston, Mass.). Immediately before the assay, ^{51}Cr -labeled target cells were washed three times with complete medium and resuspended at a concentration of 5×10^4 cells per ml. For experimental targets, PyCSP(280-288) (SYVPSAEQI) was added at a concentration of 0.025 μM . The control targets did not receive any peptide. Target cells were aliquoted at a concentration of 5,000 cells/100 μ l/well to wells containing effector cells or to control wells. Supernatants were harvested after a 12-h incubation period by using the Skatron harvesting system, and percent lysis was determined as described above.

Intracellular cytokine staining. Spleen cells (5×10^5) from immunized or control mice were aliquoted into 96-well U bottom plates containing PyCSP(280-288) peptide-pulsed P815 cells (1×10^5) and brefeldin A (2 $\mu\text{g}/\text{ml}$), in a total

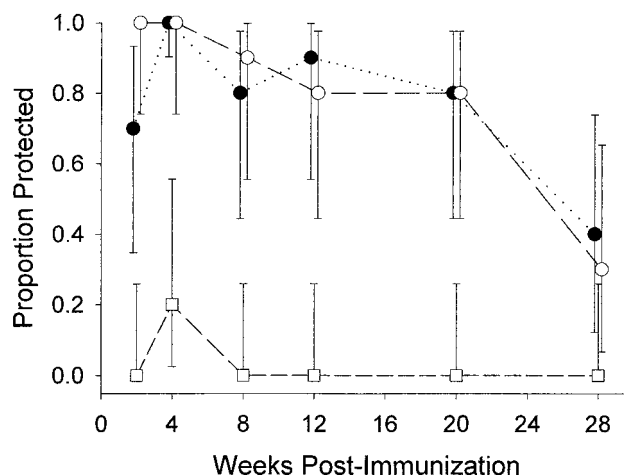


FIG. 1. Duration of protection. Mice were immunized and challenged as described in Materials and Methods. Shown is the percentage of sterile protection observed in groups of 10 mice challenged at the indicated times after vaccination with the DG-V (high-dose DNA) (●) or dg-V (low-dose DNA) (○) regimen. Nonimmunized controls (□) are also shown. Error bars represent 95% confidence intervals around the proportions of protected mice.

volume of 200 μ l of complete medium/well. Cells were cultured for 16 h at 37°C in a 5% CO_2 humidified incubator. Following culture, cells were washed with cold PBS and stained with cell surface antibodies to CD8, CD62L, CD44, CD45RB, and CD69 (Pharmingen, San Diego, Calif.) for 20 min on ice. Cells were washed twice and permeabilized in 100 μ l of Cytofix/Cytoperm buffer (Pharmingen) according to the manufacturer's directions. Cells were then stained intracellularly for IFN- γ and tumor necrosis factor alpha (TNF- α) by using phycoerythrin-conjugated monoclonal antibodies to IFN- γ or TNF- α (Pharmingen) at a concentration of 0.4 μg of antibody/ 10^6 cells. Samples were acquired by using a four-color FACSCALIBUR (Becton Dickinson, San Jose, Calif.). For each sample, 80,000 to 200,000 events were acquired. Cytokine expression on gated CD8^+ T cells was analyzed with Cellquest software (Becton Dickinson). All assays were performed in 96-well plates. Negative-control samples, consisting of cells from naive mice incubated with peptide-pulsed P815 cells or splenocytes from immunized mice incubated with P815 cells not pulsed with peptide, consistently showed $<0.2\%$ of CD8^+ T cells expressing IFN- γ or TNF- α (data not shown).

Statistical analysis. For protective efficacy, proportions of infected versus noninfected animals in control and experimental groups were compared by using the chi-square or Fisher exact (two-tailed) test as appropriate (SPSS for windows, version 8.0) and are reported with 95% confidence intervals. To compare relative immunogenicity of the vaccines, means were compared by using the independent sample *t* test (SPSS for windows, version 8.0).

RESULTS

Duration of protection. To assess the duration of protection induced by the high-dose (DG-V) and low-dose (dg-V) regimens, groups of mice were immunized with either the DG-V or the dg-V regimen as described in Materials and Methods, and samples of 10 mice from each group were challenged with 50 infectious *P. yoelii* sporozoites at various intervals after immunization. Figure 1 shows the proportion of protected mice at increasing times after immunization. For both immunization regimens, between 70 and 100% protection (mean, 87%) was obtained up to 20 weeks postboost. There was no significant difference between any of the time points up to 20 weeks for either immunization regimen. Protection was decreased to 30 to 40% at 28 weeks postboost and was significant compared

with the protection seen at 20 weeks ($P = 0.010$, two-tailed Fisher's exact test). There was no significant difference between the high-dose (DG-V) and low-dose (dg-V) immunization regimens (DG-V, 46 of 60 = 76.7%; dg-V, 48 of 60 = 80%; $P = 0.50$, chi-square test) during this period (weeks 2 to 28). These data are consistent with previous results at 2 weeks postboost (DG-V, 23 of 18 = 82%; dg-V, 13 of 18 = 72%; $P = 0.43$, chi-square test) (14). To further evaluate the longevity of protection, we selected a subset of DG-V mice shown to be protected at 20 weeks and rechallenged them at 40 weeks postboost. In this experiment, 8 out of 8 of the mice immunized with the DG-V regimen were still protected at 40 weeks (0 of 10 controls challenged in parallel were protected). Overall, the protection induced by the DG-V and dg-V regimens was maintained at a high level for 20 weeks and decreased between 20 and 28 weeks postboost. All mice protected at 20 weeks were protected at 40 weeks, indicating either persistence of protection or boosting of protective immunity by the 20-week challenge.

Protection against high-dose challenge. BALB/c mice immunized with irradiated sporozoites are protected from challenges of 5×10^3 (18) to 5×10^5 (unpublished data) *P. yoelii* sporozoites; in contrast, immunization with plasmid DNA alone provides only partial protection against inocula of 50 to 100 sporozoites (6, 12). Therefore, we next evaluated the capacity of the DG-V and dg-V regimens to protect against a higher dose challenge. Accordingly, at the time of the post-20-week challenge, we randomized mice that were protected in the 2-, 4-, 8-, or 12-week challenges into two groups and rechallenged them with either 50 or 250 sporozoites. Protection against challenge with 250 sporozoites was significant for both high-dose (8 of 13 versus 0 of 10; $P = 0.003$, two-tailed Fisher exact test) and low-dose (5 of 14 versus 0 of 10; $P = 0.05$, two-tailed Fisher exact test) regimens compared with controls. Moreover, there was essentially no difference in the capacity of the high-dose-induced protection to withstand challenge with either 250 or 50 sporozoites (8 of 13 [62%] versus 18 of 25 [72%]; $P = 0.714$, two-tailed Fisher exact test). However, the protection induced by the low-dose regimen was not as robust, and there was a trend for reduced protection against challenge with 250 versus 50 sporozoites (5 of 14 [36%] versus 15 of 23 [65%]; $P = 0.080$, chi-square test).

Immune responses of immunized mice at 2 or 28 weeks after immunization. The protection against sporozoite challenge conferred by either irradiated sporozoites or plasmid DNA is dependent on CD8⁺ T cells and IFN- γ (4, 6, 10, 20). Recent studies suggest that the protection induced by DNA priming and poxvirus boosting is also dependent on CD8⁺ T cells and IFN- γ (14); in these studies the best correlates of protection were ex vivo CTL activity and IFN- γ activity specific for the CD8⁺-T-cell epitope PyCSP(280-288) (SYVPSAEQI). We therefore characterized T cells from immunized mice in three assays, a standard restimulated CTL assay, a direct ex vivo CTL assay, and an intracellular cytokine-staining assay for IFN- γ or TNF- α . Further, since a decrease in vaccine-induced protection was observed at 28 weeks postvaccination compared with that at 2 weeks postvaccination, we evaluated T-cell responses and surface phenotype at 2 and 28 weeks postvaccination. The immunization schedule was staggered so that samples from all groups of mice were evaluated at the same time to eliminate

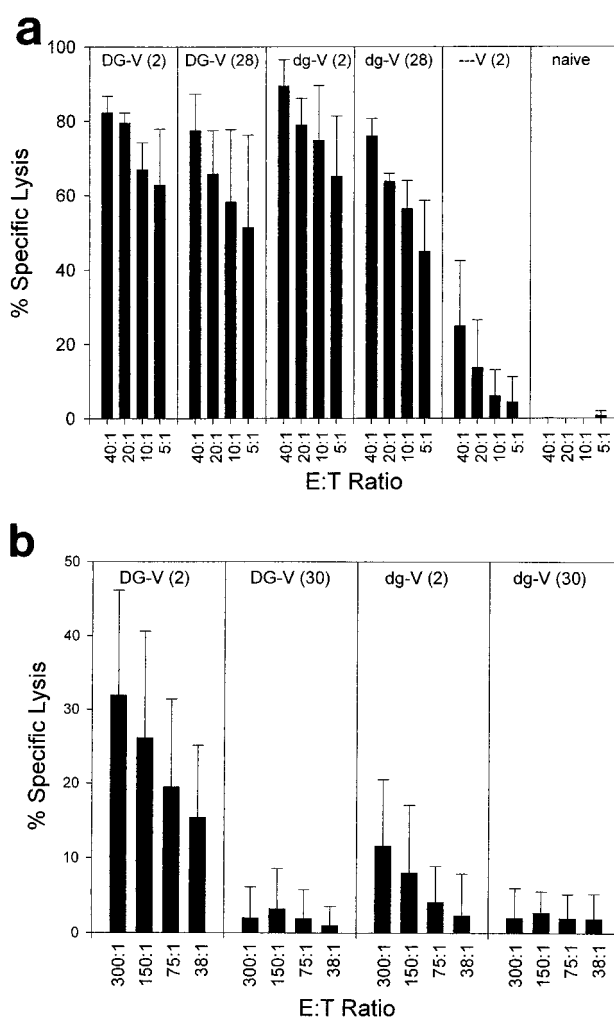


FIG. 2. CTL responses. (a) Restimulated CTL assay. Spleen cells from 3 mice from either the high (DG-V)- or low (dg-V)-dose DNA group obtained at 2 or 28 weeks postboost were restimulated in vitro for 7 days with peptide PyCSP(280-295) and used as effectors in a chromium release CTL assay with peptide PyCSP(280-288)-labeled targets as described in Materials and Methods. The percent lysis was determined according to the following formula: $(\text{experimental release} - \text{medium control release}) / (\text{maximum release} - \text{medium control release}) \times 100$. The percent specific lysis was calculated by subtracting the percent lysis of the control targets from the percent lysis of the specific peptide. Three mice per group were assayed individually, and data represent the means and standard deviations of percent specific lysis at the indicated effector-to-target-cell (E:T) ratios. (b) Ex vivo CTL assay. Freshly isolated spleen cells from 10 mice per group obtained at 2 or 30 weeks postboost were assayed individually and used as effectors as described in Materials and Methods. Data represent the means and standard deviations of percent specific lysis at the indicated effector-to-target-cell ratios.

any potential experiment-to-experiment variation. To confirm the initial results of the ex vivo CTL and intracellular cytokine staining assays, which were based on splenocytes derived from small numbers of mice, we immunized additional groups of 20 mice with the DG-V and dg-V regimens and repeated the assays on splenocytes from 10 individual mice sacrificed at either 2 or 30 weeks after the viral boost.

TABLE 1. Intracellular cytokine staining

Expt no.	Regimen (wk post-viral boost) ^a	Mean % IFN- γ ⁺ CD8 ⁺ cells (SD)	Mean MFI of IFN- γ ⁺ CD8 ⁺ cells (SD)	Mean % TNF- α ⁺ CD8 ⁺ cells (SD)	Mean MFI of TNF- α ⁺ CD8 ⁺ cells (SD)
1	DG-V (2)	12.6 (2.8)	147 (29)	8.0 (0.7)	52 (1)
	DG-V (28)	7.3 (7.2)	276 (44)	6.3 (4.7)	69 (7)
	dg-V (2)	11.9 (7.1)	143 (12)	7.6 (4.2)	55 (6)
	dg-V (28)	4.4 (0.4)	233 (20)	3.8 (0.2)	63 (6)
2	DG-V (2)	20.6 (7.4)	406 (47)		
	DG-V (30)	8.1 (5.2)	754 (99)		
	dg-V (2)	11.6 (5.7)	505 (133)		
	dg-V (30)	3.4 (1.8)	962 (152)		

^a Experiment 1, 3 mice per group; experiment 2, 10 mice per group.

CD8⁺ CTL responses. As in previous studies (14), high levels of restimulated CTL specific for the immunodominant epitope PyCSP(280-288) were detected in both high-dose and low-dose priming regimens at 2 weeks postboost (Fig. 2a); there was a slight but not significant decrease in the magnitude of restimulated CTL responses at 28 weeks compared with that at 2 weeks. There was no apparent difference in the magnitude of response between the high- and low-dose regimens at either time point (Fig. 2a).

Robust ex vivo CTL responses were also detected in both DG-V- and dg-V-immunized mice at 2 weeks postboost (Fig. 2b and data not shown), as reported previously (14). However, we were unable to detect ex vivo CTL responses in either DG-V- or dg-V-immunized mice at 28 or 30 weeks postboost (Fig. 2b and data not shown). Mice immunized with a single dose of recombinant vaccinia expressing PyCSP without DNA priming produced lower levels of CTL in both the restimulated and ex vivo CTL assays (Fig. 2a and data not shown).

We next evaluated antigen-specific production of IFN- γ and TNF- α in CD8⁺ T cells from DG-V- or dg-V-immunized mice at either early (2 weeks) or late (28 or 30 weeks) points after viral boosting. As shown in Table 1, samples from DG-V-immunized mice had marginally higher frequencies of IFN- γ - or TNF- α -secreting CD8⁺ T cells than samples from dg-V-immunized mice in experiment 1 and significantly higher frequencies of IFN- γ -secreting CD8⁺ T cells in experiment 2 (DG-V versus dg-V at 2 weeks, $P = 0.007$). At the late time points, there was a substantial decline in the fraction of CD8⁺ cells expressing IFN- γ , and to a lesser extent TNF- α , although the declines reached statistical significance only in the second experiment conducted with 10 mice per group (IFN- γ , DG-V, 2 versus 30 weeks, $P = 0.00044$; IFN- γ , dg-V, 2 versus 30 weeks, $P = 0.0014$; TNF- α , DG-V, 2 versus 28 weeks, $P = 0.58$; TNF- α , dg-V, 2 versus 28 weeks, $P = 0.27$). This decline in the percentage of antigen-responsive CD8⁺ cells corresponded to a decline in the absolute number of antigen-responsive cells in the spleen since the total number of splenocytes isolated from the spleens was reduced by 20 to 50% in the late compared to the early time points (data not shown). The cytokine-positive CD8⁺ population had the surface phenotypes CD62L^{lo}, CD69^{lo}, CD45RB^{int}, and CD44^{hi} (Fig. 3).

To further characterize the responding cell population, we quantitated the mean fluorescence intensity (MFI) of IFN- γ and TNF- α responses. As shown in Table 1, the MFI for IFN- γ was greater in the IFN- γ ⁺ CD8⁺ splenocytes taken at 28 or 30

weeks postboost than at 2 weeks for both DG-V and dg-V regimens (DG-V, $P < 0.0001$; dg-V, $P < 0.0001$; experiment 2). A similar difference was observed for the TNF- α MFI but was only significant in the case of the DG-V regimen (DG-V, $P = 0.045$; dg-V, $P = 0.159$). Thus, although the number of CD8⁺ cells expressing IFN- γ and TNF- α was lower at 28 or 30 weeks than at 2 weeks after boosting, the amount of IFN- γ and TNF- α produced per responding cell, as measured by the MFI, was higher at 28 or 30 weeks than at 2 weeks. There was no difference between the MFI for IFN- γ or TNF- α observed in cells from mice immunized with the DG-V versus dg-V regimens.

Persistence of antibody responses induced by DNA plus GM-CSF priming and poxvirus boosting. Figure 4 shows the antibody responses in mice immunized with the DG-V or dg-V regimens at 2 or 28 weeks after the viral boost, as measured by enzyme immunoassay (EIA) with either a recombinant PyCSP protein or a peptide corresponding to the immunodominant B-cell epitope (QGPGAP) or by immunofluorescence assay (IFA) against air-dried sporozoites. As measured by all three assays in both DG-V and dg-V groups, there was a modest, statistically significant, three- to sevenfold reduction in titer between weeks 2 and 28 (CSP EIA: DG-V, $P = 0.002$; dg-V, $P = 0.004$; peptide EIA: DG-V, $P < 0.001$; dg-V, $P = 0.03$; sporozoite IFA: DG-V, $P = 0.01$; dg-V, $P = 0.02$; two-tailed t test, log-transformed titers). Overall, titers were approximately threefold higher in mice immunized with the DG-V regimen than in those immunized with the dg-V regimen at both 2 and 28 weeks; however, the difference was not consistently statistically significant (CSP EIA: 2 weeks, $P = 0.05$; 28 weeks, $P = 0.003$; peptide EIA: 2 weeks, $P = 0.02$; 28 weeks, $P = 0.11$; sporozoite IFA: 2 weeks, $P = 0.19$; 28 weeks, $P = 0.05$; two-tailed t test, log-transformed titers). We further asked whether the observed antibody responses were associated with protection. We compared the geometric mean titers in all protected, immunized mice with those in all nonprotected, immunized mice; titers in protected mice were modestly, but significantly, higher in the protected mice in all three assays (CSP EIA, 32,961 versus 15,624, $P = 0.045$; peptide EIA, 1,462 versus 452, $P = 0.015$; sporozoite IFA, 70,797 versus 38,343, $P = 0.039$; two-tailed t test on log-transformed data). A similar analysis of T-cell responses and protection at the level of individual mice was not possible because the T-cell assays required the sacrifice of a subset of the animals from each group prior to challenge.

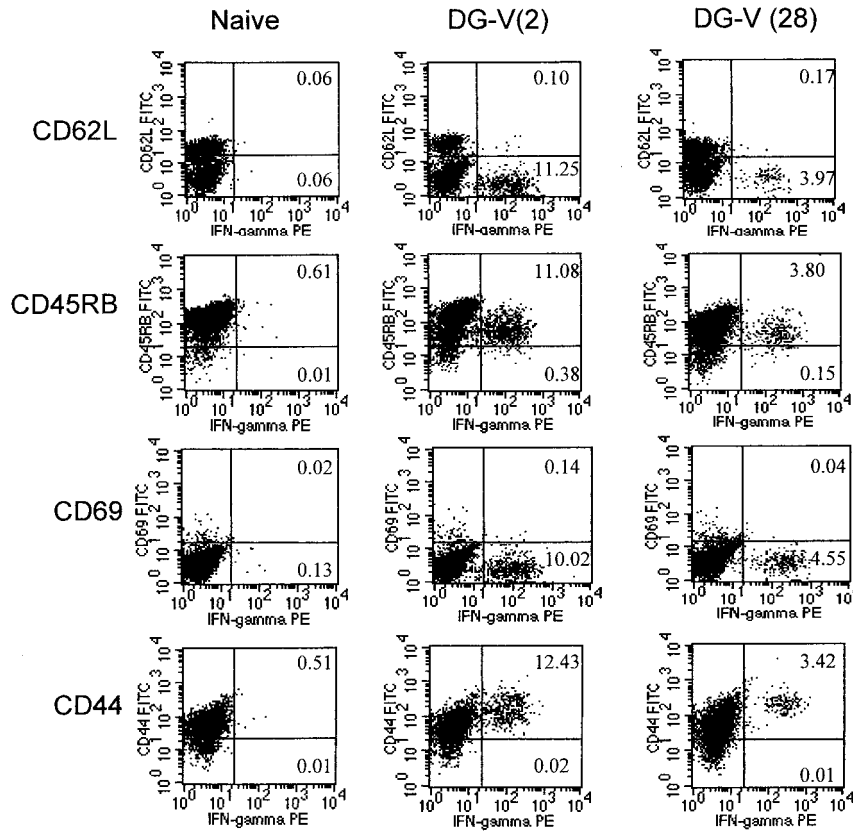


FIG. 3. Phenotype of IFN- γ -producing cells. Spleen cells from 3 mice per group obtained at 2 or 28 weeks postboost were cultured with PyCSP(280-288) peptide-pulsed P815 cells for 16 h, stained with antibodies to CD8, C62L, CD45RB, CD69, and CD44, and stained intracellularly with antibodies to IFN- γ . The phenotypes of IFN- γ -producing cells on gated CD8⁺ T cells were determined individually for each of the 3 mice per group. Representative data from one mouse are shown. PE, phycoerythrin; FITC, fluorescein isothiocyanate.

DISCUSSION

We have characterized the persistence of immunity to malaria induced by a heterologous DNA priming and poxvirus boosting regimen. In the present study, immunization with either the DG-V or dg-V regimen induced 70 to 100% sterile immunity from low-dose sporozoite challenge that persisted essentially unchanged until at least 20 weeks after boosting. Even at 28 weeks, 30 to 40% protection was seen. Further, when a subset of mice that had been protected in the 20-week challenge was rechallenged at 40 weeks, 8 out of 8 were protected. Thus, vaccine-induced protection persists for at least 28 weeks and, perhaps as a result of boosting by sporozoite challenge, may persist for as long as 40 weeks. Similar protection after a delayed challenge was recently observed in the rhesus simian-human immunodeficiency virus (SHIV) system, in which monkeys immunized with a DNA priming and poxvirus boosting regimen were challenged with infectious SHIV 29 weeks after the boost and were protected against AIDS and death, although not against the primary viremia (1). In the SHIV model, memory T cells are stimulated by a high primary viremia and have several weeks in which to develop into activated effectors to control the infection. The *P. yoelii* model is in some respects a more stringent challenge; there is only a 48-h window between challenge and the emergence from the liver of blood-stage parasites which do not express the vaccine target

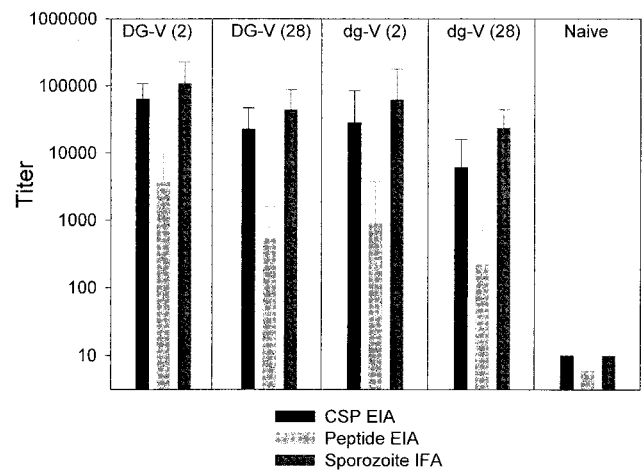


FIG. 4. Antibody responses. Shown are titers for EIA with recombinant PyCSP or synthetic peptide (QGPGAP)₄ or IFA against air-dried *P. yoelii* sporozoites as described in Materials and Methods. For the EIAs, results are reported as 0.5 optical density units (the reciprocal of the serum dilution at which the mean optical density reading is 0.5); for IFA, results are reported as the endpoint of the serum dilution at which immunofluorescence could be detected. Geometric mean titers and standard deviations from groups of 10 mice are shown.

antigen, PyCSP, and which are not affected by pre-erythrocytic stage immune responses. Thus, preexisting memory T cells have less than 2 days to become activated effectors and eliminate the liver-stage infection. It is quite remarkable, then, that 30 to 40% of mice were protected at a time when they did not have detectable effectors in the spleen, as judged by the lack of ex vivo CTL activity. It was therefore of considerable interest to characterize the memory T-cell population present 28 weeks after immunization.

There is clear evidence that protection against challenge with *P. yoelii* sporozoites in BALB/c mice induced by immunization with either irradiated sporozoites or DNA vaccine plasmids is dependent on CD8⁺ T cells and IFN- γ (5, 6, 12), and there is suggestive evidence that the same is true for the DG-V and dg-V regimens described here (14). In a previous study, the best in vitro correlates of protection were the ex vivo CTL assay used in the present study and antigen-specific IFN- γ responses measured by ELISPOT (14). We therefore characterized the CD8⁺-T-cell population responsive to the predominant K^d-restricted epitope from PyCSP, SYVPSAEQI, at early (2 weeks) or late (28 or 30 weeks) times following immunization. Two weeks after immunization, 12 to 20% of CD8⁺ splenocytes produced IFN- γ in response to incubation with SYVPSAEQI; by 28 weeks, this percentage dropped to 3 to 8%. In the absence of major histocompatibility complex class I tetramer-SYVPSAEQI reagents it is impossible to determine whether the reduction in antigen-responsive CD8⁺ T cells is due to their elimination or to their having become unresponsive. It is also possible that they emigrated from the spleen. There are important qualitative differences between the 2-week (effector) and 28- to 30-week (memory) antigen-responsive CD8⁺-T-cell populations. Both populations expressed an effector-memory surface phenotype, CD62L^{lo}, CD69^{lo}, CD45RB^{int}, and CD44^{hi}. Although the 2-week population did not express the transient activation marker, CD69, it can be identified as an effector population based on its ex vivo CTL activity. The lack of CD69 expression in CD8⁺ effectors has also been described in mice responding to infection with lymphocytic choriomeningitis virus (8). The memory population lacked detectable ex vivo CTL activity (Fig. 2b), although it did not significantly differ from the effector population in its activity in a restimulated CTL assay (Fig. 2a). Interestingly, although the percentage of antigen-responsive CD8⁺ cells declined by weeks 28 to 30, the amount of IFN- γ produced by individual responsive cells increased significantly, as judged by the increase in MFI for IFN- γ (Fig. 3b). The ability of the memory CD8⁺ cells to produce large amounts of IFN- γ in response to antigen and their inability to carry out cytolysis in a short-term ex vivo CTL assay is consistent with previous observations of different kinetics for cytokine secretion and cytolysis in memory CD8⁺ cells (2), which may reflect the greater time required for synthesis and assembly of the components for cytolytic granules than for cytokine expression (7). Finally, as has been described for memory cells induced by lymphocytic choriomeningitis virus infection (15), the memory CD8⁺ antigen-responsive cells expressed more surface CD8 than the effector cells (data not shown).

Strong antibody responses were induced by the heterologous immunization (Fig. 4) and declined only moderately between 2 and 28 weeks after immunization. Interestingly, there was a

significant correlation between antibody titer and protection at the level of individual mice. In a previous study of the DG-V regimen, Sedegah et al. found a correlation between protection and the levels of anti-PyCSP or sporozoite antibodies induced by a variety of DNA-based vaccination regimens (14). T-cell responses were well correlated with the antibody titers, and the best correlations with protection were seen with ex vivo CTL activity and IFN- γ ELISPOT activity. It seems likely that in the present study, also, the antibody titers are a surrogate for other cellular immune responses.

The differences between the high- and low-dose priming immunization regimens, DG-V and dg-V, were relatively minor. Antibody and IFN- γ responses were two- to threefold lower with the dg-V regimen. Protection against a 50-sporozoite challenge was similar. The protection induced by DG-V appeared somewhat more robust, as it provided greater protection against a second challenge with 250 sporozoites than did dg-V.

Overall, our data establish that heterologous vaccination by priming with plasmid DNA coadministered with GM-CSF followed by boosting with recombinant poxvirus induced substantial protection that persisted undiminished for 20 weeks and remained significant for at least 28 and perhaps up to 40 weeks. The antigen-specific CD8⁺ memory T cells responsible for this protection lacked measurable ex vivo CTL activity but did produce greater amounts of IFN- γ per cell than the effector cells present 2 weeks after vaccination. A detailed understanding of the development of immunity will depend on additional characterization of the effector and memory CD8⁺ populations throughout the course of immunization and challenge and, at best, will lead to more effective strategies for inducing protective, long-term CD8⁺-T-cell-mediated immunity to malaria and other infectious diseases.

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