Circumventing Genetic Restriction of Protection against Malaria with Multigene DNA Immunization: CD8$^+$ T Cell–, Interferon γ–, and Nitric Oxide–Dependent Immunity

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Summary

Despite efforts to develop vaccines that protect against malaria by inducing CD8$^+$ T cells that kill infected hepatocytes, no subunit vaccine has been shown to circumvent the genetic restriction inherent in this approach, and little is known about the interaction of subunit vaccine–induced immune effectors and infected hepatocytes. We now report that immunization with plasmid DNA encoding the *Plasmodium yoelii* circumsporozoite protein protected one of five strains of mice against malaria (H-2^d, 75%); a *PyHEP17* DNA vaccine protected three of the five strains (H-2^a, 71%; H-2^k, 54%; H-2^d, 26%); and the combination protected 82% of H-2^a, 90% of H-2^k, and 88% of H-2^d mice. Protection was absolutely dependent on CD8$^+$ T cells, IFN-γ, or nitric oxide. These data introduce a new target of protective preerythrocytic immune responses, PyHEP17 and its *P. falciparum* homologue, and provide a realistic perspective on the opportunities and challenges inherent in developing malaria vaccines that target the infected hepatocyte.

Immunization with radiation-attenuated *Plasmodium* spp. sporozoites induces sterile protective immunity against malaria in rodents, primates, and humans (for review see reference 1). Protection is dependent on CD8$^+$ T cells in some rodent models (2, 3), and CD8$^+$ T cells can protect in the absence of other immune responses (4–7). Immunity is presumably mediated via CD8$^+$ T cell recognition of parasite-derived peptides that are presented on the surface of infected hepatocytes in association with class I MHC molecules; hepatocytes are the only cell in the parasite's life cycle known to express class I MHC molecules. That CD8$^+$ CTLs can recognize an epitope from the *Plasmodium yoelii* circumsporozoite protein (PyCSP)[1] expressed on *P. yoelii*–infected hepatocytes (8), and that MHC-restricted immune-mediated elimination of infected hepatocytes that are expressing parasite Ags can protect against sporozoite-transmitted malaria (9) have been demonstrated. Accordingly, there have been efforts to develop subunit vaccines that induce protective CD8$^+$ T cells. To be effective in a heterogeneous population, however, such vaccines must contain multiple protective CD8$^+$ T cell epitopes restricted by diverse class I MHC alleles (10, 11). Only a few of such epitopes have been identified in mice, and all have been on the H-2^d background (4–7), and subunit vaccine–induced CD8$^+$ T cell–dependent protection has only been demonstrated in H-2^d mice. Furthermore, it is not known whether the CD8$^+$ T cells lyse the infected hepatocytes directly, as classical CTLs (for review see reference 12), or indirectly via mediators such as cytokines that induce the infected hepatocyte to inactivate or kill the parasite. It has been established, however, that IFN-γ is active against infected hepatocytes (2, 6, 13–17). Reports indicate that IFN-γ produced by CD8$^+$ T cells induces infected hepatocytes to produce L-arginine–derived nitrogen oxides that are toxic to the intracellular parasite (18–22).

The irradiated sporozoite vaccine is impractical for large-scale human use, and progress in developing subunit vaccines that induce protection comparable to that induced by the irradiated sporozoite vaccine has been slow (for review see reference 1). We recently reported (23) that immunization of BALB/c mice with plasmid DNA encoding the *PyCSP* induces more potent in vitro cytolytic activity against a nonamer epitope on the *PyCSP* than does immunization with irradiated sporozoites and induces CD8$^+$ T cell–dependent protection. These data raise crucial issues concerning the efficacy of the *PyCSP* DNA vaccine on non-BALB/c backgrounds as a consideration for human

[1] *Abbreviations used in this paper:* CSP, circumsporozoite protein; HEP, hepatocyte erythrocyte protein; PyCSP, *Plasmodium yoelii* CSP; PyHEP17, Plasmodium yoelii hepatocyte erythrocyte protein 17.

trials, and the host effector mechanisms responsible for the DNA-induced protective immunity.

We now report that (*a*) protective immunity induced by the *PyCSP* DNA vaccine is genetically restricted; (*b*) a DNA vaccine based on the gene encoding the recently identified 17-kD liver-stage protein designated *P. yoelii* hepatocyte erythrocyte protein (PyHEP17) (24) induces protection with a different pattern of genetic restriction; (*c*) immunization with the *PyCSP–PyHEP17* DNA combination solidly protects mice of three genetic backgrounds, circumventing the genetic restriction of protection to the individual components; and (*d*) the protective immunity directed against the parasite developing within hepatocytes is completely eliminated by in vivo depletion of $CD8^+$ T cells, treatment with anti–IFN-γ, or inhibition of nitric oxide production.

Materials and Methods

Construction of Plasmid DNA. The nkCMVintpolyli vector has been described previously (23); expression of the encoded gene is driven by a CMV immediate/early gene promoter. The *PyHEP17* DNA vaccine was constructed by cloning a 342-bp cDNA fragment representing the complete exon 1 plus 57% of exon 2 of *PyHEP17* (Doolan, D.L., R.C. Hedstrom, W.D. Rogers, Y. Charoenvit, M. Rogers, P. de la Vega, and S.L. Hoffman, manuscript submitted for publication) into the PstI site of nkCMVintpolyli. This construct does not include (Charoenvit, Y., unpublished data) the region of the PyHEP17 protein recognized by the mAb, NYLS3, used to identify PyHEP17 (24). The *PyCSP* DNA vaccine has been previously described (23).

In Vitro Expression. Expression of the *PyHEP17* plasmid DNA was assessed in vitro by two methods. First, COS cells (American Type Culture Collection [ATCC], Rockville, MD) were transfected with 10 ng of plasmid DNA by calcium phosphate coprecipitation (25). mRNA was extracted from the transfected COS cells with the Micro FastTrack mRNA isolation kit (Invitrogen, San Diego, CA). Reverse transcription (RT)–PCR was then performed using each of oligo(dT), random hexamer– and specific oligonucleotide–primed and DNAase (Boehringer Mannheim Corp., Indianapolis, IN)–treated first-strand cDNA as the template (Superscript Preamplification System for First-Strand cDNA synthesis; GIBCO BRL, Gaithersburg, MD). RT–PCR revealed that fragments of a size corresponding to the cDNA representing exon 1 and 57% of exon 2 of *PyHEP17* were amplified and were smaller than fragments amplified from genomic DNA template. The amplified fragments hybridized to *PyHEP17*-specific probes (data not presented). Second, sera from mice immunized with the *PyHEP17* DNA vaccine recognized the transfected COS cells, as demonstrated by the indirect fluorescent Ab test (data not presented).

Mice and Parasites. Female 6- to 8-wk-old BALB/cByJ, A/J, B10.BR, B10.Q, and C57BL/6 mice (the Jackson Laboratory, Bar Harbor, ME) and outbred CD-1 mice (Charles River Laboratories, Wilmington, MA) were used in all studies. *P. yoelii* (17X NL nonlethal strain, clone 1.1) was maintained by alternating passages of the parasites in *Anopheles stephensi* mosquitoes and CD-1 mice. Sporozoites were obtained 14 d after an infectious blood meal by hand-dissection of *P. yoelii* 17X NL–infected mosquito glands in M199 medium containing 5% normal mouse serum (Rockland, Inc., Gilbertsville, PA) as previously described (3) and diluted to a final concentration of 100 infectious sporozoites per 0.2-ml vol. Erythrocytic-stage parasites were obtained from heparinized blood of a BALB/CByJ mouse 10 d after a previous infection, washed in PBS, and diluted to a final concentration of 200 infected erythrocytes per 0.2-ml vol, calculated on the basis of parasitemia at the time of harvest.

Immunizations and Challenges. Female 6- to 8-wk-old mice were immunized three times at 3-wk intervals intramuscularly in each tibialis anterior muscle with 50 μg of *PyHEP17* DNA in 50 μl of saline, 20 μg of *PyCSP* DNA, a mixture of 50 μg of *PyHEP17* DNA and 20 μg of *PyCSP* DNA, or unmodified nkCMVintpolyli plasmid. 2 wk after the third immunization, mice were challenged by tail-vein injection with 100 infectious sporozoites (~50 ID_{50}; reference 26) or 200 infected erythrocytes. Giemsa-stained blood smears were examined on days 5–14, up to 50 oil-immersion fields being scanned for parasites. Protection was defined as the complete absence of blood-stage parasitemia.

Antibodies. Purified rat Ig was purchased from Rockland, Inc. The anti-$CD4^+$ mAb GK1.5 (rat IgG2a) was obtained from ATCC (TIB207) and has been previously described (27). The anti-$CD4^+$ mAb YTA3.1.2 (rat IgG2b) was provided by Dr. H. Waldmann and has been previously described (28). The anti-$CD8^+$ mAb 2.43 (mouse IgG2a) was obtained from ATCC (TIB210) and has been previously described (29). The anti–IFN-γ mAb XMG-6 (rat IgG1) was provided by Dr. F. Finkelman and has been previously described (30). All Igs were purified from ascites (Harlan Bioproducts for Science, Indianapolis, IN) by 50% ammonium sulphate precipitation, and final Ab concentrations were determined by optical density.

Depletions. Immunized mice were treated as follows. Undepleted: On days −7, −6, −5, −4, −3, −2, and 0 (relative to challenge with 100 *P. yoelii* sporozoites on day 0), mice received a single intraperitoneal dose of 1.3 mg of purified rat Ig. $CD4^+$ in 0.2 ml PBS T cell depletion: On days −7, −6, −5, −4, −3, −2, 0, and +2, mice received a single intraperitoneal dose of 1.0 mg and 0.3 mg, respectively, of the anti-$CD4^+$ mAbs GK1.5 and YTA3.1.2. $CD8^+$ T cell depletion: On days −5, −4, −3, −2, and 0, mice received a single intraperitoneal dose of 0.5 mg of the anti-$CD8^+$ mAb 2.43. Treatment with anti-IFN-γ: On days −4, −3, −2, −1 and +2, mice received a single intraperitoneal dose of 1.0 mg of the anti–IFN-γ mAb XMG-6. Treatment with aminoguanidine: Twice daily, commencing 24 h before sporozoite challenge and continuing for 72 h after challenge, mice were administered 50 mg aminoguanidine (Sigma Chemical Co., St. Louis, MO) per kg body wt in 0.5 ml PBS via gastric lavage.

FACScan® (Becton Dickinson & Co.). The extent of Ab depletion was determined by performing single-color FACS®. Approximately 10^6 cells of the population to be analyzed were stained with either anti-$CD8^+$ FITC or anti-$CD4^+$ FITC (Pharmingen, San Diego, CA) for 1 h at 4°C. Unstained and FITC controls were included for each sample. Stained cells were washed three times, resuspended in paraformaldehyde (0.5% vol/vol), and stored at 4°C until analyzed. FACScan® (FAX 4000 Royal; Becton Dickinson Immunocytometry Systems, San Jose, CA) was used for analysis of the samples.

Serology. Mice were bled for serum ~2 wk after each immunization. Sera were assayed by indirect fluorescent Ab test against air-dried *P. yoelii* sporozoites, liver-stage parasites, or blood-stage parasites as previously described (31).

Results

PyCSP DNA Vaccine Protects against Malaria on a Single Genetic Background. Both MHC and non-MHC genes con-

trol protection against *P. yoelii* in mice (32). Immune responses to *P. falciparum*, *P. vivax*, *P. yoelii*, and *P. berghei* $CD8^+$ and $CD4^+$ T cell epitopes and B cell epitopes are also known to be genetically restricted (for review see reference 33). Previously, we reported that BALB/c mice are protected against sporozoite challenge by immunization with *PyCSP* DNA (23). We wanted to assess the capacity of *PyCSP* DNA to confer protection in mice of other genetic backgrounds. Five inbred mouse strains were selected for the immunization studies on the basis of differences in both genetic background and H-2 haplotypes: BALB/cByJ ($H\text{-}2K^dI^dD^dL^d$); A/J ($H\text{-}2K^kI^kD^dL^d$); B10.BR/SgSnJ ($H\text{-}2K^kI^kD^kL^k$); B10.Q/SgJ ($H\text{-}2K^qI^qD^qL^q$); and C57BL/6J, ($H\text{-}2K^bI^bD^bL^b$). Only in BALB/c mice was protection >20% (Table 1).

The CSP is a principal candidate Ag for preerythrocytic vaccine development (34). The data presented here predict that anti-CSP $CD8^+$ T cell responses alone will, however, be inadequate to protect an outbred human population. A vaccine designed to protect humans by inducing $CD8^+$ T cells that recognize 8–10 amino acid peptides complexed with class I HLA molecules on infected hepatocytes will therefore have to include epitopes from multiple parasite proteins.

PyHEP17 DNA Vaccine Protects against Malaria on Multiple Genetic Backgrounds. Localization of the recently identified liver- and blood-stage Ag PyHEP17 in *P. yoelii*–infected hepatocytes (24) suggested that, like the CSP, this Ag may be a target of protective T cell responses directed at the infected hepatocyte. To investigate this, in vivo protection studies were carried out with a *PyHEP17* DNA vaccine in the same five inbred mouse strains differing in genetic background and H-2 haplotype studied with the *PyCSP* DNA vaccine. Immunization with *PyHEP17* DNA partially protected three of the five strains >20% against challenge with 100 infectious sporozoites (Table 1). A 2 to 6-d delay in the onset of parasitemia in some nonprotected mice (data not presented) was consistent with partial immunity, which eliminated up to 90% of infected hepatocytes.

Outbred CD-1 mice were subsequently studied. Consistent with the range of protection in the inbred strains. 40% (8 of 20) of the CD-1 mice immunized with three doses of 100 μg *PyHEP17* DNA were protected against sporozoite challenge.

Bi-gene DNA Immunization Circumvents the Genetic Restriction of Protection of the Univalent DNA Vaccines. Data indicated that protective immunity after immunization with either *PyHEP17* or *PyCSP* plasmid DNA is genetically restricted: A/J mice are high responders to *PyHEP17* and low responders to *PyCSP*, and BALB/c mice are high responders to *PyCSP* and low responders to *PyHEP17*; B10.BR mice are moderate responders to *PyHEP17* and low responders to *PyCSP*; B10.Q and C57BL/6 mice are low or nonresponders to both Ags. We therefore determined whether immunization of mice with a *PyHEP17–PyCSP* DNA combination could enhance the protection and/or protect on more than one genetic background. Both high responder

Table 1. *Genetic Restriction of the Protective Immunity Induced by Immunization with PyHEP17DNA, PyCSPDNA, or the Combination*

DNA vaccine	Strain	H-2 haplotype	IFAT titers (1/x)	No. protected out of no. challenged	Percent protection
PyCSP	BALB/c	d	10,240	18 of 24	75
	A/J	a	10,240	6 of 32	19
	B10.BR	k	320–640	1 of 12	8
	B10.Q	q	40–160	0 of 7	0
	C57 BL/6	b	10,240	0 of 17	0
PyHEP17	BALB/c	d	0–160	9 of 34	26
	A/J	a	0–40	25 of 35	71
	B10.BR	k	0–80	20 of 37	54
	B10.Q	q	0–80	2 of 12	17
	C57 BL/6	b	160–640	0 of 19	0
PyHEP17 + *PyCSP*	BALB/c	d		15 of 17	88
	A/J	a		22 of 27	82
	B10.BR	k		27 of 30	90
	B10.Q	q		1 of 7	14
	C57 BL/6	b		0 of 7	0

Data represent accumulated results of up to four separate experiments with a total of 585 mice. All naive mice (149 mice) and all control mice immunized with the nkCMVintpolyli plasmid (119 mice) became infected.
PyHEP17 antibody titers were assessed by IFAT against *P. yoelii* blood-stage parasites. PyCSP Ab titers were assessed by IFAT against *P. yoelii* sporozoites. Data represent the range of titers obtained from individual mouse sera.

strains (88% of BALB/c, H-2^d and 82% of A/J, H-2^a) and 90% of the moderate responder B10.BR (H-2^k) mice (Table 1) were protected with the combination. Although no C57BL/6 (H-2^b) and only 14% of B10.Q (H-2^q) mice were protected, in most mice a delay in the onset of parasitemia of up to 4 d was noted with the combination, compared with either of the vaccines alone (data not presented).

Thus, bivalent DNA immunization protected mice of diverse genetic backgrounds and H-2 haplotypes, circumventing the genetic restriction of protection found after immunization with the *PyCSP* or *PyHEP17* DNA vaccines delivered alone. The results are not related to the dosage of DNA, since the quantity of DNA used in the combination experiments reported here (100 μg *PyHEP17* plus 40 μg *PyCSP*) is lower than that used in previous studies with either the *PyHEP17* DNA (200 μg) or *PyCSP* DNA (200 μg), where no effect of the increased DNA dose was observed (data not presented).

DNA-induced Protection Is Dependent upon CD8$^+$ T Cells. The protective immune responses induced by the *PyHEP17* DNA vaccine must be directed against the infected hepatocyte. First, immunity cannot be directed against sporozoites in the circulation, since PyHEP17 is not expressed in sporozoites and is first expressed within 6 h of sporozoite invasion of hepatocytes (24). Second, immunity is not directed against the erythrocytic stage of the parasite life cycle, which commences when the parasites are released from infected hepatocytes, since there was no protection against challenge with 200 infected erythrocytes (data not presented). Third, sera from mice immunized with the *PyHEP17* DNA did not recognize *P. yoelii* liver-stage parasites and recognized *P. yoelii* erythrocytic-stage parasites by IFAT only at very low levels (Table 1), and there was no correlation between Ab titers and protection against sporozoite challenge (Table 1).

To determine whether a specific subset of T cells was required for the DNA-induced protection, we conducted in vivo depletion studies. In six of the eight different groups of mice, CD4$^+$ T cell depletion did not lead to any reduction in protection (Table 2). However, in B10.BR mice immunized with *PyHEP17* DNA, protection was reduced from 75 to 50% by CD4$^+$ T cell depletion, and in BALB/c mice immunized with the *PyHEP17–PyCSP* DNA combination, protection was reduced from 80 to 40% after CD4$^+$ depletion (Table 2). These data may reflect the variability of the protection after DNA immunization in general or may be indicative of a role for CD4$^+$ T cells in DNA vaccine–induced protection in these mice. Since the reduction only occurred in two of the eight groups, and the differences were not statistically significant (Fisher's exact *P* value: B10.BR mice immunized with the *PyHEP17* vaccine, 0.377; BALB/c mice immunized with the combination vaccine, 0.1698), we favor the former explanation. In marked contrast, however, CD8$^+$ T cell depletion completed eliminated protection in all groups of immunized mice (Table 2). Therefore, protective immunity after immunization with *PyHEP17* DNA and with the *PyHEP17–PyCSP* DNA combination is entirely dependent on CD8$^+$ T cells. Consistent with this finding, MHC-restricted Ag-specific CD8$^+$ CTLs are induced in mice by *PyHEP17* DNA immunization (Doolan, L.D., unpublished data).

DNA-induced Protection Is Dependent upon IFN-γ and Nitric Oxide. We reasoned that DNA vaccine-induced anti-PyCSP and PyHEP17 CD8$^+$ T cells are eliminating infected hepatocytes, as demonstrated with irradiated sporozoite–induced CD8$^+$ T cells (8, 9). In vitro and in vivo data indicate that IFN-γ produced by malaria-specific CD8$^+$ T cells stimulate *Plasmodium*-infected hepatocytes to eliminate the parasite by producing nitric oxide (19, 20, 22). To determine whether the *PyHEP17*, *PyCSP*, or *PyHEP17-PyCSP* combination DNA vaccines induce the same pathway, B10.BR mice were immunized and treated before sporozoite challenge with either anti–IFN-γ mAb or aminoguanidine, an

Table 2. *Elimination of Protective Immunity by In Vivo Depletion of CD8$^+$ T Cells*

DNA vaccine	Strain	Percent protection (no. protected out of no. challenged)					
		Undepleted		CD4$^+$ depleted		CD8$^+$ depleted	
PyHEP17	A/J	43	(3 of 7)	78	(7 of 9)	0	(0 of 9)
	BALB/c	22	(2 of 9)	22	(2 of 9)	0	(0 of 9)
	B10.BR	75	(9 of 12)	50	(5 of 10)	0	(0 of 10)
PyCSP	A/J	11	(1 of 9)	11	(1 of 9)	0	(0 of 9)
	BALB/c	56	(5 of 9)	56	(5 of 9)	0	(0 of 9)
PyHEP17 + PyCSP	A/J	80	(8 of 10)	90	(9 of 10)	0	(0 of 10)
	BALB/c	80	(8 of 10)	40	(4 of 10)	0	(0 of 10)
	B10.BR	90	(9 of 10)	90	(9 of 10)	0	(0 of 10)

One mouse per group was killed on the day of challenge to assess depletion with FACScan. The efficiencies of CD4$^+$ and CD8$^+$ T cell depletion were estimated as 99 and 96%, respectively.

Table 3. *Elimination of Protective Immunity by In Vivo Treatment with Anti–IFN-γ or Aminoguanidine*

Vaccine	Strain	Percent protection (no. protected out of no. challenged)		
		Undepleted	IFN-γ depleted	iNOS depleted
PyHEP17	B10.BR	75 (9 of 12)	0 (0 of 10)	0 (0 of 10)
PyHEP17 + PyCSP	B10.BR	90 (9 of 10)	0 (0 of 10)	0 (0 of 10)

inhibitor of the inducible isoform of nitric oxide synthase (iNOS) (35). The results show that there is an absolute requirement for both IFN-γ and nitric oxide in the DNA vaccine–induced protective immunity (Table 3).

Discussion

The data presented here identify PyHEP17 (24) as a new target of protective immune responses directed against the infected hepatocyte and systematically define the process whereby effector $CD8^+$ T cells induced by immunization with a single gene or bi-gene DNA malaria vaccine recognize the infected hepatocyte and eliminate the capacity of that cell to sustain a viable plasmodial infection. The data also emphasize the absolute requirement for such a vaccine to induce protective T cell responses against multiple parasite proteins if it is to be effective in a heterogeneous population.

Genetic restriction of protective $CD8^+$ T cell responses is expected, since the T cell receptor can only recognize its target peptide when that 8–10-amino acid peptide is complexed with a class I MHC molecule, with specific amino acid restrictions on the formation of that complex (for review see reference 36). Nonetheless, we were surprised that immunization with the *PyCSP* DNA vaccine only protected one of five strains of inbred mice (see Table 1). The *PyCSP* gene encodes a protein of 391 amino acids and we expected, apparently inappropriately, that the *PyCSP* would contain protective $CD8^+$ T cell epitopes recognized by class I MHC molecules from multiple strains of mice. Inbred mice are different from outbred humans; however, this finding certainly suggests that it may be inappropriate to construct a human malaria vaccine designed to induce protective $CD8^+$ T cell responses directed against only the CSP.

The studies presented here demonstrate that it is possible to circumvent genetic restriction of $CD8^+$ T cell–mediated protective immunity against individual *Plasmodium* spp. proteins expressed in infected hepatocytes by immunizing with more than one protein. This may appear obvious, but there are only a few examples of this phenomenon after immunization with subunit vaccines (37–39). Furthermore, immunization with two peptides representing defined $CD8^+$ CTL epitopes on the CSP often leads to less cytolytic activity against either one than does immunization with the individual peptide (Franke, E.D., personal communication). Additional work is required to determine whether additive protection will be obtained with all DNA vaccines, or whether it is restricted to the responses to certain Ags.

The irradiated sporozoite vaccine has been the model and gold standard for preerythrocytic stage malaria vaccine development. In BALB/c mice, immunization with irradiated sporozoites, but not with DNA, can confer 100% sterile protective immunity against massive sporozoite challenge (Sedegah, M., unpublished data). In contrast, Weiss et al. (32) reported that only 31% of B10.BR mice were protected against challenege with 5,000 *P. yoelii* sporozoites after immunization with irradiated sporozoites; this protection was not dependent on $CD8^+$ T cells. Here, we report that the *PyCSP–PyHEP17* combination protected 90% of B10.BR mice against challenge with 100 *P. yoelii* sporozoites after DNA immunization with the *PyCSP–PyHEP17* combination (see Table 1); this protection was completely dependent on $CD8^+$ T cells (see Table 2). Therefore, in B10.BR mice, the bi-gene DNA vaccine may provide better $CD8^+$ T cell–dependent protection against *P. yoelii* than does the whole organism vaccine. It is unlikely that the bi-gene DNA vaccine contains more $CD8^+$ T cell epitopes recognized by $H\text{-}2^k$ B10.BR mice than does the irradiated sporozoite vaccine, or that "cryptic" epitopes are exposed by DNA immunization. It is more probable that, as demonstrated for *PyCSP* in $H\text{-}2^d$ BALB/c mice (23), the DNA vaccine is inducing a much more potent $CD8^+$ T cell response against epitopes presented by both systems than is the irradiated sporozoite vaccine. Certainly, such protection may be better than that afforded by repeated exposure to malaria in the field, where sterile immunity does not occur (40) and malaria-specific $CD8^+$ CTL responses are low (41–43).

Despite a large literature referring to protective $CD8^+$ CTLs (for review see reference 1), the phenomenon of classical cytolytic activity with direct contact between the effector T cell and the infected target cell and subsequent lysis of the target cell by $CD8^+$ CTLs (for review see reference 12) has never been established in malaria. Although CSP- and sporozoite surface protein 2–specific $CD8^+$ T cell clones with cytolytic activity can adoptively transfer protection (4–7), it is not known whether the activity against the infected hepatocytes is cytolytic. In fact, it has been shown that the protective activity of one such anti-PyCSP CTL clone is abrogated by in vivo treatment of mice with anti–IFN-γ (6), and that in two strains of mice, the protective immunity induced by immunization with irradiated *P. berghei* sporozoites is reversed by in vivo treatment of the mice with anti–IFN-γ (2, 22). The data presented here suggest that the activity of protective $CD8^+$ T cells against *Plasmodium*-infected hepatocytes is not via a

classical cytolytic mechanism. The protection is completely reversed by in vivo depletion of $CD8^+$ T cells, by treatment with anti–IFN-γ, or by inhibiting the inducible isoform of nitric oxide synthase. We interpret these findings to indicate that the $CD8^+$ T cell activated by interacting with the MHC–peptide complex on the surface of the infected hepatocyte secretes IFN-γ. The IFN-γ then induces the infected hepatocyte to produce nitric oxide that renders the parasite noninfectious. This may in part explain the multitude of reports in both rodent and human systems showing that the induction of CTLs against *Plasmodium* spp. proteins does not correlate with protection of the host (44–48).

This study lays the foundation for multigene DNA vaccines (for reviews see references 49 and 50) designed to attack infected human hepatocytes and identifies the newly discovered *P. falciparum* homologue of PyHEP17 (Doolan, D.L., et al. manuscript submitted for publication) as a candidate component of any such multivalent vaccine. However, it must be placed in perspective. Some strains of mice were not protected, and all challenge work was done with a parasite clone. If there is any hope of producing long-lasting protection in heterogeneous human populations, additional genes will have to be included to overcome the MHC restriction of T cell responses and the antigenic polymorphism of the parasite at T cell epitopes. The exquisite dependency of this protection on each of the individual effectors—$CD8^+$ T cells, IFN-γ, and nitric oxide—underscores the long-term complexity of developing an effective hepatocyte-targeted malaria vaccine. It seems obvious that an effective vaccine that functions by killing infected hepatocytes will have to be as complex and elegant as its parasite opponent.

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