INFECTION AND IMMUNITY, May 2005, p. 2863–2872 Vol. 73, No. 5
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.5.2863–2872.2005

Boosting of DNA Vaccine-Elicited Gamma Interferon Responses in Humans by Exposure to Malaria Parasites

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Received 23 August 2004/Returned for modification 13 October 2004/Accepted 11 December 2004

A mixture of DNA plasmids expressing five *Plasmodium falciparum* pre-erythrocyte-stage antigens was administered with or without a DNA plasmid encoding human granulocyte-macrophage colony-stimulating factor (hGM-CSF) as an immune enhancer. After DNA immunization, antigen-specific gamma interferon (IFN-γ) responses were detected by ELISPOT in 15/31 volunteers to multiple class I- and/or class II-restricted T-cell epitopes derived from all five antigens. Responses to multiple epitopes (≤7) were detected simultaneously in some volunteers. By 4 weeks after challenge with *P. falciparum* parasites, 23/31 volunteers had positive IFN-γ responses and the magnitude of responses was increased from 2- to 143-fold. Nonetheless, none was protected against malaria. Volunteers who received hGM-CSF had a reduced frequency of IFN-γ responses to class I peptides compared to those who only received plasmids expressing *P. falciparum* proteins before challenge (3/23 versus 3/8; $P = 0.15$) or after parasite challenge (4/23 versus 5/8; $P = 0.015$) but not to class II peptides before or after challenge. The responses to one antigen (*P. falciparum* circumsporozoite protein [PfCSP]) were similar among volunteers who received the five-gene mixture compared to volunteers who only received the PfCSP DNA plasmid in a previous study. In summary, DNA-primed IFN-γ responses were boosted in humans by exposure to native antigen on parasites, coadministration of a plasmid expressing hGM-CSF had a negative effect on boosting of class I-restricted IFN-γ responses, and there was no evidence that immunization with PfCSP DNA in the mixture reduced T-cell responses to PfCSP compared to when it was administered alone.

The development of a malaria vaccine is one of the highest priorities in infectious disease research, as such a vaccine could be enormously helpful in reducing the 500 million new *Plasmodium* infections and 1 to 3 million deaths due to malaria annually. Administration of radiation-attenuated *Plasmodium falciparum* sporozoites by the bite of infected mosquitoes provides >90% protection against *P. falciparum* for at least 10 months (14). It is likely that this excellent protective immunity is directed against many antigens expressed at the sporozoite and liver stages of the parasite life cycle (13). In contrast, almost all malaria vaccine development efforts have been directed against a single antigen, such as the *P. falciparum* circumsporozoite protein (PfCSP), or a mixture of a few antigens. This approach has not resulted in protection closely comparable to that elicited by the irradiated sporozoite vaccine (21). For this reason we have focused efforts on developing a vaccine that elicits antibody and T-cell responses against multiple parasite antigens from the sporozoite, liver, and asexual erythrocyte stages of the parasite life cycle (5, 16). Because of the ease of construction and early promising animal studies in mice and monkeys by others (10, 15, 22, 25, 26, 32) and our group (5, 23, 27–30), we have focused on DNA vaccine technology as a primary component of our vaccine development strategy. We have demonstrated that a single-gene DNA vaccine encoding the *P. falciparum* circumsporozoite protein (PfCSP) is well tolerated and induces $CD8^+$ T-cell-dependent cytotoxic T lymphocytes (CTLs) and Tc1 gamma interferon (IFN-γ) responses in humans (28, 29) and that immune responses can be improved by sequential immunization with DNA and recombinant protein (9, 30). However, the antibody and cellular responses elicited in humans by DNA vaccines alone have not been optimal. We have shown that coadministration of a plasmid expressing murine granulocyte-macrophage colony-stimulating factor (GM-CSF) enhances the immunogenicity and protective efficacy against malaria in mice of a *Plasmodium yoelii* CSP DNA vaccine when administered alone (33) or as part of a sequential immunization regimen in which the first dose is PyCSP DNA and the second dose is a recombinant poxvirus expressing PyCSP (24).

In this study, we immunized human volunteers with a mixture including five DNA plasmids expressing different *P. falciparum* pre-erythrocyte-stage antigens called MuStDO 5 (Multi-Stage DNA Vaccine Operation 5 antigens). In an attempt to improve T-cell responses, a plasmid encoding human GM-CSF (hGM-CSF) (20) was added to the mixture. We administered to volunteers three doses of the mixture of *P. falciparum* plasmids with no, 20 μg, 100 μg, or 500 μg of the

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TABLE 1. *P. falciparum*-specific MHC class I-restricted CTL epitopes tested in the study

MuStDO5 component or other parameter	No. of peptides restricted by each HLA allele							No. of CTL epitopes tested/antigen
	A1	A2	A3/11	B7	B8	B35	B53	
PfCSP	1	4	1	1	1	1		9
PfSSP2		3	1	1	1			6
PfEXP-1		3	1					4
PfLSA-1		2	4	1		1	1	9
PfLSA-3		3			1		2	6
No. of CTL epitopes tested/HLA allele	1	15	7	3	3	2	3	34[a]
No. of volunteers tested/HLA allele	10	15	19	7	5	6	4	
No. of control volunteers tested/HLA allele	2	4	7	3	1	4	2	

[a] Total number of CTL epitopes tested in this study (1, 4).

plasmid expressing hGM-CSF. To determine if malaria infection could boost the immune responses induced by multivalent DNA vaccine, all volunteers were exposed to five *P. falciparum*-infected mosquitoes' bites after three doses of DNA immunization. IFN-γ responses of peripheral blood mononuclear cells (PBMCs) were assessed before and after immunization and challenge.

MATERIALS AND METHODS

DNA vaccines. We assessed the safety, immunogenicity, and protective efficacy against sporozoite challenge of a native sequence, multigene DNA vaccine in phosphate-buffered saline, with each 1-ml dose containing 500 μg each of five plasmids encoding *P. falciparum* (3D7 strain) antigens PfCSP, *P. falciparum* sporozoite surface protein 2 (PfSSP2), *P. falciparum* exported protein 1 (PfEXP-1), N′ C terminus of *P. falciparum* liver-stage antigen 1 (PfLSA-1), and *P. falciparum* liver-stage antigen 3 (PfLSA-3). All six plasmids expressed in the DNA vector VCL-25 were manufactured by Vical, Inc. (San Diego, CA).

Human volunteers and study design. This was a phase 1/2a clinical trial conducted at the Naval Medical Research Center (NMRC) Malaria Program Clinical Trials Center. Malaria sporozoite challenge was performed at the Walter Reed Army Institute of Research Insectary in Silver Spring, MD. The protocol for this study was approved by the NMRC Institutional Review Board and the U.S. Navy Surgeon General in accordance with U.S. Navy regulations (SECNAVINST3900.39B) governing the use of human subjects in medical research. A total 47 healthy adult male and female and malaria-naïve volunteers were enrolled in this phase 1/2a trial. All were negative for antibodies to five *P. falciparum* DNA vaccine components (CSP, SSP2, EXP-1, LSA-1, and LSA-3), human immunodeficiency virus (HIV), hepatitis B virus core antigen, hepatitis C virus, and double-stranded DNA. HLA class I and class II typing was carried out (data not presented). Volunteers who did not express HLA alleles for which *P. falciparum*-specific peptides were available were excluded. Forty-seven volunteers were enrolled in the study: 32 volunteers were vaccinated with MuStDO 5 alone (8 volunteers) or with MuStDO 5 plus one of the three doses of hGM-CSF-encoding plasmid (24 volunteers); 13 volunteers served as unvaccinated controls; and 2 additional volunteers were enrolled after the completion of vaccination in order to replace two control volunteers who withdrew prior to challenge.

HLA types were distributed evenly among the four cohorts, due to the requirement for measuring genetically restricted T-cell responses to the vaccine and the limited repertoire of HLA class I alleles for which strongly binding peptides had been identified from the five antigens (Table 1). The distribution was as follows: HLA-A*0201 (15 HLA-A*0201-restricted peptides available from the five antigens; at least three vaccinees and one control per cohort); HLA-A*0301 or -A*1101 (seven HLA-A*0301/*1101-restricted peptides available; at least two vaccinees and one control per cohort); HLA-A*0101, -B*0701, -B*0801, -B*3501, and -B5301 (total of 12 peptides restricted by these five HLA-A and -B alleles available; as many as possible to fill the remaining slots).

Vaccinees in cohort 1 received 2,500 μg of the pentavalent DNA vaccine mixture (500 μg of each *P. falciparum* protein expressed in plasmid), and vaccinees in cohorts 2, 3, and 4 received 2,500 μg of the mixture of five DNA plasmids plus 20, 100, or 500 μg of plasmid encoding recombinant hGM-CSF, respectively. Each formulation was administered intramuscularly by jet injection (Biojector; Bioject, Inc.) at 0, 4, and 8 weeks. All vaccinees and control volunteers were challenged by exposure to five *P. falciparum* (3D7 clone)-infected mosquitoes 18 days after the third immunization, as described previously (14). One volunteer in cohort 3 withdrew from the study after the first immunization due to a family member request. One control volunteer in cohort 1 and one control volunteer in cohort 4 did not want to participate in the parasite challenge study, and they were replaced with two backup control volunteers.

The primary objective in this study was to assess the safety, immunogenicity, and protective efficacy of the *P. falciparum* multivalent pre-erythrocyte-stage DNA vaccine in malaria-naïve individuals. The focus of this report was to demonstrate that a multivalent malaria DNA vaccine could induce T-cell responses in humans and that the DNA-induced T-cell responses could be boosted by parasite challenge. Antigen-specific T-cell responses were evaluated by determining IFN-γ-producing cells by ELISPOT assays. The safety, antibody, and protective efficacy results will be reported elsewhere.

Synthetic peptides. A total of 51 synthetic peptides were studied in this trial, including defined $CD4^+$ or $CD8^+$ T-cell epitopes expressed on five *P. falciparum* DNA vaccine components, based on the sequence of clone 3D7. Of 51 peptides, 34 included the defined major histocompatibility complex (MHC)-restricted $CD8^+$ T-cell epitopes at 8 to 10 amino acids in length mentioned above (1, 4), and 17 peptides included defined or potential $CD4^+$ T-cell epitopes at 20 to 23 amino acids in length (see Table 3, below, which lists the peptide sequences positively responded to by vaccinees) (4, 7). All peptides were purchased from AnaSpec (San Jose, CA) and were at >90% purity. Of the 34 defined class I-restricted epitopes tested in this study, 9 were derived from CSP, 6 were from SSP2, 4 were from EXP-1, 9 were from LSA-1, and 6 were from LSA-3; they are restricted by seven different HLA-A and -B alleles or allelic supertypes (Table 1). Of 17 HLA-DR-restricted epitopes, 4 were derived from CSP, 3 were from SSP2, 2 were from EXP-1, 5 were from LSA-1, and 3 were from LSA-3. As previously described (29, 30), peptides containing a known HLA-A*0201-restricted epitope from influenza virus matrix protein (residues 58 to 66 [GILGFVFTL]) and a known HLA-A*0301-restricted epitope from influenza virus nuclear protein (residues 265 to 273 [ILRGSVAHK]) were used as positive controls, and a peptide containing a known HLA-A*0201-restricted epitope from the HIV gag protein (residues 77 to 85 [SLYNTVATL]) was used as a negative control.

Quantification of IFN-γ-producing cells by ELISPOT assay. ELISPOT assays were first conducted using fresh PBMCs collected preimmunization (day zero), 2 weeks after the second immunization, and 2 weeks after the third immunization, by stimulating with individual peptides. After the trial was completed, we performed additional ELISPOT assays with blinded, frozen PBMCs from preimmunization (day zero), prechallenge (approximately 1 week prechallenge), and samples from 4 weeks and 9 weeks after the parasite challenge simultaneously, this time stimulating with 15 separate peptide pools due to a limited availability of PBMCs. Peptides were pooled on the basis of their antigenic specificity and MHC restriction, with three distinct sets of peptides defined for each of the five vaccine antigens: one set included defined HLA-A*0201-restricted epitopes, the second set included non-A2 class I-restricted epitopes (HLA-A*0101, -A*0301, -B*0701, -B*0801, and -B*3501/*5301 alleles), and the third set included HLA-DR-restricted epitopes. For each assay, the number of antigen-specific IFN-γ-producing cells was determined by ELISPOT after 36 h in vitro incubation in the presence of 10 μg/ml of individual peptide or of a peptide pool containing 5 μg/ml of each peptide. The number of spots corresponding to cytokine-producing cells in wells (spot-forming cells [SFCs]) was enumerated with an automated spot counting system (Scanalytics, Fairfax, VA). Responses were expressed as the mean number of SFCs/10^6 PBMCs, and the conserved criteria for discrimination of positive from negative responses used in our previous malaria DNA vaccine trials were also used in this study (29, 30). Assays were all conducted in quadruplicate. In brief, responses were considered significant if (i) the mean number of cells in quadruplicate wells with experimental peptide was significantly greater ($P < 0.05$) than in wells with control peptide, (ii) the net SFCs/well (mean SFCs in experimental peptide wells –mean SFCs in control peptide wells) was ≥5 SFCs/well, and (iii) the stimulation index (the ratio of mean SFCs in experimental peptide wells to mean SFCs in control peptide wells) was greater than 2.0. Furthermore, if cells obtained before immunization had a positive response to any of five *P. falciparum* antigen-specific peptides as

TABLE 2. Effect of coinjection of hGM-CSF plasmid on induction of IFN-γ responses in volunteers immunized with a DNA vaccine mixture encoding five *P. falciparum* pre-erythrocyte-stage antigens

Cohort	hGM-CSF (μg/dose)	No. of IFN-γ responders/no. tested to each of five vaccine components[a]					Total
		CSP	SSP2	EXP-1	LSA-1	LSA-3	
1	0	6/8	2/8	4/8	3/8	1/8	6/8
2	20	4/8	3/8	4/8	4/8	3/8	4/8
3	100	2/7	1/7	1/7	2/7	0/7	2/7
4	500	2/8	1/8	3/8	3/8	0/8	3/8
Total responders/total vaccinees		14/31 (45.2%)	7/31 (22.6%)	12/31 (38.7%)	12/31 (38.7%)	4/31 (12.9%)	15/31 (48.4%)
Total responders/total controls (*P* value[b])		0/12 (0.005)	0/12 (0.07)	0/12 (0.011)	0/12 (0.011)	0/12 (0.19)	0/12 (0.003)

[a] Number of IFN-γ responders represents the positive IFN-γ responses detected at 2 weeks after the second immunization and 2 weeks after the third immunization.
[b] *P* values were calculated using a chi-square test (two tailed).

defined above, the response to the same peptide after the immunization was not considered positive.

Statistical analysis. Since this was the first safety and immunogenicity study in humans of a multivalent DNA malaria vaccine with or without a recombinant plasmid expressing hGM-CSF, the sample size of each study group was limited by safety considerations and was not calculated with regard to power for demonstrating a specified level of efficacy with a specified confidence interval. The frequency of peptide-specific IFN-γ responses was assessed using the chi-square test (two-tailed, uncorrected) except when the cell value was less than five, in which case Fisher's exact test was employed (two-tailed), and the magnitude of responses was assessed using Student's *t* test (two-tailed). Analysis was conducted using SPSS version 8.0 (SPSS Inc., Chicago, IL) or Epi Info version 6.04b (Centers for Disease Control and Prevention, Atlanta, GA). The level of significance was a *P* value of <0.05. No corrections were made for multiple comparisons.

RESULTS

IFN-γ responses to multiple $CD4^+$ and $CD8^+$ T-cell epitopes derived from all five vaccine components. Fresh PBMCs were used to compare IFN-γ responses before and after DNA immunization. Overall, IFN-γ responses to *P. falciparum*-specific peptides were detected in 15/31 (48.4%) immunized volunteers (6/8 volunteers in cohort 1, 4/8 volunteers in cohort 2, 2/7 volunteers in cohort 3, and 3/8 in cohort 4), compared with 0/12 (0%) controls ($P = 0.003$) (Table 2) (one volunteer dropped out of cohort 3 following the first immunization due to a family member request). There were no statistically significant differences between the four cohorts receiving DNA with or without different doses of GM-CSF in terms of the frequency (Table 2) or magnitude of IFN-γ responses. Comparing antigens, IFN-γ responses were detected to CSP-specific peptides in 14/31 ($P = 0.005$), to EXP-1-specific peptides in 12/31 ($P = 0.011$), to LSA-1-specific peptides in 12/31 ($P = 0.011$), to SSP2-specific peptides in 7/31 ($P = 0.07$), and to LSA-3-specific peptides in 4/31 ($P = 0.19$) immunized volunteers (Table 2).

The magnitude of IFN-γ responses was measured as the net number of SFCs per 10^6 PBMCs. (The net number of SFCs was the number of SFCs after the background was subtracted.) To be considered positive, the results had to meet our strict criteria for positivity (see Materials and Methods). Positive responses ranged from 12 to 96 net SFCs/10^6 (geometric mean [geomean], 23.4) overall and from 13.3 to 96.3 (geomean, 23.3) for CSP, 16.3 to 42.1 (geomean, 26.1) for SSP2, 14.4 to 96 (geomean, 27.4) for EXP-1, 12.1 to 41.3 (geomean, 20.0) for LSA-1, and 13.8 to 27.5 (geomean, 17.4) for LSA-3 (Table 3).

Breadth of DNA-induced IFN-γ responses. Of 15 positive responders who had *P. falciparum*-specific IFN-γ responses in fresh PBMCs, 7 responded to defined class I-restricted peptides (comprising 13/34 class I peptides tested) and all 15 showed IFN-γ responses to defined class II-restricted peptides (comprising 16/17 class II peptides tested). Overall, IFN-γ responses were detected to 8/15 HLA-A2 peptides tested, derived from four of five vaccine components (CSP, EXP-1, LSA-1, and LSA-3), 5/19 non-A2 class I peptides tested (three HLA-A3 restricted and one HLA-B8 restricted), derived from three of five proteins tested (CSP, SSP2, and LSA-1), and 16/17 DR-binding peptides tested, derived from all five proteins (Table 3).

The highest percentages of responses were detected with two DR-binding peptides, EXP. DR.71 (10/15 responders [66.7%]) and CSP. DR.375 (8/15 responders [53.3%]) (Table 3). The magnitudes of responses to these two peptides were not greater than those of the responses to the other peptides eliciting positive responses (Table 3).

Responses to multiple peptides were detected simultaneously in the same volunteer. Of 15 responders, 1 recognized seven peptides from three components (four derived from CSP, two from EXP-1, and one from SSP2), 3 responders recognized six peptides, 6 recognized four or five peptides from all five components, and 5 responders recognized two or three peptides from four proteins (except LSA-3).

All four multi-peptide responders who had IFN-γ responses to six or seven peptides and three of six volunteers who had IFN-γ responses to four or five peptides were HLA-A2 (*0201) positive, and the other three volunteers who had IFN-γ responses to four or five peptides were HLA-A1/A3, -A3/A68, or -A3/A11 positive, respectively. Overall, 10/15 (67%) HLA-A2, 4/10 (40%) HLA-A1, and 5/19 (26%) HLA-A3/A11 vaccinees were positive responders to two to seven *P. falciparum*-specific peptides. A larger proportion of HLA-A2-positive volunteers responded to multiple peptides than did HLA-A1- or A3/11-positive volunteers, but the difference only reached statistical significance when compared with A2 and A3/11 volunteers ($A2^+$ [10/15] versus $A1^+$ [4/10], $P = 0.2$; $A1^+$ [4/10] versus $A3/11^+$ [5/19], $P = 0.4$; $A2^+$ [10/15] versus $A3/11^+$ [5/19], $P = 0.02$). However, there are more defined HLA-A2-restricted T-cell epitopes than other HLA class I-restricted T-cell epitopes expressed on the five antigens tested (Table 1), so this result is expected and does not indicate an intrinsic difference

TABLE 3. IFN-γ responses to *P. falciparum*-derived peptides in DNA-immunized volunteers

Vaccine component	Peptide code	Predicted HLA restriction	Sequence	Residues	No. responders/ no. tested (% positive responders)	Range of net SFCs/10^6 PBMCs (geomean)
Defined class I-restricted T epitopes						
PfCSP	CSP.A2.386	HLA-A2 supertype	GLIMVLSFL	386–394	1/15 (6.7)	70.8
	CSP.A2.319	HLA-A2.1	YLNKIQNSL	319–327	2/15 (13.3)	12–96 (30.3)
	CSP.B8.86	HLA-B8	LRKPKHKKL	86–94	1/5 (20)	22.5
PfSSP2/TRAP	SSP.A3.522	A3 supertype	LLACAGLAYK	522–531	1/19 (5.3)	23.8
PfEXP-1	EXP.A2.2	HLA-A2 supertype	KILSVFFLA	2–10	1/15 (6.7)	42.1
	EXP.A2.83	HLA-A2 supertype	GLLGNVSTV	83–91	3/15 (20)	14–17 (15.2)
PfLSA-1	LSA1.A2.1655	HLA-A2	RLEIPAIEL	1655–1663	2/15 (13.3)	12–38 (21.5)
	LSA1.A2.4	HLA-A2	ILYISFYFI	4–12	1/15 (6.7)	13–19 (15.3)
	LSA1.A3.94	HLA-A3 supertype	QTNFKSLLR	94–102	1/19 (5.3)	17.5
	LSA1.A3.105	HLA-A3 supertype	GVSENIFLK	105–113	2/19 (10.5)	18–41 (26.9)
	LSA1.A3.11	HLA-A3 supertype	FILVNLLIFH	11–20	2/19 (10.5)	16–20 (18.1)
PfLSA-3	LSA3.A2.981	HLA-A2.1	VLDKVEETV	981–989 la26	1/15 (6.7)	14.4
	LSA3.A2.113	HLA-A2.1	DLLEEGNTL	113–121 la20	1/15 (6.7)	16.9
Defined or potential class II-restricted T epitopes						
PfCSP	CSP.DR/A2.1	HLA-DR, A2	MMRKLAILSVSSFLFVEALF	1–20	3/31 (9.7)	12–23 (17.9)
	CSP.DR/A2.318	HLA-DR, A2	EYLNKIQNSLSTEWSPCSVT	318–337	5/31 (16.1)	13–69 (25.4)
	CSP.DR.363	HLA-DR	DIEKKICKMEKCSSVFNVVNS	363–383	1/31 (3.2)	16.9
	CSP.DR/A2.375	HLA-DR, A2	SSVFNVVNSSIGLIMVLSFLFLN	375–397	8/31 (25.8)	13–28 (21.0)
PfSSP2/TRAP	SSP.DR.223	HLA-DR	VKNVIGPFMKAVCVE	223–237	2/31 (6.5)	28–30 (29.0)
	SSP.DR.527	HLA-DR	GLAYKFVVPGAATPY	527–541	4/31 (12.9)	16–42 (25.2)
	SSP.DR.509	HLA-DR	KYKIAGGIAGGLALL	509–523	3/31 (9.7)	17–39 (27.0)
PfEXP-1	EXP.DR.82	HLA-DR	AGLLGNVSTVLLGGV	82–96	3/31 (9.7)	21–31 (25.7)
	EXP.DR.71	HLA-DR	KSKYKLATSVLAGLL	71–85	10/31 (32.3)	21–96 (34.0)
PfLSA-1	LSA1.DR.13	HLA-DR	LVNLLIFHINGKIIKNS	13–27	1/31 (3.2)	27.9
	LSA1.DR.94	HLA-DR	QTNFKSLLRNLGVSENIFLK	94–113	2/31 (6.5)	13–25 (16.5)
	LSA1.DR.84	HLA-DR	LTMSNVKNVQTNFKSLLRNLGVS	84–107 (T1)	3/31 (9.7)	19–22 (20.4)
	LSA1.DR.1813	HLA-DR	NENLDDLDEGIEKSSEELSEEKI	1813–35 (T3)	3/31 (9.7)	18–25 (22.5)
	LSA1.DR.1888	HLA-DR	DNEILQIVKELSEKITKYFMKL	1888–1909 (T5)	3/31 (9.7)	19–20 (19.1)
PfLSA-3	LSA3.II.142	Potential	LLSNIEEPKENIIDNLLNNI	142–161 (CT1)	1/31 (3.2)	27.5
	LSA3.II.200	Potential	LEESQVNDDIFNSLVKSVQQEQQHNV	200–225 (NRII)	1/31 (3.2)	13.8

TABLE 4. *P. falciparum*-specific IFN-γ responses induced by a multiple DNA vaccine and enhanced by exposure to sporozoite-infected mosquitoes

Cohort	hGM-CSF (μg/dose)	Volunteers with positive IFN-γ responses before challenge		Volunteers with evidence of boosting of IFN-γ responses after challenge			
		1 wk before challenge		4 wks after challenge[b]		9 wks after challenge[b]	
		Vaccinees	Controls	Vaccinees	Controls	Vaccinees	Controls
1	0	6/8[a]	0/3	7/8	1/3	4/8	1/3
2	20	4/8	0/3	8/8	1/3	4/8	0/3
3	100	2/7	1/3	2/7	1/3	3/7	1/3
4	500	3/8	0/4	6/8	0/4	4/8	0/4
Total		15/31 (48.4%)	1/13 (7.7%)	23/31 (74.2%)	3/13 (23.1%)	15/31 (48.4%)	2/13 (15.4%)
P value[c]		0.01		0.002		0.04	

[a] No. responders/no. tested
[b] IFN-γ responses were considered to be boosted by parasite challenge only if the number of IFN-γ SFCs/1 × 10^6 PBMCs was at least two times higher after challenge than before challenge.
[c] Comparison of vaccinees and controls in each challenge group (1, 4, or 9 weeks).

between haplotypes in their ability to induce genetically restricted T-cell responses to an appropriate epitope.

IFN-γ responses to CSP were comparable in humans immunized with CSP either as univalent or multivalent vaccines. To determine if antigenic competition among the multiple components interfered with the T-cell responses to individual components, we compared the IFN-γ responses to CSP in individuals who received univalent CSP DNA vaccine in a previous study (29) with the individuals who received the pentavalent DNA vaccine including the identical CSP DNA plasmid in this study. The ELISPOT assays were done separately, and the assays used to detect IFN-γ responses in both sets of samples were the same. Because all volunteers in the previous study were HLA-A*0201 positive, we restricted the comparison to HLA-A*0201-positive individuals in the current study so that responses to identical sets of peptides could be compared. In addition, we restricted the comparison to those volunteers in the previous study who had received the vaccine by the same intramuscular Biojector delivery method as in the current study. IFN-γ responses in both groups ($n = 5$ for each) were assessed in freshly isolated PBMCs stimulated with the same set of eight CSP-specific peptides restricted by HLA-A*0201 and/or HLA-DR alleles. IFN-γ responses to CSP were detected to eight of eight CSP peptides in five of five univalent DNA-immunized volunteers, compared to responses to six of eight CSP peptides in four of five pentavalent DNA-immunized volunteers. The magnitude of responses ranged from 12.0 to 156.3 SFC/10^6 (geomean, 26.9) in univalent DNA-immunized volunteers and from 13.1 to 96.3 SFC/10^6 (geomean, 30.3) in pentavalent DNA-immunized volunteers ($P = 0.84$). In these studies, which were done at different times, both the frequency and magnitude of the IFN-γ responses to CSP were similar whether the plasmid was administered as part of a pentavalent mixture or as a univalent vaccine. This is particularly notable as the dose of CSP plasmid was only 500 μg per immunization in the pentavalent mixture compared with 2,500 μg in the earlier monovalent vaccine study.

Enhancement of IFN-γ responses by exposure to *P. falciparum* parasites after DNA immunization. The 31 volunteers who received three doses of vaccine were challenged with *P. falciparum* sporozoites (strain 3D7) 18 days after the third immunization. The sporozoites were delivered via the bites of five infected *Anopheles stephensi* mosquitoes (14). Four separate challenges were required for the four cohorts because of the staggered immunization schedules (Table 4). Sporozoite infectivity was verified by including three or four nonimmunized control volunteers in each challenge. Because all 44 challenge recipients developed patent parasitemias, the opportunity was provided to determine if antigen-specific IFN-γ responses primed by immunization with a DNA vaccine were enhanced following exposure of volunteers to *P. falciparum* sporozoite-, liver-, and blood-stage infection. This situation paralleled what might happen to individuals traveling in an endemic area following immunization with a DNA vaccine against malaria who are exposed to malaria and do or do not acquire a malaria blood-stage infection.

Frozen PBMCs, collected from the volunteers preimmunization and 1 week prechallenge or 4 and 9 weeks post-sporozoite challenge, were assayed for antigen-specific IFN-γ release simultaneously in blinded fashion. PBMCs were stimulated with 15 peptide pools comprising three sets of pools (A2-restricted peptides, non-A2 class I-restricted peptides, and DR-binding peptides) for each of the five vaccine components (five vaccine components × three different HLA groups = 15 pools of peptides).

All 15 volunteers determined to be responders by ELISPOT assay using fresh cells also had positive responses in frozen cells collected approximately 1 week before parasite challenge (Table 4). IFN-γ responses were considered to be boosted by parasite challenge if the number of IFN-γ SFCs/10^6 PBMCs was at least two times higher following challenge than before challenge.

At 4 weeks after sporozoite challenge, IFN-γ responses were boosted to all five vaccine components, as shown by doubling of responses to at least one and up to five of the peptide pools in PBMCs from 23/31 (74.2%) DNA-immunized volunteers (Table 4). IFN-γ responses were elicited to three antigens (CSP, EXP-1, and LSA-1) in 3/13 (23.1%) nonimmunized control volunteers ($P = 0.002$). The 23 positive responders post-challenge included 14 of the 15 individuals who had responded prechallenge and 9 of the 16 individuals who had not been responders prechallenge when using fresh PBMCs. At 9 weeks

after challenge, IFN-γ responses were still at least double the prechallenge levels in 15/23 (65.2%) DNA-immunized volunteers who had shown boosting at 4 weeks (Table 4).

IFN-γ responses to CSP (12/31 volunteers), LSA-1 (12/31), EXP-1 (10/31), SSP2 (2/31), or LSA-3 (2/31) were boosted by sporozoite challenge in immunized volunteers. In comparison the frequencies of responses in nonimmunized, infectivity control volunteers were lower (1/13, 1/13, 2/13, 0/13, and 0/13, respectively, for the five proteins). IFN-γ responses to class I-restricted peptide pools were boosted in 9 of the 23 positive responders after challenge (detected with four peptide pools derived from CSP [A2-CSP and I-CSP pools] or LSA-1 [A2-LSA-1 and I-LSA-1 pools]), and responses to the class II peptide pools were boosted in 21 of the 23 initial responders (detected with five peptide pools derived from all five proteins). Furthermore, 7/23 responders produced IFN-γ in response to both class I and II peptide pools, 2/23 produced IFN-γ in response only to class I, and 14/23 produced IFN-γ in response only to class II peptide pools.

IFN-γ responses to class I and/or II peptides were absent in 8/23 immunized, week 4 responders at 9 weeks after the challenge. The magnitude of response to the class II peptides was lower at 9 weeks than at 4 weeks. The number of IFN-γ SFCs/10^6 PBMCs to the five class II peptide pools at 4 weeks versus 9 weeks after challenge ranged from 29.6 to 187.5 (geomean, 59) versus 13.1 to 93.8 (geomean, 33.3) for all proteins ($P = 0.007$), 33.3 to 94.4 (60.2) versus 28.1 to 93.8 (52.5) for CSP ($P = 0.78$), 32.7 to 123.8 (53.6) versus 14.4 to 38.1 (23.5) for EXP-1 ($P = 0.007$), and 29.6 to 187.5 (63.3) versus 13.1 to 88.8 (35.6) for LSA-1 ($P = 0.11$).

IFN-γ responses to the 10 class I peptide pools were also boosted after challenge, and the magnitude of the responses was similar at 9 weeks (range of SFCs/10^6 PBMCs, 24.4 to 88.8 [geomean, 41.3]) and at 4 weeks after challenge (range, 13.1 to 139.4 [geomean, 33.5]) ($P = 0.9$).

When comparing peaks and durations of the IFN-γ responses boosted by sporozoite challenge among all vaccinees and controls, the following four distinguishable patterns were revealed: pattern 1, IFN-γ responses that were positive after DNA immunization and before challenge were increased by 4 weeks and absent or decreased dramatically at 9 weeks postchallenge; pattern 2, no detectable IFN-γ responses after DNA immunization and before challenge, positive responses at 4 weeks and absent or dramatically decreased at 9 weeks postchallenge; pattern 3, no or low detectable IFN-γ responses after DNA immunization and before challenge, positive responses at 4 weeks, and responses were still sustained at 9 weeks postchallenge; pattern 4, no detectable IFN-γ responses after DNA immunization and before challenge nor by 4 weeks after challenge, and there were responses at 9 weeks after challenge. Overall, DNA-induced or primed IFN-γ responses to class II peptides derived from all five components increased by parasite challenge fell mostly into pattern 1 or 2 (26/30 [87%] positive assays). In contrast, a majority of DNA-primed IFN-γ responses to class I peptides increased by parasite challenge fell into pattern 3 or 4 (7/11 [64%] positive assays). Of the 23 immunized volunteers showing enhanced responses following challenge, 18 fell into patterns 1 and 2 (3/7 in cohort 1, 7/7 in cohort 2, 2/3 in cohort 3, and 6/6 in cohort 4), and 5 fell into patterns 3 and 4 (4/7 in cohort 1 and 1/3 in cohort 3). The three naïve control individuals with increased IFN-γ responses after challenge fell into pattern 2 or 3 (3/3 positive assays).

Effects of GM-CSF on IFN-γ responses enhanced by exposure to *P. falciparum* parasites after DNA immunization. To determine the effect of GM-CSF on IFN-γ responses induced by the pentavalent DNA vaccine, we compared responses in cohort 1 receiving DNA alone with the responses in the other three cohorts receiving DNA with different doses of GM-CSF. There was a higher frequency of volunteers showing positive IFN-γ responses after three doses of DNA immunization in cohort 1 (6 responders out of 8 volunteers, 75%) than in the other three cohorts combined (9 responders out of 23 volunteers, 39%), although this difference did not attain statistical significance ($P = 0.11$) (Table 2). Among these positive responders, there were no differences in the proportion of IFN-γ responses attributable to class I or II peptides when comparing cohort 1 with cohorts 2 to 4 (class I, 3/6 versus 3/9, $P = 0.52$; class II, 5/6 versus 9/9, $P = 0.20$). Similarly, there were no differences in the proportion of IFN-γ responses attributable to class I or class II peptides when comparing proportions between cohort 1 and cohorts 2 to 4 for all volunteers tested (class 1, 3/8 [38%] versus 3/23 [13%], $P = 0.15$; class II, 5/8 [63%] versus 9/23 [39%], $P = 0.25$).

A different finding emerged when we assessed the boosting of vaccine-induced IFN-γ responses by exposure to parasites in individuals who had and had not received the vaccine with GM-CSF. There was a statistically significant difference in the numbers of individuals with positive IFN-γ responses to class I peptides between cohort 1 (no GM-CSF) and cohorts 2 to 4 (20 to 500 μg of GM-CSF plasmid) (5/8 [63%] in cohort 1 versus 4/23 [17%] in cohorts 2 to 4; $P = 0.015$) (see Fig. 2). This difference was not seen in response to class II peptides (6/8 [75%] in cohort 1 versus 15/23 [65%] in cohorts 2 to 4; $P = 0.6$). Thereafter, with challenge there was no difference in the frequency of IFN-γ responses to class I compared to class II peptides in cohort 1 volunteers (5/8 [63%] to class I versus 6/8 [75%] to class II peptides). However, there was a significant difference in the frequency of responses to class I compared to class II peptides in cohort 2 to 4 volunteers (4/23 [17%] responded to class I versus 15/23 [65%] to class II peptides in cohorts 2 to 4; $P = 0.002$). GM-CSF may have led to $CD4^+$ T-cell-dependent responses similar to those observed in the absence of GM-CSF but reduced $CD8^+$ T-cell-dependent type 1 IFN-γ responses to parasite challenge.

DISCUSSION

DNA vaccines have been studied in animal models as a strategic approach to delivering multivalent vaccines against various infectious agents, based on their ease of production, stability, and simplicity of combination. Multivalency may be particularly important for inducing protective responses against complex organisms such as *P. falciparum*, as suggested by the high level of efficacy provided by the irradiated sporozoite vaccine. Previously, we demonstrated that multivalent *P. falciparum* DNA vaccines are immunogenic in mice and nonhuman primates (12, 27). For example, administration of a mixture of four plasmids encoding *P. falciparum* pre-erythrocyte-stage proteins (CSP, SSP2, EXP-1, and LSA-1) was effective in inducing CTL responses in rhesus monkeys and was not

associated with reduced responses to any component when administered as part of the mixture compared to administration alone (27). A primary purpose of the current study was to determine if a similar, DNA vaccine encoding five *P. falciparum* pre-erythrocyte-stage proteins could induce cell-mediated immune responses in humans. Because all volunteers developed parasitemia following challenge, the study also afforded the opportunity to determine if the responses induced by a multivalent DNA vaccine primed the immune system for enhanced immune responses (boosting) following exposure to the malaria parasite. The fact that DNA immunization did effectively provide antigen-specific priming in this study may indicate the potential for a multivalent DNA vaccine to prime and possibly protect residents in areas where malaria is endemic who are naturally exposed to repeated malaria infections. Field trials will be required to see if DNA priming leads to definitive reductions in the severity of infection, since the goal in the field is a reduction of burden of infection and of morbidity associated with infection. It is quite possible that a vaccine that does not prevent infection in its entirety and is boosted by natural exposure may reduce the burden of infection and morbidity. A recent example has been published in *Lancet* (2). The vaccine, which is not boosted by natural exposure, reduced the incidence of new parasitemia by 10% during 6 months but was reported to reduce the incidence of severe disease by 57%.

A total of 51 *P. falciparum*-specific peptides, including 34 MHC class I-restricted and 17 class II-restricted T-cell epitopes derived from all five antigens, were used to stimulate fresh PBMCs from volunteers before and after the DNA vaccination. The results indicated that each of the five plasmids was immunogenic and that immunization with the plasmids as a mixture induced IFN-γ responses to all components of the vaccine. IFN-γ responses to *P. falciparum*-specific peptides were only detected in the DNA-immunized volunteers (15/31; 48.4%) and not in any of the 12 controls. IFN-γ responses to CSP occurred with the greatest frequency among DNA-immunized volunteers (14/31), followed by EXP-1 and LSA-1 (12/31 each), LSA-3 (7/31), and SSP2 (4/31) (Table 2). Positive responses were detected to 38.2% (13/34) of the class I-restricted peptides and to 94.1% (16/17) of the class II-restricted peptides tested. The 29 peptides eliciting positive responses were derived from all five antigens. Furthermore, multiple, peptide-specific, HLA allele-restricted responses were induced simultaneously in single volunteers, and 9/15 (60%) responders produced IFN-γ to both class I and II (DR-binding) peptides. These results support our approach of immunizing humans with a multivalent DNA vaccine encoding sufficiently diverse epitopes to circumvent genetic restriction of T-cell responses based on polymorphism of human HLA molecules (i.e., to provide broad coverage of the host population) and to eliminate genetically heterogeneous parasites that may vary at T-cell epitopes (i.e., to provide broad coverage of parasite populations) (11).

The two peptides inducing the highest frequencies of responses in this study were derived from EXP-1 and CSP. The responses to EXP. DR.71 (10/31 responders [32.3%]) were comparable to the responses detected in humans who have been naturally exposed to *P. falciparum* infection as a result of living in endemic areas (7, 19). The second peptide, CSP. DR.375 (8/31 responders [25.8%]), was the peptide inducing the best responses in humans immunized with the monovalent CSP DNA vaccine in a previous study (29). Immune responses to such high-responder peptides may prove useful as surrogate markers for monitoring and evaluating cellular immune responses to multivalent vaccines in future clinical trials.

Determination of whether mixing of plasmids inhibits the immunogenicity of each component is crucially important to our goal of enhancing protection by using multiantigen formulations, a goal based on the supposition that the robust protection induced by the irradiated sporozoite vaccine relies upon inducing multiple immune responses to multiple antigens. Here, we compared the IFN-γ responses to CSP in HLA-A*0201-positive individuals who received univalent CSP DNA vaccine in a previous study (29) to the IFN-γ responses to CSP shown by individuals who received the pentavalent DNA vaccine including the CSP gene in this study. Thus, if mixing plasmids interfered with their immunogenicity, we would expect that responses to CSP would be relatively inhibited in the current study. We found, however, that the frequency and magnitude of the IFN-γ responses to CSP were not suppressed by administering the plasmid as part of a pentavalent mixture. This was found despite the dose difference between the two groups: the volunteers immunized with the univalent vaccine received three doses of 2,500 μg of the plasmid encoding CSP, and the volunteers immunized with the pentavalent vaccine received three doses of 500 μg of CSP plasmid mixed into 2,500 μg of the five-gene mixture. The near equivalence of the IFN-γ responses identified in the two studies despite the reduced dosage in the mixture indicates that, at least for CSP and this particular antigen combination, interference is not a concern. This finding is consistent with our previous report that CTL responses to all four components of a tetravalent DNA plasmid cocktail were generated in rhesus monkeys regardless of whether the plasmids were administered singly or in various combinations (27). However, it has been reported that the dose of DNA and route of DNA administration have significant effects on T-cell and/or antibody responses against HIV and other infectious diseases in nonhuman primate studies (3). The fact that our multivalent DNA vaccine failed to induce primary antibody responses in this study may have been due to the relatively low doses (500 μg) of each plasmid that we used. The route and method of administration may have also impacted outcome.

This study also allowed us to determine if the IFN-γ responses in those primed by DNA immunization could be boosted by exposure to malaria infection, as all vaccinees and controls received a similar exposure to *P. falciparum* sporozoites, liver stages, and blood stages. We challenged the volunteers via the bites of five infected mosquitoes and report here for the first time for a DNA vaccine assessed in humans that IFN-γ responses to multiple (all five) antigens were higher following challenge in vaccinees than in the controls. Since 3/13 controls also mounted antigen-specific IFN-γ responses after parasite challenge, this indicated that challenge can prime the antigen-specific responses in controls. Whether boosting would still have occurred had the vaccine been effective in preventing blood-stage infection cannot be determined. However, since three of the five antigens (CSP, SSP2, and LSA-1) are not expressed by the asexual erythrocyte stage of *P.*

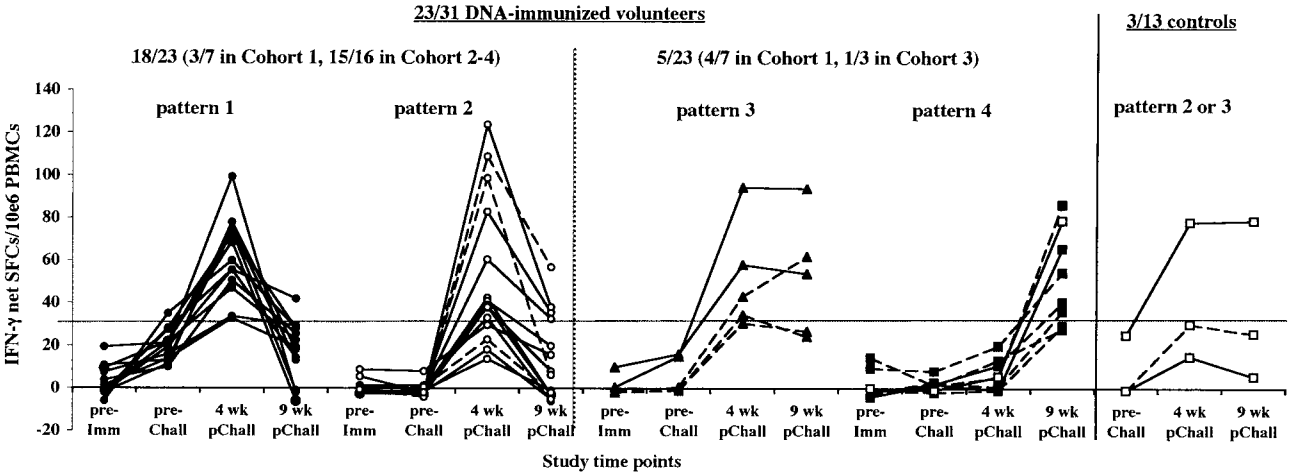

FIG. 1. DNA-primed IFN-γ responses were increased by exposure of the immunized volunteers to *P. falciparum*-infected mosquitoes. ELISPOT assays were conducted with frozen PBMCs from volunteer samples taken before immunization with DNA vaccine (pre-Imm), 3 weeks after the last dose of DNA and 1 week before challenge (pre-Chall), and 4 and 9 weeks after challenge (pChall). IFN-γ responses to class I (dotted line) or class II (solid line) peptide pools in immunized and challenged volunteers were divided into four different patterns, as follows. Pattern 1, IFN-γ responses were positive after DNA immunization and before challenge, increased at 4 weeks postchallenge, and were absent or decreased at 9 weeks postchallenge. Pattern 2, IFN-γ responses were not detectable after DNA immunization and before challenge, increased at 4 weeks postchallenge, and were absent or decreased at 9 weeks postchallenge. Pattern 3, IFN-γ responses were low or not detectable after DNA immunization and before challenge, increased at 4 weeks postchallenge, and were sustained at 9 weeks postchallenge. Pattern 4, IFN-γ responses were not detectable after DNA immunization and before challenge or at 4 weeks postchallenge but were increased at 9 weeks after challenge.

falciparum, the responses to these proteins could not have been boosted by blood-stage infection.

Four different patterns characterized the responses to *P. falciparum*-specific, MHC-restricted epitopes that were induced or primed by DNA vaccination and boosted by parasite challenge (Fig. 1). DNA-induced IFN-γ responses to class II peptides were, in general, boosted rapidly at 4 weeks after challenge and were significantly decreased or absent at 9 weeks after challenge (patterns 1 and 2; 85% of responses in these two patterns were to class II peptides from EXP-1, LSA-1, and CSP). In contrast, DNA-primed IFN-γ responses ("primed" here meaning no measurable responses after immunization but with boosting shown following challenge) to class I peptides were boosted slowly and lasted longer than 9 weeks after challenge (for patterns 3 and 4, 60% responses in these two patterns were boosted to class I peptides from CSP, LSA-1, and SSP2), a pattern also shown in three unvaccinated control volunteers, albeit at a much lower magnitude of response in two of the three (Fig. 2). Responses to EXP-1 typically fell into pattern 1 or 2, with boosting at 4 weeks and reduced or absent responses at 9 weeks after challenge, while responses to CSP and LSA-1 were sometimes enhanced at 9 weeks after challenge, indicating that the duration of the responses boosted by parasite exposure could be partially antigen dependent. In addition, our results may indicate that the effector cells present at the time of challenge are primarily $CD4^+$ T cells, whereas the antigen-specific $CD8^+$ memory T cells might undergo a clonal expansion after parasite challenge to provide detectable functional responses.

In spite of strong enhancement of IFN-γ responses after challenge, all vaccinees developed parasitemia, suggesting that immunization with DNA alone, at least if formulated as the antigen mixture and delivered according to the regimen selected for this study, provides little protection against malaria. Encouragingly, cellular immune responses were amplified significantly as measured at 4 and 9 weeks after challenge against multiple class I and II peptides. Furthermore, postchallenge antibody responses were also amplified, even though antibodies primed by DNA were not detected after DNA immunization and prior to challenge (T. L. Richie et al., unpublished data). If natural infection boosted T-cell and antibody responses as effectively as observed in this study following experimental challenge, and particularly if responses to blood-stage antigens were also boosted, then DNA vaccination could accelerate the acquisition of naturally acquired immunity in endemic areas and significantly reduce the morbidity and mortality associated with malaria infection.

It has now been reported that immunization of volunteers with a DNA vaccine encoding multiple *P. falciparum*-derived epitopes followed by a recombinant poxvirus vaccine expressing the same epitopes induces excellent T-cell responses against many of these epitopes. However, this did not elicit any antibody responses and did not prevent volunteers from developing malaria after experimental challenge (18). The volunteers in this study who were immunized with DNA alone had much lower IFN-γ responses than did the volunteers in the study referred to above, and they did not develop antibodies against the *P. falciparum* proteins. Thus, it is not surprising that there was no protection.

In attempt to improve immune responses induced by the pentavalent DNA vaccine, we added to the mixture a plasmid encoding hGM-CSF, tested for adjuvant effect through a dose escalation design. We demonstrated here that coadministration of GM-CSF with the DNA vaccine at 20, 100, and 500 μg had no strong effect on induction of IFN-γ responses, although there was a trend toward diminished frequency of responses in the cohorts that received GM-CSF (cohorts 2 to 4) relative to the cohort that did not receive GM-CSF (cohort 1). There

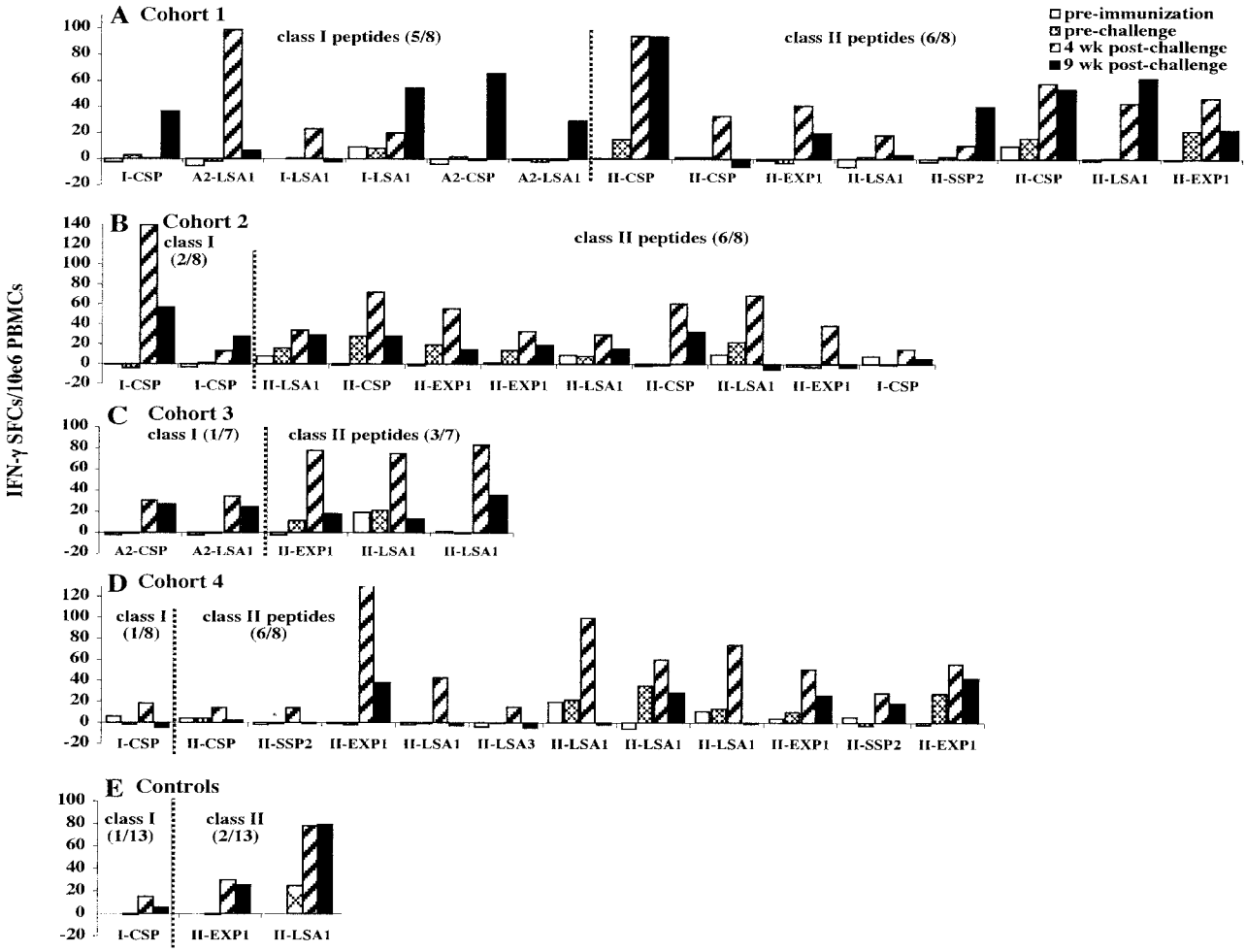

FIG. 2. IFN-γ responses after challenge among volunteers in cohorts 1 to 4 and among controls. (A) Cohort 1 received DNA vaccine alone without GM-CSF. (B, C, and D) Cohorts 2, 3, and 4 received DNA with GM-CSF at 20, 100, and 500 μg per dose, respectively. Controls did not receive vaccine and GM-CSF but were challenged with *P. falciparum*. The frequency of individuals with positive IFN-γ responses to class I peptides was significantly greater in cohort 1 (did not receive the GM-CSF plasmid) than in cohorts 2 to 4 (did receive the GM-CSF plasmid) (5/8 versus 4/23; $P = 0.027$; Fisher's exact test, two-tailed [2/8 in cohort 2, 1/7 in cohort 3, and 1/8 in cohort 4]). There was no difference in the number of responders to class II peptides between cohort 1 and cohorts 2 to 4 (6/8 versus 15/23, respectively [6/8 in cohort 2, 3/7 in cohort 3, and 6/8 in cohort 4]). In addition, there was no difference in the number of volunteers with IFN-γ responses to class I compared to class II peptides in cohort 1 (5/8 versus 6/8). However, there were significantly lower numbers of volunteers who responded to class I compared to class II peptides in volunteers in cohorts 2 to 4 (4/23 versus 15/23; $P = 0.002$; Fisher's exact test, two-tailed).

were no statistically significant differences in IFN-γ responses to class I- or II-restricted peptides between groups receiving DNA with or without GM-CSF. Interestingly, GM-CSF did significantly impact on the enhancement of IFN-γ responses to class I, but not to class II peptides by parasite challenge (Fig. 2). In volunteers receiving DNA alone, IFN-γ responses to both class I and class II peptides were comparably boosted after challenge (Fig. 2A). In volunteers receiving DNA with GM-CSF, however, the responses to class II but not to class I peptides were significantly boosted after challenge. In pharmacology studies conducted in mice, GM-CSF could only be detected in the muscle but not in the serum following intramuscular administration of the vaccine (20), suggesting that enhancement of recruitment of antigen-presenting cells in the vaccine injection site may have been the role of GM-CSF through an exogenous antigen presentation pathway in enhancing Th1-type IFN-γ responses to parasite challenge. A profound enhancement of DNA-induced $CD8^+$ cytotoxic T-cell responses may require a systemic effect of GM-CSF as reported in other systems (8, 17, 31). We are currently investigating ways to deliver DNA plasmid vaccines so as to induce vigorous T-cell and antibody responses in the same recipients.

ACKNOWLEDGMENTS

This work was supported by the Naval Medical Research and Development Command work units 61102A.S13.F.A0009, 62787A.870.F.A0010, 63002A.810.F.A0011, 603792N.01889.135.A0039, and 60000.000.000.A0062, by the U.S. Agency for International Development, and by the Office of Naval Research Advanced Technology Demonstration.

The opinions and assertions herein are the private ones of the authors and are not to be construed as official or as reflecting the views of the Navy Department.

We thank the staff of the Clinical Trials Center, Malaria Program, Naval Medical Research Center, for their professional performance. We extend our thanks to the volunteers, without whom this study would not have been possible.

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Editor: W. A. Petri, Jr.