CLINICAL AND VACCINE IMMUNOLOGY, Nov. 2006, p. 1267–1277 Vol. 13, No. 11
1556-6811/06/$08.00+0 doi:10.1128/CVI.00162-06

A DNA Vaccine for Ebola Virus Is Safe and Immunogenic in a Phase I Clinical Trial▽†

Julie E. Martin,[1] Nancy J. Sullivan,[1] Mary E. Enama,[1] Ingelise J. Gordon,[1] Mario Roederer,[1] Richard A. Koup,[1] Robert T. Bailer,[1] Bimal K. Chakrabarti,[1] Michael A. Bailey,[1] Phillip L. Gomez,[1] Charla A. Andrews,[1] Zoe Moodie,[2] Lin Gu,[2] Judith A. Stein,[1] Gary J. Nabel,[1] Barney S. Graham,[1]* and the VRC 204 Study Team[1]‡

Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 40 Convent Drive, Bethesda, Maryland 20892-3017,[1] and Statistical Center for HIV/AIDS Research and Prevention, Fred Hutchinson Cancer Research Center, Seattle, Washington 98109-1024[2]

Received 2 May 2006/Returned for modification 9 June 2006/Accepted 20 August 2006

Ebola viruses represent a class of filoviruses that causes severe hemorrhagic fever with high mortality. Recognized first in 1976 in the Democratic Republic of Congo, outbreaks continue to occur in equatorial Africa. A safe and effective Ebola virus vaccine is needed because of its continued emergence and its potential for use for biodefense. We report the safety and immunogenicity of an Ebola virus vaccine in its first phase I human study. A three-plasmid DNA vaccine encoding the envelope glycoproteins (GP) from the Zaire and Sudan/Gulu species as well as the nucleoprotein was evaluated in a randomized, placebo-controlled, double-blinded, dose escalation study. Healthy adults, ages 18 to 44 years, were randomized to receive three injections of vaccine at 2 mg ($n = 5$), 4 mg ($n = 8$), or 8 mg ($n = 8$) or placebo ($n = 6$). Immunogenicity was assessed by enzyme-linked immunosorbent assay (ELISA), immunoprecipitation-Western blotting, intracellular cytokine staining (ICS), and enzyme-linked immunospot assay. The vaccine was well-tolerated, with no significant adverse events or coagulation abnormalities. Specific antibody responses to at least one of the three antigens encoded by the vaccine as assessed by ELISA and $CD4^+$ T-cell GP-specific responses as assessed by ICS were detected in 20/20 vaccinees. $CD8^+$ T-cell GP-specific responses were detected by ICS assay in 6/20 vaccinees. This Ebola virus DNA vaccine was safe and immunogenic in humans. Further assessment of the DNA platform alone and in combination with replication-defective adenoviral vector vaccines, in concert with challenge and immune data from nonhuman primates, will facilitate evaluation and potential licensure of an Ebola virus vaccine under the Animal Rule.

Outbreaks of infection with Ebola virus result in a rapid and severe disease with high mortality, for which there is currently no licensed antiviral treatment or vaccine. While outbreaks remain unpredictable, they have occurred with increasing frequency in equatorial Africa, west of the Rift Valley, where they have infected both humans and nonhuman primates and have significantly depleted chimpanzee and gorilla populations in Central Africa. Although a potential reservoir has been suggested (15), the risk of zoonotic transmission remains high and unpredictable (1). Announcements of Ebola virus outbreaks cause widespread fear and have socioeconomic consequences beyond the direct impact on infected persons. Outbreaks of Ebola virus infection have become more frequent since its discovery in 1976 and reemergence in 1995, and there are now areas in which the infection appears to be endemic. Although there have been no human outbreaks of Ebola virus in the United States, the virus caused an outbreak in imported laboratory nonhuman primates in Reston, Va., in 1989. It is also considered to be a potential bioweapon. For these reasons, vaccine development for Ebola virus and other filoviruses has become a priority.

Outbreaks of hemorrhagic fever caused by the Ebola virus are associated with high mortality rates. The highest lethality is associated with the Zaire subtype, one of four species identified to date (9, 21). An outbreak of hemorrhagic fever reported in October 2000 in the Gulu district of Uganda was confirmed as Ebola virus and resulted in the deaths of dozens of people (6). Another outbreak in Gabon and the Republic of Congo likely involved several independent introductions. It continued from 2001 through 2003 and resulted in more than 100 deaths (3, 28). The triggers for such outbreaks are not understood, although there may be a correlation with climatic changes (31). These periodic but devastating outbreaks underscore the difficulty in controlling this virus that emerges periodically via uncertain primary transmission routes and then disappears into an unclearly defined natural reservoir.

Infection with Ebola virus initially results in an influenza-like syndrome that progresses to severe illness manifested by co-

* Corresponding author. Mailing address: Vaccine Research Center, NIAID/NIH, 40 Convent Drive, MSC-3017, Room 2502, Bethesda, MD 20892-3017. Phone: (301) 594-8468. Fax: (301) 480-2771. E-mail: bgraham@nih.gov.

† Supplemental material for this article may be found at http://cvi.asm.org/.

‡ The VRC 204 Study Team includes Margaret M. McCluskey, Brenda Larkin, Sarah Hubka, Lasonji Holman, Laura Novik, Pamela Edmonds, Steve Rucker, Michael Scott, Colleen Thomas, LaChonne Stanford, Ed Tramont, Woody Dubois, Tiffany Alley, Erica Eaton, Sandra Sitar, Ericka Thompson, Andrew Catanzaro, Joseph Casazza, Janie Parrino, Laurence Lemiale, Rebecca Sheets, Ellen Turk, Laurie Lamoreaux, Jennifer Fischer, Mara Abashian, John Rathmann, and Adrienne McNeil.

▽ Published ahead of print on 20 September 2006.

agulation abnormalities, disseminated intravascular coagulation, multi-organ system involvement, and an exaggerated but nonprotective inflammatory response (1). Fatality rates range from 50 to 90%, and death is frequently due to bleeding and hypotensive shock (22). The rapid advancement of severe disease following Ebola virus infection allows little opportunity to develop natural immunity, and there is no effective antiviral therapy currently available. Vaccination offers a propitious intervention to prevent infection and limit spread as well as an important public health benefit for health care workers involved in care of patients and the containment of outbreaks.

Because of the potential safety concerns associated with using conventional vaccination strategies, such as attenuated or inactivated Ebola virus as an immunogen, the vaccination strategies that have been evaluated in published preclinical studies to date for Ebola virus have focused on the use of live and replication-defective vectors and virus-like particles. Published studies have included expression of Ebola virus protein subunits in live vaccinia virus vectors, selected DNA plasmids, Ebola virus-like particles, replication-competent vesicular stomatitis virus, Venezuela equine encephalitis virus replicons, recombinant parainfluenza virus type 3, replication-defective recombinant adenovirus (rAd), and DNA combined with rAd prime-boost strategies (5, 12, 14, 19, 22, 24, 26, 31). While many of these approaches have been evaluated in a nonhuman primate model (10), only DNA/rAd-, rAd-, or vesicular stomatitis virus-based vaccines have shown efficacy in primates.

DNA vaccination conferred an Ebola virus-specific immune response in guinea pigs and mice (4, 25, 32) that protected against a challenge with Ebola virus adapted to produce lethal infection in rodents (1, 4, 8). Humoral and T-cell-mediated immunity were elicited in these animal models, but antibody titers appeared to correlate better with protection following immunization with plasmids carrying genes for Ebola virus Zaire proteins. DNA vaccination followed by a boost with recombinant adenoviral vectors encoding Ebola viral proteins uniformly protected nonhuman primates from an otherwise-lethal dose of Ebola virus (25). Protection correlated with the development of Ebola virus-specific $CD8^+$ T-cell and antibody production. The vaccinated animals remained protected and asymptomatic following challenge with a lethal dose of the highly pathogenic, wild-type, 1976 Mayinga strain of Ebola virus Zaire (22, 24, 25).

Ideally, an effective vaccine would provide immunity to the multiple Ebola virus species that have been isolated in human infections and may require multiple antigenic specificities. There is a concern that combining multiple expression vectors in the same vaccine may result in interference of expression of some of the constructs (23). However, a multigene vaccine containing antigens for Ebola virus glycoproteins from the Zaire, Ivory Coast, and Sudan viruses induced specific immune responses to all three subtypes without evidence of interference in an animal model (24, 25). Additionally, a series of studies showed that both GP and sGP (a soluble form of the glycoprotein) conferred optimal protection in the guinea pig model (25, 32). We report the results of the first human clinical trial of a candidate Ebola virus DNA vaccine and show that plasmids expressing Ebola virus GP (Zaire [Z]), GP (Sudan/Gulu [S/G]), and NP (Z) are safe and well-tolerated and induce Ebola virus-specific antibody and T-cell responses in healthy adults.

MATERIALS AND METHODS

Study design. Protocol VRC 204 was a single-site, phase I, randomized, placebo-controlled, double-blinded, dose escalation study to examine safety and tolerability, dose, and immune response to an investigational Ebola virus plasmid DNA vaccine. Healthy adult volunteers 18 to 44 years of age were recruited at the NIAID Vaccine Research Center Clinic, National Institutes of Health (Bethesda, Md.). Human experimental guidelines of the U.S. Department of Health and Human Services were followed in the conduct of clinical research, and the protocol was reviewed and approved by the National Institute of Allergy and Infectious Diseases (NIAID) Institutional Review Board. Three sequential groups of volunteers were enrolled between November 2003 and July 2004 to receive placebo or vaccine at doses of 2.0 mg, 4.0 mg, and 8.0 mg, respectively. Group 1 subjects ($n = 7$) were randomized in a ratio of 5 vaccine/2 placebo; group 2 ($n = 10$) and group 3 ($n = 10$) subjects were randomized in a ratio of 8 vaccine/2 placebo. Total enrollment was 27 volunteers (21 vaccine, 6 placebo). In all groups, the vaccine was administered on study days 0, 28 ± 7, and 56 ± 7 (with at least 21 days between injection days).

Safety through at least 2 weeks after the second injection at each dose level was reviewed by a data and safety monitoring board prior to enrolling volunteers into the next dose level group. For the 2.0-mg and 4.0-mg immunizations, a single dose of vaccine or placebo required a volume of 1.0 ml, administered by intramuscular injection in the lateral deltoid muscle. The maximum concentration of vaccine formulation is 4 mg/ml; therefore, two 1.0-ml injections (one intramuscular injection in each deltoid muscle) of this formulation were necessary to deliver the 8.0-mg dose. Placebo was administered the same way as vaccine for each dosage group. All intramuscular injections were administered with the Biojector 2000 needle-free injection management system. Adverse reactions were evaluated by laboratory and clinical evaluations at scheduled study visits, coded using the *Medical Dictionary for Regulatory Activities*, and severity graded using a scale of 0 to 5. Solicited reactogenicity was collected by study subject report on 7-day diary cards. Subjects were followed for a total of 12 months, and the study was completed in August 2005.

Vaccine. This multigene plasmid DNA vaccine is a mixture of three plasmids in equal concentrations that were constructed to produce three Ebola virus proteins designed to elicit broad immune responses to multiple Ebola virus subtypes. These proteins include the nucleoprotein (NP) derived from the Zaire strain of Ebola virus, which exhibits highly conserved domains, as well as two glycoproteins (GP), which mediate viral entry, from the Zaire strain (homologous to the Ivory Coast strain) and the Sudan/Gulu species (associated with recent outbreaks of hemorrhagic fever in Africa). The Ebola virus GP genes expressed by plasmid DNA constructs in this vaccine contain deletions in the transmembrane region of GP that were intended to eliminate potential cellular toxicity observed in the in vitro experiments using plasmids expressing the full-length wild-type GPs (33). In addition, the Ebola virus GP inserts have been modified to optimize expression in human cells. The three plasmids in this vaccine are incapable of replication in animal cells and would not permit the generation of an infectious virion even if recombination or gene duplication were to occur.

The vaccine plasmids were prepared by cloning the Ebola virus gene sequences into the VR-1012 expression vector produced by Vical, Inc. (San Diego, CA) (13). The VR-1012 expression vector is very similar to the vector backbone used in a plasmid DNA-based malaria vaccine (29) and a multiclade human immunodeficiency virus (HIV) DNA vaccine that has been tested in humans (11). To generate the vaccine (EBODNA012-00-VP) tested in this clinical trial, Ebola virus GP gene sequences were subcloned into a slightly modified VR-1012 plasmid backbone containing the human T-cell leukemia virus 1 R region translational enhancer for improved expression (2). The CMV/R expression vector has been tested in a clinical trial of a multiclade HIV DNA vaccine (11) and in other candidate vaccines currently undergoing evaluation in clinical studies by the Vaccine Research Center and the Division of AIDS, NIAID, National Institutes of Health.

The DNA plasmids were produced in bacterial cell cultures containing a kanamycin selection medium. The process involved *Escherichia coli* fermentation, purification, and formulation as a sterile liquid injectable dosage form for intramuscular injection. Following growth of bacterial cells harboring the plasmid, the plasmid DNA was purified from cellular components.

The vaccine was produced by Vical, Inc. (San Diego, CA), under current Good Manufacturing Practices conditions and met lot release specifications prior to administration. This naked DNA product involves no lipid, viral, or cellular vector components. A phosphate-buffered saline placebo control, pH 7.2, was

TABLE 1. Demographic characteristics at enrollment

Characteristic and subcategory	No. (%) of subjects with characteristic in dose group				
	2 mg (n = 5)	4 mg (n = 8)	8 mg (n = 8)	Placebo (n = 6)	Overall (n = 27)
Gender					
Male	5 (100.0)	5 (62.5)	5 (62.5)	3 (50.0)	18 (66.7)
Female	0 (0.0)	3 (37.5)	3 (37.5)	3 (50.0)	9 (33.3)
Age[a] (yrs)					
18–20	0 (0.0)	0 (0.0)	1 (12.5)	0 (0.0)	1 (3.7)
21–30	1 (20.0)	3 (37.5)	3 (37.5)	0 (0.0)	7 (25.9)
31–44	4 (80.0)	5 (62.5)	4 (50.0)	6 (100.0)	19 (70.4)
Mean (SD)	34.0 (6.4)	36.4 (8.8)	28.3 (8.4)	34.7 (1.9)	33.1 (7.6)
Range	24–41	24–44	18–43	32–37	18–44
Race					
White	5 (100.0)	8 (100.0)	7 (87.5)	6 (100.0)	26 (96.3)
Black or African American	0 (0.0)	0 (0.0)	1 (12.5)	0 (0.0)	1 (3.7)
Asian	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
American Indian/Alaskan Native	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Native Hawaiian or other Pacific Islander	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Multiracial	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Other/unknown	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Ethnicity					
Non-Hispanic/Latino	5 (100.0)	8 (100.0)	8 (100.0)	5 (83.3)	26 (96.3)
Hispanic/Latino	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	1 (3.7)
BMI[b]					
Under 18.5	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
18.5–24.9	4 (80.0)	6 (75.0)	3 (37.5)	3 (50.0)	16 (59.3)
25.0–29.9	0 (0.0)	1 (12.5)	3 (37.5)	2 (33.3)	6 (22.2)
30.0 or over	1 (20.0)	1 (12.5)	2 (25.0)	1 (16.7)	5 (18.5)
Mean (SD)	23.0 (4.0)	25.8 (7.1)	27.0 (7.9)	25.5 (5.3)	25.6 (6.4)
Range	20.1–30.1	21.0–42.2	19.5–39.2	20.6–34.7	19.5–42.2
Education					
Less than high school graduate	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
High school graduate/GED	1 (20.0)	2 (25.0)	2 (25.0)	0 (0.0)	5 (18.5)
College/university	3 (60.0)	3 (37.5)	5 (62.5)	2 (33.3)	13 (48.1)
Advanced degree	1 (20.0)	3 (37.5)	1 (12.5)	4 (66.7)	9 (33.3)

[a] Age at enrollment day.
[b] Height and weight (used for BMI) were from the screening evaluation.

produced under current Good Manufacturing Practices conditions by Bell-More Labs, Inc. (Hampstead, MD).

Measurement of antibody responses: enzyme-linked immunosorbent assay (ELISA). Endpoint titers of antibodies directed against Ebola virus antigens NP (Z), GP (S/G), and GP (Z) were determined using 96-well Immulon 2 plates (Dynex Technologies) coated with a preparation of purified recombinant proteins according to methods adapted from those described previously (11). Biotin-labeled anti-human immunoglobulin G (IgG), IgA, or IgM and streptavidin conjugated with horseradish peroxidase and 3,5′,5,5′-tetramethylbenzidine substrate was used to develop the reaction, which was detected on a Spectramax microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). The endpoint titer was calculated as the most dilute serum concentration that gave an optical density reading of >0.2 above background.

Measurement of antibody responses by immunoprecipitation and Western blot analysis. Antibody responses were measured in a semiquantitative assay combining immunoprecipitation (IP) of crude cell-free supernatants containing GP (Z) or GP (S/G) or cell lysates containing NP protein with volunteer sera, followed by Western blotting for GP or NP as previously described (7). Briefly, sera (10 μl) from immunized individuals were used to immunoprecipitate Ebola virus proteins either from 100 μl of cell-free supernatant or from cell lysates of 293 cells (100 μl of cell lysate is equivalent to 300 to 400 μg of total protein) transfected with vectors encoding transmembrane-deleted Ebola virus GP (Z) or transmembrane-deleted Ebola virus GP (S/G) or Ebola virus NP (Z). Immune complexes were separated by sodium dodecyl sulfate–7.5% polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by immunoblotting using the following Ebola virus protein-specific antibodies: mouse monoclonal 12B5 (generous gift from Mary Kate Hart, USAMRIID) against GP (Z), rabbit polyclonal B83 (generous gift from Barton Haynes, Duke University) against GP (S/G), or mouse monoclonal 1C9 (generous gift from Barton Haynes, Duke University) against NP. Preimmune sera (10 μl) from those individuals were used as controls. The gels were scanned, and the intensity of each band was quantified by densitometry using the program ImageQuant; results are presented graphically to facilitate comparisons among groups.

Measurement of T-cell responses and cell preparation. Peripheral blood mononuclear cells (PBMC) were prepared by standard Ficoll-Hypaque density gradient centrifugation (Pharmacia, Uppsala, Sweden). PBMC were frozen in heat-inactivated fetal calf serum containing 10% dimethyl sulfoxide in a Forma CryoMed cell freezer (Marietta, OH). Cells were stored at −140°C. All immunogenicity assays were performed on thawed specimens; average viability was >95%.

Antibodies. Unconjugated mouse anti-human CD28, unconjugated mouse anti-human CD49d, allophycocyanin-conjugated mouse anti-human CD3, fluorescein isothiocyanate-conjugated mouse anti-human CD8, peridinin chlorophyll protein-conjugated mouse anti-human CD4, and a mixture of phycoerythrin-conjugated mouse anti-human gamma interferon (IFN-γ) and interleukin 2 (IL-2) monoclonal antibodies were obtained from Becton Dickinson Immunocytometry Systems (BDIS; San Jose, CA). All reagents were independently titrated to determine the optimum concentrations for staining.

Peptides and cell stimulation. Peptides 15 amino acids in length, overlapping by 11, and corresponding to the vaccine inserts were synthesized at >85% purity as confirmed by high-performance liquid chromatography (24). Peptides were pooled for each protein, NP (Z), GP (S/G), and GP (Z), and used at a final

concentration of 500 ng per stimulation. Cell stimulation was performed as described previously (11). Briefly, one million PBMC in 200 μl R-10 medium (RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, and 1.7 mM sodium glutamate) were incubated with 1 μg/ml each of costimulatory anti-CD28 and -CD49d monoclonal antibodies and 2.5 μg/ml of each peptide in wells of 96-well V-bottom plates. Cells incubated with only costimulatory antibodies were included in every experiment to control for spontaneous production of cytokine and activation of cells prior to addition of peptides. Staphylococcal enterotoxin B (10 μg/ml; Sigma-Aldrich) was used as a positive control for lymphocyte activation. Cultures were incubated at 37°C in a 5% CO_2 incubator for 6 h in the presence of brefeldin A (10 μg/ml; Sigma, St. Louis, MO).

Intracellular cytokine and immunofluorescence staining. Cells were permeabilized for 7 min in 200 μl of a solution containing 67 μl Tween 20 (Sigma), 106 μl deionized water, and 27 μl of 10× FACS-Lyse solution (BDIS) at room temperature, washed twice in cold Dulbecco's phosphate-buffered saline containing 1% fetal bovine serum and 0.02% sodium azide (FACS [fluorescence-activated cell sorting] buffer), and stained directly with conjugated anti-human CD3, anti-human CD4, anti-human CD8, and anti-human IFN-γ and IL-2 antibodies for 15 min on ice. Stained cells were then immediately washed twice with cold FACS buffer. The cells were resuspended in Dulbecco's phosphate-buffered saline containing 1% paraformaldehyde (Electron Microscopy Systems, Fort Washington, PA) and stored at 4°C until analysis. Four-parameter flow cytometric analysis was performed on a FACSCalibur flow cytometer (BDIS). Following intracellular cytokine staining (ICS), between 50,000 and 250,000 events were acquired, gated on small lymphocytes, and assessed for CD3, CD8, CD4, and IFN-γ/IL-2 expression. Results were analyzed using FlowJo software (Tree Star Software, Ashland, OR). The same cytokine, CD4, and CD8 gates were used for the entire trial.

ELISPOT. Vaccine-induced T-cell responses were also detected by enzyme-linked immunospot assays (ELISPOT) according to a modification of previously published methods (11) using a commercially available ELISPOT kit (BD Biosciences). PBMC were stimulated overnight at 37°C in triplicate wells at a density of 2×10^5 cells/well for all stimulations other than staphylococcal enterotoxin B, which was conducted at 5×10^4 cells/well. Following incubation, cells were lysed, and the wells were washed and incubated for 2 h at room temperature in the presence of biotinylated IFN-γ detection antibodies. Subsequently, the wells were incubated with an avidin-horseradish peroxidase solution for 1 h at room temperature, followed by a 20-min incubation with the AEC substrate solution. The plate was air dried for a minimum of 2 hours prior to spot quantitation on a CTL ELISPOT image analyzer (Cellular Technology Ltd., Cleveland, OH). Results were expressed as mean spot-forming cells per million PBMC.

Statistical methods. Positive response rates to any antigen (GP [S/G], GP [Z], or NP [Z]) and to each individual antigen were used to summarize the T-cell response data; exact two-sided 95% confidence intervals (29) are reported. The positivity criteria for the ICS data consisted of a statistical hypothesis test for a difference in the stimulated and unstimulated wells followed by the requirement of a minimal level of response. For an individual's response to be categorized as positive, it had to be statistically significant and had to exceed the threshold for positivity. Positivity thresholds were based on an ICS validation study of HIV peptides completed at the VRC. The thresholds were selected to give a 1% false-positive rate across PBMC from 34 HIV type 1-seronegative individuals stimulated with eight HIV peptide pools in the validation data set. Only 2 of the 272 samples (0.007) had responses exceeding the thresholds. The validation study results using HIV peptides are expected to be relevant for the Ebola virus peptides; hence, in addition to the statistical hypothesis test for positivity, the same thresholds were used. For the ICS responses, Fisher's exact test was applied to each antigen-specific response versus the negative control response, with a Holm adjustment for the multiple comparisons. The nominal significance level was $\alpha = 0.01$, and the minimum threshold for background-corrected percent positive response was 0.0241 for $CD4^+$ and 0.0445 for $CD8^+$. To determine positivity of the ELISPOT responses, a permutation test was applied to each antigen-specific response versus negative control responses using the Westfall-Young approach to adjust for the multiple comparisons. The nominal significance level was $\alpha = 0.05$. In addition, for the sample to be categorized as positive, the result had to achieve a statistically significant difference and be above a predetermined cutoff set at a false-positive rate of <1% (i.e., the mean difference in the antigen-stimulated wells and the negative control wells had to be greater than or equal to 10 spot-forming cells per 2×10^5 PBMC). A variance filter for the antigen-specific responses was also used: samples with a ratio of antigen-well variance (median, +1) greater than or equal to 100 were discarded from the analysis; no such samples were found in the data set. SAS (version 9.1; SAS Institute) and Splus (version 6.0; Insightful) were used for all analyses.

TABLE 2. Local and systemic reactogenicity[a]

Symptom and intensity	No. (%) of patients with reaction in dose group			
	2 mg (n = 5)	4 mg (n = 8)	8 mg (n = 8)	Placebo (n = 6)
Local symptoms				
Pain or tenderness				
None	0 (0.0)	1 (12.5)	2 (25.0)	3 (50.0)
Mild	5 (100.0)	5 (62.5)	3 (37.5)	3 (50.0)
Moderate	0 (0.0)	2 (25.0)	3 (37.5)	0 (0.0)
Severe	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Induration				
None	3 (60.0)	4 (50.0)	2 (25.0)	6 (100.0)
Mild	2 (40.0)	4 (50.0)	6 (75.0)	0 (0.0)
Moderate	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Severe	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Skin discoloration				
None	2 (40.0)	1 (12.5)	2 (25.0)	5 (83.3)
Mild	3 (60.0)	7 (87.5)	6 (75.0)	1 (16.7)
Moderate	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Severe	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Any local symptom				
None	0 (0.0)	1 (12.5)	1 (12.5)	3 (50.0)
Mild	5 (100.0)	5 (62.5)	4 (50.0)	3 (50.0)
Moderate	0 (0.0)	2 (25.0)	3 (37.5)	0 (0.0)
Severe	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Systemic symptoms				
Malaise				
None	4 (80.0)	6 (75.0)	3 (37.5)	4 (66.7)
Mild	1 (20.0)	0 (0.0)	4 (50.0)	0 (0.0)
Moderate	0 (0.0)	2 (25.0)	0 (0.0)	2 (33.3)
Severe[b]	0 (0.0)	0 (0.0)	1 (12.5)	0 (0.0)
Myalgia				
None	5 (100.0)	7 (87.5)	5 (62.5)	6 (100.0)
Mild	0 (0.0)	1 (12.5)	3 (37.5)	0 (0.0)
Moderate	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Severe	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Headache				
None	4 (80.0)	5 (62.5)	6 (75.0)	5 (83.3)
Mild	1 (20.0)	3 (37.5)	1 (12.5)	1 (16.7)
Moderate	0 (0.0)	0 (0.0)	1 (12.5)	0 (0.0)
Severe	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Nausea				
None	4 (80.0)	6 (75.0)	3 (37.5)	5 (83.3)
Mild	1 (20.0)	2 (25.0)	4 (50.0)	1 (16.7)
Moderate	0 (0.0)	0 (0.0)	1 (12.5)	0 (0.0)
Severe	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Fever				
None	5 (100.0)	7 (87.5)	8 (100.0)	6 (100.0)
Mild	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Moderate	0 (0.0)	1 (12.5)	0 (0.0)	0 (0.0)
Severe	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Any systemic symptom				
None	4 (80.0)	5 (62.5)	3 (37.5)	3 (50.0)
Mild	1 (20.0)	1 (12.5)	3 (37.5)	1 (16.7)
Moderate	0 (0.0)	2 (25.0)	1 (12.5)	2 (33.3)
Severe[b]	0 (0.0)	0 (0.0)	1 (12.5)	0 (0.0)

[a] The local injection site reactions were recorded by clinicians at 30 to 45 min postinjection and were then recorded as self-assessments at home by subjects on a 7-day diary card. Systemic reactions were recorded as self-assessments at home by subjects on a 7-day diary card following each injection.

[b] A single severe systemic symptom (malaise) was related to a foot fracture which occurred 6 days following vaccination.

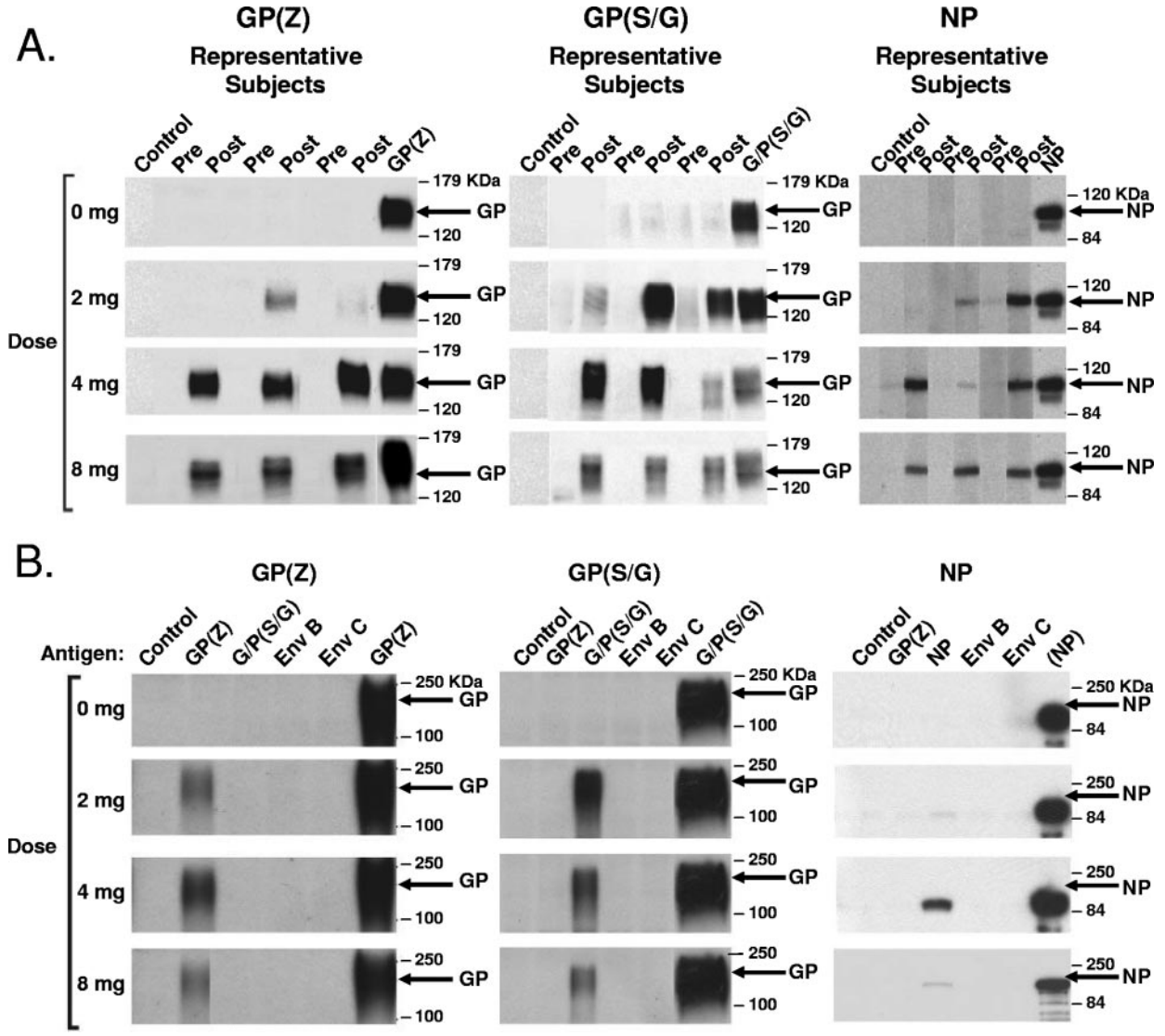

FIG. 1. Specific antibody responses to all vaccine components by IP-Western blot analysis. Sera from three representative subjects from each vaccine dose group are shown for each antigen (A). Sera were drawn at week 12, 4 weeks following the third vaccination. Antibody responses were specific and not cross-reactive to other vaccine antigens based on immunoblotting with monoclonal antibodies (B).

RESULTS

Study population demographics. A total of 27 healthy adult volunteers were enrolled, with 5 in the 2-mg dose group, 8 each in the 4-mg and 8-mg dose groups, and 6 in the placebo group. Table 1 includes demographic data regarding subject gender, age, race/ethnicity, body mass index (BMI), and educational level at the time of enrollment. The subject population was 66.7% male and 33.3% female with a mean age of 33.1 years (range of 18 to 44 years). Subjects were predominantly white (96.3%) and non-Hispanic/Latino (96.3%). The mean BMI was 25.6 (range, 19.5 to 42.2). All subjects had an educational level of high school or higher, with 48.1% having college level degrees and 33.3% holding advanced degrees.

Vaccine safety. Due to a theoretical concern over GP-mediated cytopathicity (22), coagulation parameters of study subjects were closely monitored. At enrollment and throughout the study, D-dimer, prothrombin time, partial thromboplastin time, fibrinogen, complete blood count, and red blood cell smears were evaluated. There were no reportable coagulation laboratory abnormalities.

Two subjects were withdrawn from the vaccination schedule due to serious adverse events that were assessed as "possibly" related to vaccination: a grade 4 creatine phosphokinase elevation 2 weeks after first vaccination and a grade 2 herpes zoster thoracic dermatome eruption 3 weeks after the second vaccination, both in 8-mg recipients. Of note, the grade 4 creatine phosphokinase elevation was associated with vigorous exercise. These events resolved without sequelae, and these subjects continued to participate in the study and attended all study visits. Although only six of eight subjects in the 8-mg dose group received all three injections, the immunogenicity and safety laboratory values for all subjects are included in the

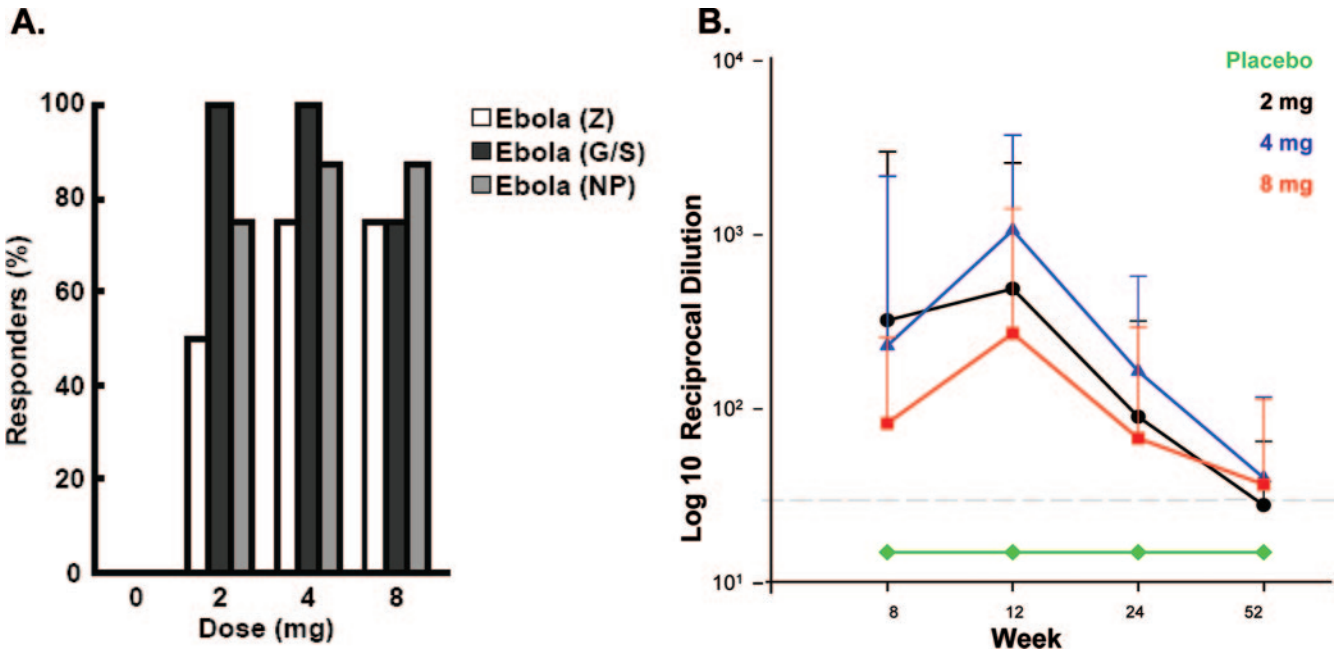

FIG. 2. Kinetics and frequency of antibody responses. (A) Percentages of responders following the third vaccination by the IP-Western assay for all subjects are shown. The *y* axis represents the percentage of responders with a positive assay, and the *x* axis represents the vaccine dose group. White bars, GP (Z); black bars, GP (S/G); gray bars, NP (Z). (B) Kinetics of the antibody response for all subjects is shown over the 52 weeks of the study. The geometric mean titer of the $\log_{10}$ reciprocal dilution and standard deviation of the antibody response to GP (S/G) are plotted against the number of weeks after initial vaccination for each of the three dose levels. Vaccinations were given at 0, 4, and 8 weeks. The threshold for positivity in this assay was a reciprocal dilution of 30 and is shown as a dashed line. Of note, only six of eight subjects in the 8-mg dose group received all three vaccinations in the series, yet all vaccinees are included in the immunogenicity analysis.

analyses. One subject in the 2-mg dose group chose to withdraw after the second vaccination; another subject (in the placebo group) withdrew after the third injection. Neither of these subjects returned for further visits and, therefore, they were not included in the immunogenicity analysis due to a lack of samples at time points following their withdrawal. As a result, 20 of 21 vaccinees had immune responses assessed. All subjects are represented in the safety data through the time points available.

The diary cards showed that 90.5% (19/21) of subjects who received vaccine (at any dose level) experienced at least one local injection site symptom (mild to moderate pain/tenderness, mild induration, or mild skin discoloration) following a vaccination. The systemic symptoms recorded on diary cards included malaise, myalgia, headache, nausea, and fever, as well as local injection site symptoms (Table 2). The study vaccinations were well-tolerated and safe in healthy subjects, ages 18 to 44 years.

Antibody responses. Ebola virus-specific humoral responses were detected in all vaccinees. GP- and NP-specific antibody responses were detected by IP-Western blot analysis (Fig. 1). Initial analysis of three representative subjects at different vaccine doses, 4 weeks following the third dose of vaccine (week 12), revealed antibodies specific for GP (Zaire) or GP (Sudan/Gulu) (Fig. 1A, left and middle panels) and to NP (Fig. 1A, right panel). These data demonstrate that specific antibodies to each antigen can be induced by the vaccine independently and are not cross-reactive (Fig. 1B). All (100%) of the 2-mg and 4-mg recipients and 75% (6/8) of the 8-mg recipients made GP antibodies. Three-fourths (75%) of the 2-mg and 87.5% of the 4-mg and 8-mg recipients produced an NP-specific antibody response (Fig. 2A). All (100%) vaccinees made a specific antibody response detected by ELISA to at least one of the three antigens encoded by the vaccine, with 19 of 20 vaccinees producing a GP (Z)- and GP (S/G)-specific antibody response at one or more time points (data not shown). This antibody response was detected after the second dose of vaccine in some subjects, peaked after the third dose (week 12), and waned over the course of 1 year (Fig. 2B). Antibody titers (reciprocal dilution) at week 12 ranged from undetectable to 4,000 for either GP antigen (see Tables S1 and S2 in the supplemental material). Ebola virus-specific neutralizing antibody, measured by a pseudotyped virus neutralization assay (23), was not detected in any study subject (data not shown), as might be expected with DNA vaccination in the absence of rAd boosting.

T-cell responses. $CD4^+$ and $CD8^+$ T-cell responses were assessed by ICS for all three antigens encoded by the vaccine for all study subjects. GP (S/G) was the stronger T-cell immunogen of the two GP antigens encoded by the vaccine, and NP induced the weakest response of the three antigens but was still measurable in the majority of vaccinees. An Ebola virus-specific $CD4^+$ T-cell response was demonstrated in all vaccinees by ICS, and many of these responses occurred by week 4, following just one dose of vaccine. $CD4^+$ T-cell responses for GP (S/G) were detected in 100% of vaccinees by week 10. By week 12, 100% of 2-mg (4/4) and 88% of 4-mg (7/8) and 8-mg (7/8) recipients produced a $CD4^+$ T-cell GP (Z)-specific response; by week 52, 100% of 2-mg (4/4) and 4-mg (8/8) recip-

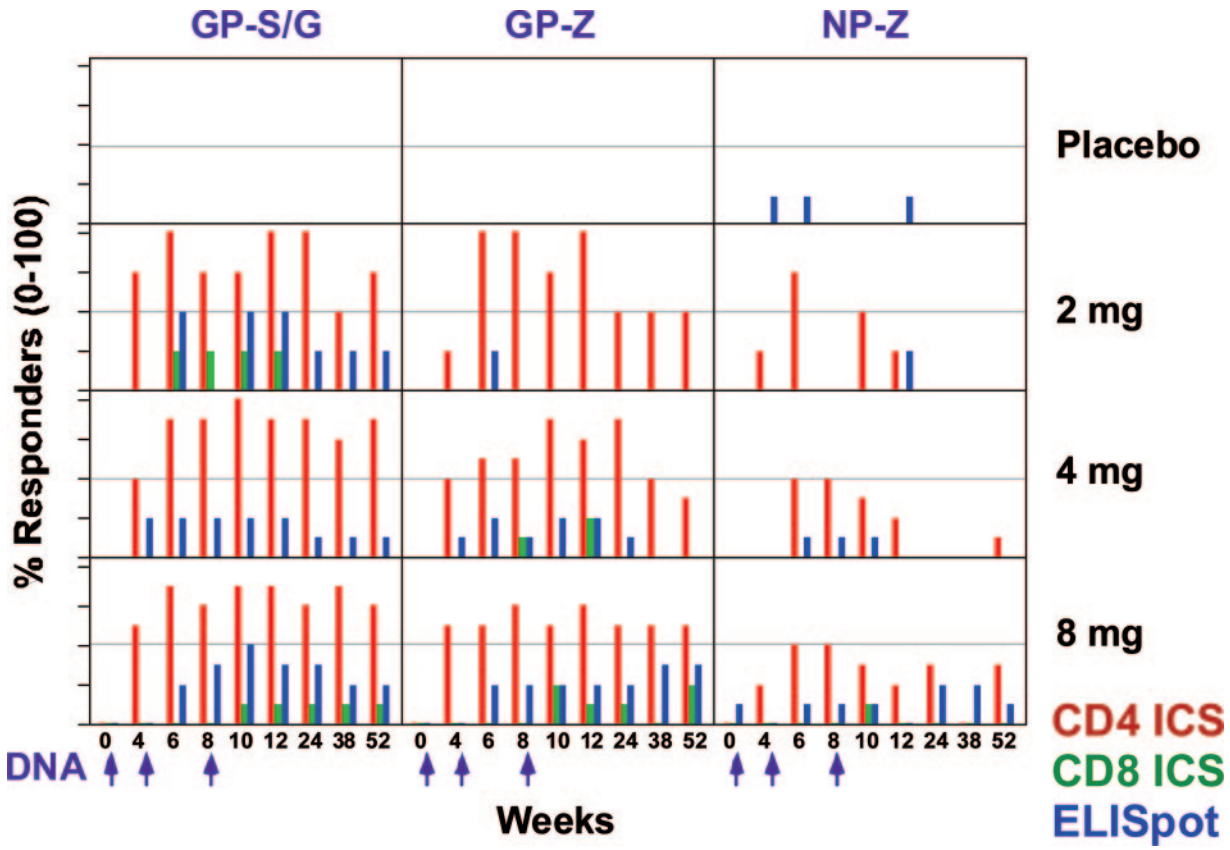

FIG. 3. Frequency of $CD4^+$ and $CD8^+$ T-cell responses by ICS and ELISPOT analysis. Frequency (percent responders) is represented on the left *y* axis. The week of analysis is shown on the lower *x* axis, the antigen assessed is shown on the upper *x* axis, and vaccine dose group is shown on the right *y* axis. The frequency of $CD4^+$ ICS responses is shown by red bars, the frequency of $CD8^+$ ICS responses is shown by green bars, and the frequency of positive ELISPOT responses is shown with blue bars. The schedule of the three DNA vaccinations is represented by arrows along the lower *x* axis.

ients and 88% (7/8) of 8-mg recipients produced a $CD4^+$ T-cell response to the GP (Z) antigen. By week 10, 75% (3/4) of 2-mg and 4-mg recipients (6/8) and 100% of 8-mg (8/8) recipients developed a $CD4^+$ T-cell response to NP (Fig. 3).

$CD8^+$ T-cell Ebola-specific responses were detected less frequently than $CD4^+$ T-cell responses but were present in 30% (6/20) of all vaccinees by ICS. By week 10, 25% (1/4) of 2-mg, none of the 4-mg, and 13% (1/8) of 8-mg recipients produced a $CD8^+$ T-cell response to GP (S/G). By week 12, none of the 2-mg and 25% (4/16) of the 4-mg and 8-mg recipients generated a $CD8^+$ T-cell response to GP (Z). None of the 2-mg or 4-mg vaccinees and only one of the 8-mg vaccinees produced a measurable $CD8^+$ T-cell response to the NP antigen by week 10 as assessed by ICS (Fig. 3).

Analyses were also performed on all study subjects for all three antigens by ELISPOT. Consistent with the ICS results, the dominant antigen was GP (S/G). By week 12, 50% (2/4) of 2-mg, 63% (5/8) of 4-mg, and 63% (5/8) of 8-mg recipients developed a positive ELISPOT response to GP (S/G). By week 12, 50% of 2-mg (2/4), 50% of 4-mg (4/8), and 38% of 8-mg (3/8) recipients had a positive ELISPOT response to GP (Z) as well. By week 24, 25% (1/4) of 2-mg, 13% (1/8) of 4-mg, and 63% (5/8) of 8-mg recipients displayed positive ELISPOT responses to the NP antigen (Fig. 3).

The magnitude of the $CD8^+$ T-cell response was slightly less than that seen in the $CD4^+$ T-cell analysis as assessed by ICS. The GP (S/G) immunogen induced slightly higher-magnitude $CD4^+$ T-cell responses compared to the other immunogens in the vaccine. The magnitude of the GP (Z)-specific $CD4^+$ response was 70% of the GP (S/G) response, while the magnitude of the NP-specific $CD4^+$ response was 16% of the GP (S/G) response. A correlation was not seen in the low number of positive responses as assessed by ICS for $CD8^+$ T cells (Fig. 4). Analysis of the kinetics of the T-cell responses revealed that the responses peaked between weeks 10 and 12 and, in general, detectable responses were not sustained, although there was a trend in the higher dose group toward a slightly greater duration of detectable responses (Fig. 5). Consistent with the ICS and antibody responses, the ELISPOT response was of greatest magnitude for the GP (S/G) antigen (see Table S3 in the supplemental material).

DISCUSSION

The rapid progression of severe disease after Ebola virus infection allows little opportunity to develop protective immunity, and there is currently no effective antiviral therapy. Therefore, vaccination offers a promising intervention to pre-

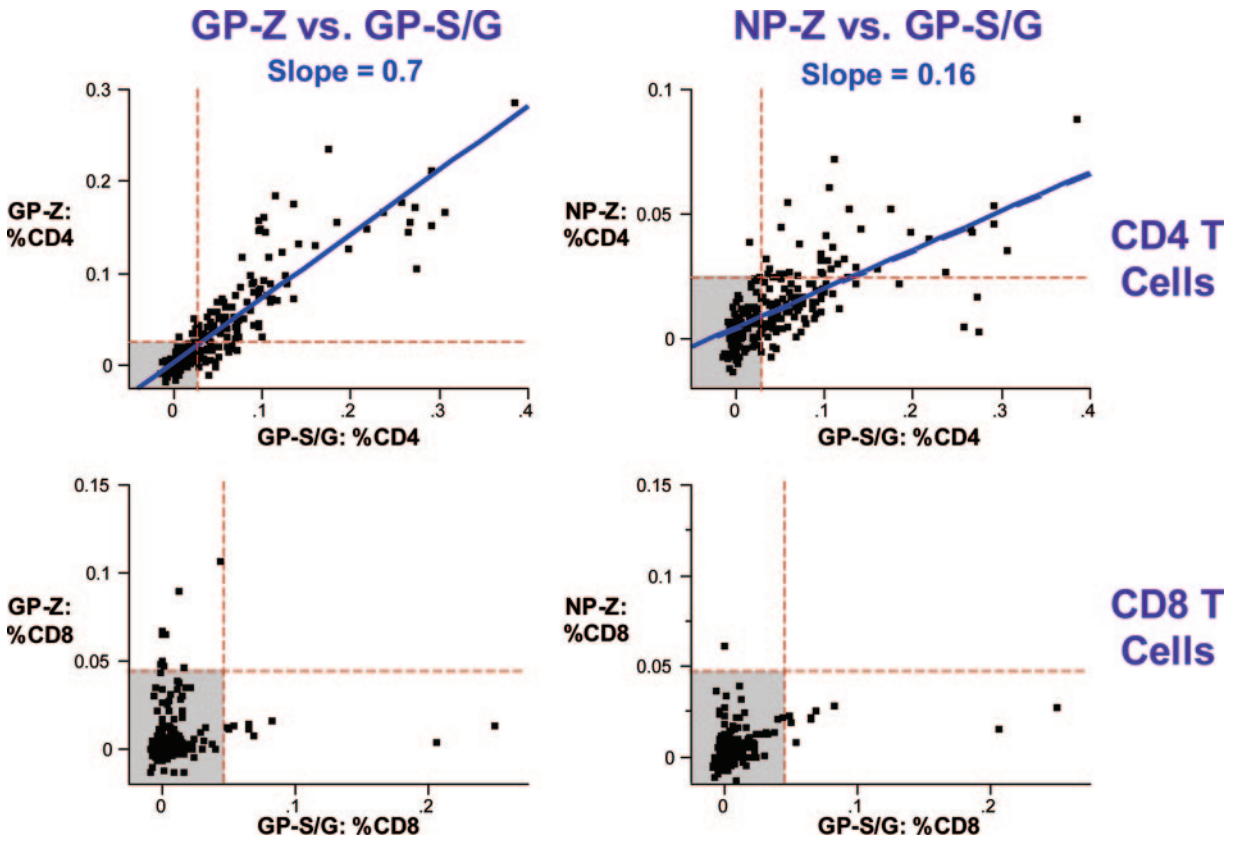

FIG. 4. Magnitude of antigen-specific T-cell responses to vaccine components. GP (S/G) (*x* axes) are shown in relation to each of the other two antigens, GP (Z) and NP (Z) (*y* axes). All antigen-specific $CD4^+$ and $CD8^+$ T-cell responses for all subjects (vaccine and placebo recipients) as assessed by ICS are shown. $CD4^+$ and $CD8^+$ T-cell responses are shown as a percentage of total $CD4^+$ or $CD8^+$ T cells on the *x* and *y* axes. CD4 responses are shown in the upper graphs, and $CD8^+$ responses are shown in the lower graphs. The red dashed line represents the threshold of positivity (0.0445% for $CD8^+$ T cells and 0.0241% for $CD4^+$ T cells).

vent infection or severe disease and limit spread to contacts and would be an important public health benefit for health care workers involved in the care of patients and containment of outbreaks. Another compelling reason for accelerated development of an Ebola virus vaccine relates to its contribution to biodefense (17). The Centers for Disease Control and Prevention (6) Category A agents are highly contagious and largely lack effective vaccines or treatments (18) and include the filoviruses (Ebola and Marburg viruses).

Gene-based vaccine technology provides a safe avenue for producing candidate vaccines for select agents without the need for extreme biocontainment. Gene-based vectors for filoviruses are particularly attractive vaccine approaches because of their capacity to induce both humoral and cell-mediated immune responses, both of which may be important for protection. The concept of using bacterium-derived plasmid DNA to deliver vaccine antigens has many attractive features, including (i) ease and flexibility of construction, (ii) scalable manufacturing capacity, (iii) stability, (iv) intracellular production of vaccine antigen, (v) transient expression, (vi) no induction of antivector immunity, (vii) induction of both $CD4^+$ and $CD8^+$ T-cell responses as well as antibody, and (viii) lack of local or systemic reactogenicity. However, DNA vaccines have not performed well enough to be considered as a vaccine platform in humans until recently. A hepatitis B virus DNA vaccine administered by a needle-free particle-mediated delivery was shown to be safe and immunogenic in a phase I clinical trial (20). Additionally, DNA vaccination against malaria was shown to be safe and immunogenic, especially as a priming vaccination in a prime-boost regimen (16, 30). Recently, a multiclade HIV DNA vaccine based on a similar design to the Ebola virus DNA vaccine described here was shown to be safe and immunogenic in healthy adults (11).

The broad immunogenicity of this Ebola virus DNA vaccine suggests that immunization by plasmid DNA delivery is a viable platform and merits further development. The consistent immunogenicity of the Ebola virus DNA vaccine described here likely reflects a combination of factors, including optimization of vector design, manufacturing methods, delivery, sample processing, and immunological assays. Additional work is needed to further improve the efficiency and consistency of DNA vaccination.

Nonhuman primate studies have shown that an rAd5 vaccine effectively prevents disease, and DNA vaccination prior to boosting with rAd5 also confers protection and markedly increases the magnitude of the immune response (22, 24, 25). Further vector and construct optimization may further increase protective immunity of this DNA vaccine. Recently, the importance of the GP transmembrane region in the design of the immunogen has been described. Reduced protection with

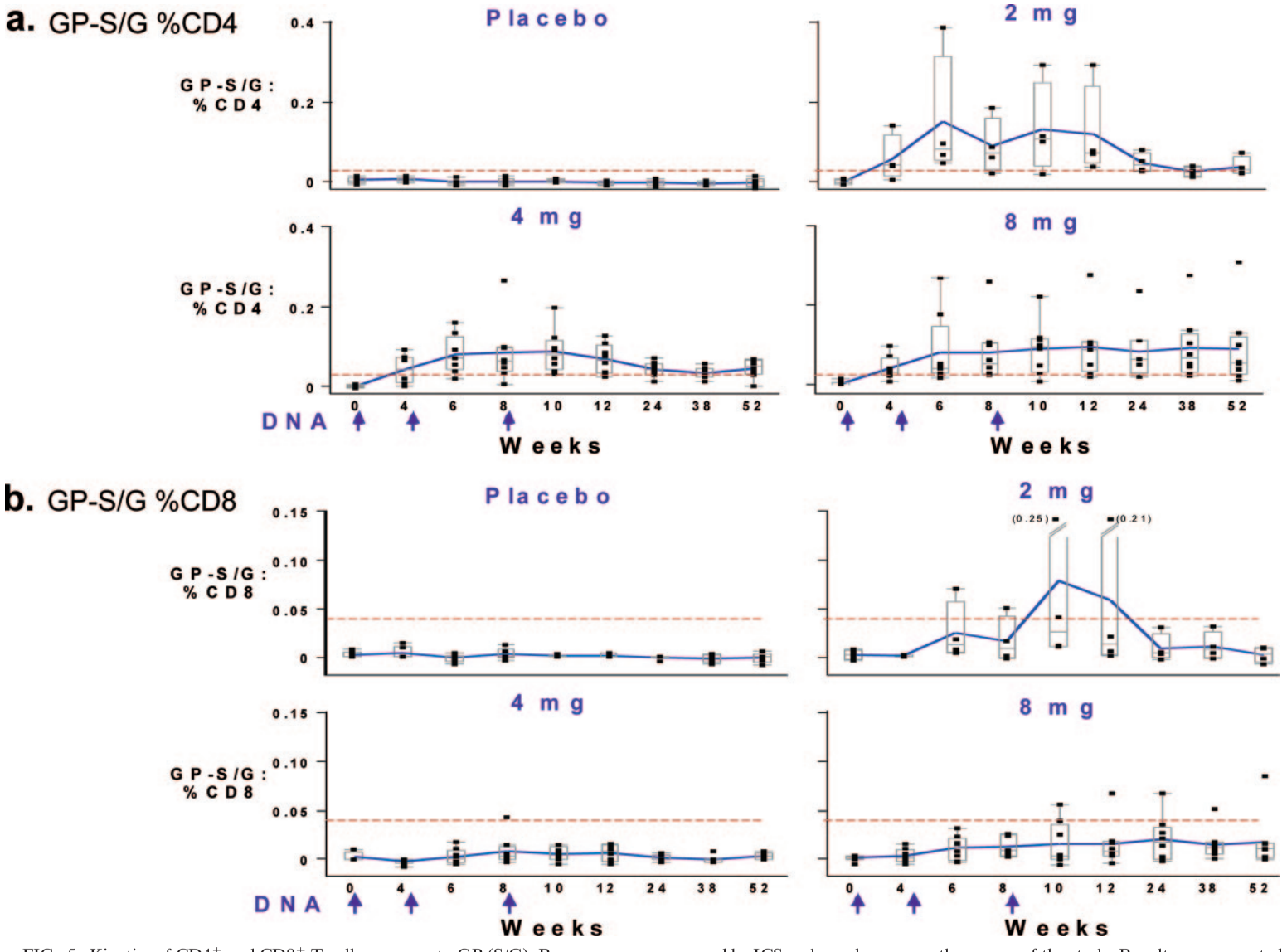

FIG. 5. Kinetics of $CD4^+$ and $CD8^+$ T-cell responses to GP (S/G). Responses were assessed by ICS and are shown over the course of the study. Results are presented by dose group, with a blue line indicating the mean response over time for all subjects in a given group. The median of the response is represented in the box and whisker plots at each time point. The red dashed lines represent the positivity threshold for the ICS assay.

an Ebola virus rAd immunogen containing a GP transmembrane region deletion compared to a point mutation in this region or wild-type GP constructs has been found (22). Additionally, it was found that the NP gene is dispensable for immune protection, and the addition of NP in a candidate vaccine may diminish the immune response to Ebola virus GP (23). Therefore, future formulations of this DNA product will include multiple GP constructs encoding GP in either its wild-type form or a modified form to optimize vaccine potency. Because Ebola virus from Ivory Coast has been observed in only one limited outbreak and is closely related to Ebola virus Zaire, it is not included in vaccine formulations.

This is the first report of an evaluation of a candidate Ebola virus vaccine in humans. This three-plasmid DNA candidate Ebola virus vaccine was safe and well-tolerated in 21 healthy adults. Importantly, DNA immunization induced both Ebola virus-specific antibody and T-cell responses to the GP and NP antigens. While Ebola virus-specific neutralizing antibody could not be detected in vaccinees, the range of antibody titers measured by ELISA was similar to those seen in nonhuman primates following vaccination with similar vaccine constructs (24). Recently, in a series of nonhuman primate studies demonstrating protection from Ebola virus with vaccine constructs expressing similar antigens as used in this clinic trial, IgG as measured by ELISA correlated with survival. In functional assays, serum antibodies were neither neutralizing nor enhancing, suggesting that IgG levels may reflect the overall level of immune stimulation. Although antibody-dependent enhancement of Ebola virus replication has been observed in tissue culture, there is no evidence of antibody-dependent enhancement in humans or in animal studies, and only protection, rather than enhancement, has been observed in animal studies evaluating DNA or rAd-based vaccine strategies (23, 27). In the clinical trial described here, the vaccine-induced antibody and T-cell-mediated immune responses were greatest to the GP immunogens, with a less frequent response to the NP immunogen, and Ebola virus-specific $CD4^+$ T-cell responses were more frequent than $CD8^+$ T-cell responses. While the presence of Ebola virus GP-specific IgG seems to predict survival in nonhuman primates, the definite correlate(s) of protection from Ebola virus infection is not known, and it is possible that T-cell responses also contribute to protection. Therefore, we believe it is important that a candidate Ebola virus vaccine be capable of eliciting both Ebola virus-specific antibody and T-cell responses.

Further studies are needed to determine the optimal preventive gene-based Ebola virus vaccine strategy. Our development plan includes evaluation of DNA vaccination alone, rAd5 vaccination alone, and a heterologous prime-boost strategy of DNA priming followed by rAd boosting. Even if the optimal strategy were determined to be heterologous prime-boost, the potential vaccines would need to be independently demonstrated as safe and immunogenic. Since the prophylactic efficacy of an Ebola virus vaccine cannot feasibly or ethically be demonstrated in a human trial, the combination of safety and immunogenicity data from phase I, II, and III human trials and efficacy data from nonhuman primate studies will ultimately need to be utilized to obtain licensure of an Ebola virus vaccine under the Animal Rule.

The successful evaluation of a DNA vaccine to multiple Ebola virus subtypes reported here provides the opportunity for further clinical evaluation of candidate Ebola virus DNA vaccines alone or in combination with Ebola virus rAd vaccines as a heterologous prime-boost strategy. Evaluation of gene-based candidate vaccines in humans will continue in parallel with efforts to define immunological correlates of vaccine-induced protection in nonhuman primate models of Ebola virus infection. Together, these studies will provide the scientific basis for identifying a vaccine strategy for the prevention of Ebola virus and other filovirus infections in humans.

ACKNOWLEDGMENTS

We thank the study volunteers who graciously gave their time and understand the importance of finding a safe and effective Ebola virus vaccine. We also thank NIH Clinical Center staff, NIAID staff, PRPL and OCPL staff, the members of the Intramural NIAID DSMB, EMMES Corporation (Phyllis Zaia, Lihan Yan, and others), Vical Incorporated (David Kaslow and others), Biojector, Inc. (Richard Stout and others), and other supporting staff (Richard Jones, Kathy Rhone, Katina Bryan, Theodora White, Ariella Blejer, and Monique Young) who made this work possible. We are grateful as well for the advice and important preclinical contributions of VRC investigators and key staff, including Daniel Douek, Yue Huang, Wing-Pui Kong, Peter Kwong, Norman Letvin, Abraham Mittelman, Steve Perfetto, Srini Rao, Robert Seder, Jessica Wegman, Richard Wyatt, Ling Xu, and Zhi-yong Yang.

This work was funded by the National Institute of Allergy and Infectious Diseases.

REFERENCES

1. **Baize, S., E. M. Leroy, M. C. Georges-Courbot, M. Capron, J. Lansoud-Soukate, P. Debre, S. P. Fisher-Hoch, J. B. McCormick, and A. J. Georges.** 1999. Defective humoral responses and extensive intravascular apoptosis are associated with fatal outcome in Ebola virus-infected patients. Nat. Med. **5:**423–426.
2. **Barouch, D. H., Z. Y. Yang, W. P. Kong, B. Korioth-Schmitz, S. M. Sumida, D. M. Truitt, M. G. Kishko, J. C. Arthur, A. Miura, J. R. Mascola, N. L. Letvin, and G. J. Nabel.** 2005. A human T-cell leukemia virus type 1 regulatory element enhances the immunogenicity of human immunodeficiency virus type 1 DNA vaccines in mice and nonhuman primates. J. Virol. **79:** 8828–8834.
3. **Birmingham, K., and S. Cooney.** 2002. Ebola: small, but real progress. Nat. Med. **8:**313.
4. **Bray, M., K. Davis, T. Geisbert, C. Schmaljohn, and J. Huggins.** 1998. A mouse model for evaluation of prophylaxis and therapy of Ebola hemorrhagic fever. J. Infect. Dis. **178:**651–661.
5. **Bukreyev, A., L. Yang, S. R. Zaki, W. J. Shieh, P. E. Rollin, B. R. Murphy, P. L. Collins, and A. Sanchez.** 2006. A single intranasal inoculation with a paramyxovirus-vectored vaccine protects guinea pigs against a lethal-dose Ebola virus challenge. J. Virol. **80:**2267–2279.
6. **Centers for Disease Control and Prevention.** 2001. Outbreak of Ebola hemorrhagic fever Uganda, August 2000–January 2001. Morb. Mortal. Wkly. Rep. **50:**73–77.
7. **Chakrabarti, B. K., W. P. Kong, B. Y. Wu, Z. Y. Yang, J. Friborg, X. Ling, S. R. King, D. C. Montefiori, and G. J. Nabel.** 2002. Modifications of human immunodeficiency virus envelope glycoprotein enhance immunogenicity for genetic immunization. J. Virol. **76:**5357–5368.
8. **Connolly, B. M., K. E. Steele, K. J. Davis, T. W. Geisbert, W. M. Kell, N. K. Jaax, and P. B. Jahrling.** 1999. Pathogenesis of experimental Ebola virus infection in guinea pigs. J. Infect. Dis. **179**(Suppl. 1)**:**S203–S217.
9. **Feldmann, H., S. T. Nichol, H. D. Klenk, C. J. Peters, and A. Sanchez.** 1994. Characterization of filoviruses based on differences in structure and antigenicity of the virion glycoprotein. Virology **199:**469–473.
10. **Geisbert, T. W., and P. B. Jahrling.** 2003. Towards a vaccine against Ebola virus. Expert Rev. Vaccines **2:**777–789.
11. **Graham, B. S., R. A. Koup, M. Roederer, R. Bailer, M. E. Enama, F. Z. Moodie, J. E. Martin, M. M. McCluskey, B. K. Chakrabarti, L. Lamoreaux, C. A. Andrews, P. L. Gomez, J. R. Mascola, G. J. Nabel, and VRC 004. Study Team.** Phase I safety and immunogenicity evaluation of a multiclade HIV-1 candidate DNA vaccine. J. Infect. Dis., in press.
12. **Hart, M. K.** 2003. Vaccine research efforts for filoviruses. Int. J. Parasitol. **33:**583–595.
13. **Hartikka, J., M. Sawdey, F. Cornefert-Jensen, M. Margalith, K. Barnhart, M. Nolasco, H. L. Vahlsing, J. Meek, M. Marquet, P. Hobart, J. Norman,**

and M. Manthorpe. 1996. An improved plasmid DNA expression vector for direct injection into skeletal muscle. Hum. Gene Ther. **7:**1205–1217.
14. **Jones, S. M., H. Feldmann, U. Stroher, J. B. Geisbert, L. Fernando, A. Grolla, H. D. Klenk, N. J. Sullivan, V. E. Volchkov, E. A. Fritz, K. M. Daddario, L. E. Hensley, P. B. Jahrling, and T. W. Geisbert.** 2005. Live attenuated recombinant vaccine protects nonhuman primates against Ebola and Marburg viruses. Nat. Med. **11:**786–790.
15. **Leroy, E. M., B. Kumulungui, X. Pourrut, P. Rouquet, A. Hassanin, P. Yaba, A. Delicat, J. T. Paweska, J. P. Gonzalez, and R. Swanepoel.** 2005. Fruit bats as reservoirs of Ebola virus. Nature **438:**575–576.
16. **Moorthy, V. S., E. B. Imoukhuede, S. Keating, M. Pinder, D. Webster, M. A. Skinner, S. C. Gilbert, G. Walraven, and A. V. Hill.** 2004. Phase 1 evaluation of 3 highly immunogenic prime-boost regimens, including a 12-month re-boosting vaccination, for malaria vaccination in Gambian men. J. Infect. Dis. **189:**2213–2219.
17. **National Institute of Allergy and Infectious Diseases.** 2002, posting date. NIAID biodefense research agenda for CDC category A agents. [Online.] http://www.niaid.nih.gov/biodefense/research/biotresearchagenda.pdf.
18. **Niiler, E.** 2002. Bioterrorism—biotechnology to the rescue? Nat. Biotechnol. **20:**21–25.
19. **Olinger, G. G., M. A. Bailey, J. M. Dye, R. Bakken, A. Kuehne, J. Kondig, J. Wilson, R. J. Hogan, and M. K. Hart.** 2005. Protective cytotoxic T-cell responses induced by Venezuelan equine encephalitis virus replicons expressing Ebola virus proteins. J. Virol. **79:**14189–14196.
20. **Roy, M. J., M. S. Wu, L. J. Barr, J. T. Fuller, L. G. Tussey, S. Speller, J. Culp, J. K. Burkholder, W. F. Swain, R. M. Dixon, G. Widera, R. Vessey, A. King, G. Ogg, A. Gallimore, J. R. Haynes, and D. Heydenburg Fuller.** 2000. Induction of antigen-specific $CD8^+$ T cells, T helper cells, and protective levels of antibody in humans by particle-mediated administration of a hepatitis B virus DNA vaccine. Vaccine **19:**764–778.
21. **Sanchez, A., S. G. Trappier, B. W. Mahy, C. J. Peters, and S. T. Nichol.** 1996. The virion glycoproteins of Ebola viruses are encoded in two reading frames and are expressed through transcriptional editing. Proc. Natl. Acad. Sci. USA **93:**3602–3607.
22. **Sullivan, N., Z. Y. Yang, and G. J. Nabel.** 2003. Ebola virus pathogenesis: implications for vaccines and therapies. J. Virol. **77:**9733–9737.
23. **Sullivan, N. J., T. W. Geisbert, J. B. Geisbert, D. J. Shedlock, L. Xu, L. Lamoreaux, J. H. Custers, P. M. Popernack, Z. Y. Yang, M. G. Pau, M. Roederer, R. A. Koup, J. Goudsmit, P. B. Jahrling, and G. J. Nabel.** 2006. Immune protection of nonhuman primates against Ebola virus with single low-dose adenovirus vectors encoding modified GPs. PLoS Med. **3:**e177.
24. **Sullivan, N. J., T. W. Geisbert, J. B. Geisbert, L. Xu, Z. Yang, M. Roederer, R. A. Koup, P. B. Jahrling, and G. J. Nabel.** 2003. Accelerated vaccination for Ebola virus haemorrhagic fever in non-human primates. Nature **424:**681–684.
25. **Sullivan, N. J., A. Sanchez, P. E. Rollin, Z. Y. Yang, and G. J. Nabel.** 2000. Development of a preventive vaccine for Ebola virus infection in primates. Nature **408:**605–609.
26. **Swenson, D. L., K. L. Warfield, D. L. Negley, A. Schmaljohn, M. J. Aman, and S. Bavari.** 2005. Virus-like particles exhibit potential as a pan-filovirus vaccine for both Ebola and Marburg viral infections. Vaccine **23:**3033–3042.
27. **Takada, A., and Y. Kawaoka.** 2003. Antibody-dependent enhancement of viral infection: molecular mechanisms and in vivo implications. Rev. Med. Virol. **13:**387–398.
28. **Thacker, P. D.** 2003. An Ebola epidemic simmers in Africa: in remote region, outbreak shows staying power. JAMA **290:**317–319.
29. **Wang, R., J. Epstein, F. M. Baraceros, E. J. Gorak, Y. Charoenvit, D. J. Carucci, R. C. Hedstrom, N. Rahardjo, T. Gay, P. Hobart, R. Stout, T. R. Jones, T. L. Richie, S. E. Parker, D. L. Doolan, J. Norman, and S. L. Hoffman.** 2001. Induction of $CD4^+$ T cell-dependent $CD8^+$ type 1 responses in humans by a malaria DNA vaccine. Proc. Natl. Acad. Sci. USA **98:**10817–10822.
30. **Wang, R., T. L. Richie, M. F. Baraceros, N. Rahardjo, T. Gay, J. G. Banania, Y. Charoenvit, J. E. Epstein, T. Luke, D. A. Freilich, J. Norman, and S. L. Hoffman.** 2005. Boosting of DNA vaccine-elicited gamma interferon responses in humans by exposure to malaria parasites. Infect. Immun. **73:**2863–2872.
31. **Wilson, J. A., C. M. Bosio, and M. K. Hart.** 2001. Ebola virus: the search for vaccines and treatments. Cell. Mol. Life Sci. **58:**1826–1841.
32. **Xu, L., A. Sanchez, Z. Yang, S. R. Zaki, E. G. Nabel, S. T. Nichol, and G. J. Nabel.** 1998. Immunization for Ebola virus infection. Nat. Med. **4:**37–42.
33. **Yang, Z. Y., H. J. Duckers, N. J. Sullivan, A. Sanchez, E. G. Nabel, and G. J. Nabel.** 2000. Identification of the Ebola virus glycoprotein as the main viral determinant of vascular cell cytotoxicity and injury. Nat. Med. **6:**886–889.