Published in final edited form as:
Toxicol Sci. 2006 June ; 91(2): 620–630.

Toxicological Safety Evaluation of DNA Plasmid Vaccines against HIV-1, Ebola, Severe Acute Respiratory Syndrome, or West Nile Virus Is Similar Despite Differing Plasmid Backbones or Gene-Inserts

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Abstract

The Vaccine Research Center has developed a number of vaccine candidates for different diseases/infectious agents (HIV-1, Severe Acute Respiratory Syndrome virus, West Nile virus, and Ebola virus, plus a plasmid cytokine adjuvant—IL-2/Ig) based on a DNA plasmid vaccine platform. To support the clinical development of each of these vaccine candidates, preclinical studies were performed to screen for potential toxicities (intrinsic and immunotoxicities). All treatment-related toxicities identified in these repeated-dose toxicology studies have been confined primarily to the sites of injection and seem to be the result of both the delivery method (as they are seen in both control and treated animals) and the intended immune response to the vaccine (as they occur with greater frequency and severity in treated animals). Reactogenicity at the site of injection is generally seen to be reversible as the frequency and severity diminished between doses and between the immediate and recovery termination time points. This observation also correlated with the biodistribution data reported in the companion article (Sheets *et al.*, 2006), in which DNA plasmid vaccine was shown to remain at the site of injection, rather than biodistributing widely, and to clear over time. The results of these safety studies have been submitted to the Food and Drug Administration to support the safety of initiating clinical studies with these and related DNA plasmid vaccines. Thus far, standard repeated-dose toxicology studies have not identified any target organs for toxicity (other than the injection site) for our DNA plasmid vaccines at doses up to 8 mg per immunization, regardless of disease indication (i.e., expressed gene-insert) and despite differences (strengths) in the promoters used to drive this expression. As clinical data accumulate with these products, it will be possible to retrospectively compare the safety profiles of the products in the clinic to the results of the repeated-dose toxicology studies, in order to determine the utility of such toxicology studies for signaling potential immunotoxicities or intrinsic toxicities from DNA vaccines. These data build on the biodistribution studies performed (see companion article, Sheets

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et al., 2006) to demonstrate the safety and suitability for investigational human use of DNA plasmid vaccine candidates for a variety of infectious disease prevention indications.

Keywords

DNA vaccines; HIV/AIDS; SARS; WNV; Ebola; DNA vaccine toxicology; plasmid vaccines

Before regulatory agencies approve initiation of human clinical trials of novel vaccines, a basis for their safety must be established. Generally, this is accomplished by the conduct of a general toxicological "screening" protocol to identify potential toxicological signals or target organs to guide clinical safety monitoring. The purpose of such a study is to identify both intrinsic toxicity of the product as well as immunotoxicity arising from the host immune response to the vaccine. Rabbits are often used for such "screening" protocols because while they may not be the perfect animal model for all products, they represent a species large enough to receive a full human dose of vaccine. This is important since the immunogenicity/pharmaceutical effect of vaccines does not scale directly on body weight or body surface area, as may be the case for most drugs which disseminate through the blood to other parts of the body. Vaccines act at the local site of injection (if delivered parenterally) to induce an immune response that traffics systemically. Thus, delivery of a full human dose to the animal model is deemed the most suitable means of addressing potential human toxicities (CBER, 2000; Chang *et al.*, 2003).

Concern about the potential for vaccines to induce immunotoxicity has been a regulatory concern for decades. The seriousness of immunotoxicity is perhaps most strikingly illustrated by the occurrence of rheumatic heart disease, which develops as a consequence of the host immune response to group A streptococcus (GAS) infections. Antigens presented by GAS molecularly mimic cardiac myosin, and thus, the host immune response to GAS also attacks the rheumatic valve, leading to chronic rheumatic heart disease (reviewed by Guilherme *et al.*, 2006). Because of the concern that biological products containing GAS antigens, including potential vaccine candidates against GAS, would have the potential to also result in such immunotoxicity to the heart, clinical testing for them was proscribed by regulation, until recently (21 CFR 610.19; Department of Health & Human Services, Food & Drug Administration, 2005). Another immunotoxic disease that has been found to be sufficiently associated with vaccination to result in warnings in vaccine package inserts is Guillain-Barre Syndrome (GBS), an immune-mediated peripheral nervous syndrome disease resulting from direct destruction of the myelin sheath or of the axon itself. Sufficient epidemiological evidence linking development of GBS with "swine-flu" vaccinations of 1976 resulted in the suspension of the mass immunization program that year (reviewed in Schattner, 2005).

DNA vaccines are a novel product class with limited (though ever-increasing) clinical experience, beginning with clinical trials in the late 1990's (Ulmer *et al.*, 1996). Thus, their development represents a unique preclinical challenge. Preclinical testing recommended by U.S. Food and Drug Administration (FDA) to support conduct of clinical trials includes repeated-dose toxicology studies and biodistribution and integration analyses. DNA vaccines consist of a closed circular bacterial plasmid containing a bacterial origin of replication, (generally) a selectable marker such as an antibiotic resistance gene, and a gene-insert expressing a vaccine immunogen or cytokine adjuvant under the control of a (generally) strong mammalian promoter (often one from a human or animal virus) and polyadenylation signals for good transcriptional expression in the vaccinee. Frequently, the gene-insert has been codon optimized for efficient translation in the species to be vaccinated.

The Vaccine Research Center (VRC) is developing several vaccine candidates for human diseases based on a DNA vaccine platform. These various candidate vaccines are intended for

use in prevention of diseases from emergent viruses, such as the HIV/AIDS, Severe Acute Respiratory Syndrome (SARS) virus, or West Nile virus (WNV), as well as for counterterrorism measures, such as vaccines against Ebola virus. An immune response is generated by the *in vivo* expression of the viral proteins after inoculation with the plasmid vaccine candidates encoding them. In addition, one plasmid described herein expresses a cytokine adjuvant. Therefore, the authors have performed numerous similar biodistribution and repeated-dose toxicology studies on similar DNA vaccines expressing a variety of vaccine immunogens and a cytokine adjuvant to support the preclinical development of each of these vaccine candidates. The experience thereby gained has permitted refinements of protocols over time, as well as eventual efficiencies in preparing regulatory submissions by providing more data on the safety of various candidate vaccines. These similarities are observed even with vaccines for differing disease indications, built on similar, although not identical, plasmid vaccine platforms. This has expedited the clinical development of newer candidates with significant savings in time and resources. This is crucial given the public health burden of such diseases.

A companion article in this issue provides the results of biodistribution and integration analyses performed on the same vaccines for which the toxicology studies are described in this article. The biodistribution analyses of the plasmid vaccine candidates showed that they remained at the sites of injection and did not distribute to any organs to suggest targets for toxicity. Thus, we would conclude that any toxicology findings outside of the sites of inoculation, had there been any, would likely be attributable to the immune response to inoculation rather than to the intrinsic actions of the vaccines themselves.

MATERIALS AND METHODS

The vaccine products and delivery device are described in the companion article on biodistribution (Sheets, 2006). All studies were conducted in compliance with Good Laboratory Practices (21 CFR 58). The clinical protocols these studies were intended to support generally delivered the DNA plasmid vaccine intramuscularly by Biojector 2000 (referred to hereafter as Biojector) at doses up to 4 mg or up to 8 mg at three time points (on a schedule of 0, 1, and 2 months). Thus, the toxicology studies reported herein delivered either three inoculations or four (n + 1, as is the current regulatory guidance) inoculations to mimic the human dosing albeit on an accelerated schedule, which is acceptable to the regulatory authorities so long as the animals have a sufficient time between doses to develop immune responses.

Study designs (see Table 1) and methods

Groups of animals inoculated for biodistribution studies, reported in the companion article (Sheets *et al.*, 2006), were different from those inoculated for repeated-dose toxicology studies for the following reasons: In the biodistribution analyses, the animals were only inoculated once to determine where the inoculum subsequently biodistributed, whereas in the repeated-dose toxicology studies, the animals were repeatedly dosed. The timing of terminations in the biodistribution studies (generally 1 week, 1 month, and 2 months post-inoculation) was markedly different from the repeated-dose inoculations and timing of terminations (generally 2 days and 2 weeks after last inoculation) in the repeated-dose toxicology studies.

Vaccines were compared to a true placebo (PBS) in each study to evaluate treatment-related effects against a background rate of safety observations. Unlike viral vectors, DNA plasmid vectors are not susceptible to preexisting vector immunity and have not been found to generate anti-DNA antibodies.

Studies I, III, V, VI, and VII were performed by GeneLogic (formerly TherImmune) (Table 1). With the exception of study III (which was a three-armed study), each study compared the test vaccine to a placebo (PBS). Study III compared either Product 6 (HIV-1) or Product 2 (HIV-1) adjuvanted with Product 4 (cytokine adjuvant) to the PBS placebo. Either 5 (studies I and III) or 10 (studies V, VI, and VII) New Zealand white rabbits per gender (in study III, one rabbit was mis-sexed at the outset of the study resulting in six males and four females rather than five of each gender being inoculated with Product 2 [HIV-1] adjuvanted with Product 4 [cytokine adjuvant]) were inoculated intramuscularly by Biojector with 4 mg total (studies I, VI, and VII) or 8 mg total (studies III and V) of DNA vaccine in two (or four) 0.5-ml inoculations at each injection time point on a schedule of 1, 28, and 56 days (study I, i.e., every 4 weeks for 3 time points, number of inoculations equal to that in the clinical trial); 1, 29, 57, and 85 days (study III, group receiving adjuvanted vaccine) or 29, 57, and 85 days (study III, group receiving unadjuvanted vaccine; i.e., every 4 weeks for 3 or 4 time points—number of inoculations equal to that in the clinical trial); or a schedule of 1, 22, 43, and 64 days (for studies V, VI, and VII; i.e., every 3 weeks for 4 time points—this represents one more inoculation than proposed for clinical trials). In studies I and III, all the animals were terminated 2 weeks after the last inoculation. In studies V, VI, and VII, half of the animals per gender per group were terminated 2 days after the last inoculation (immediate) and the remaining animals were terminated 2 weeks after the last inoculation (recovery). Animals were monitored for mortality and morbidity (cageside and clinical observations), Draize observations, body weight and weight changes, food consumption, ophthalmology, and clinical pathology (hematology, chemistries, and coagulation parameters—see Table 2) during the in-life portion of the study and immediately prior to or at the time of termination. Necropsies included organ weights and ratios and gross and histopathology (tissues listed in Tables 3–5). Samples were taken for immunogenicity assays (studies V, VI, and VII) to confirm that the rabbits were inoculated with an active ingredient (i.e., dosing confirmation). In addition, in study V, bone marrow specimens were examined.

Studies II and IV were performed by BioReliance (Rockville, MD). These studies compared two test vaccines to a placebo (PBS). Five New Zealand white rabbits of each gender were inoculated by Biojector intramuscularly with PBS or one or the other vaccine at 8 mg (study II; split into four 0.5-ml inoculations—two in the right thigh 1 inch apart and 2 in the left thigh 1 inch apart) or 4 mg (study IV; split into two 0.5-ml inoculations—one in the right thigh and the other in the left thigh) at each injection time point, on a schedule of study day (SD) 1, 29, and 57 (with number of doses equal to that of the clinical protocol). Animals were terminated at SD71. Animals were monitored for mortality and morbidity, body weights, ophthalmology, Draize scores, and clinical pathology (hematology and clinical chemistries—see Table 2) during the in-life portion of the study and immediately prior to or at the time of termination. Necropsies included organ weights and gross and histopathology (tissues are listed in Tables 3–5).

Statistical analyses

In studies II and IV, for each gender, analysis of variance (ANOVA) tests were conducted on body weight, hematology, clinical chemistry, and organ weight data. If a significant F ratio was obtained (≤ 0.05), Dunnett t-test was used for pairwise comparisons between the test animals and the control animals. Frequency data such as incidence of mortality and gross necropsy observations were compared by Fisher exact test or chi-square analysis, as necessary. In study I, tests for homogeneity of variances, ANOVA, and the Dunnett t-test for individual differences were evaluated at the 5% one-tailed probability level. Evaluations were conducted on body weights, food consumption, organ weights and ratios, hematology, and clinical chemistry parameters. In studies III, V, VI, and VII, quantitative results were analyzed using the Kolmogorov-Smirnov test for normality, the Levene median test for equal variance, and

the one-way ANOVA. If either the normality or the equal variance test indicated non-normal distribution, then the analysis was continued using the nonparametric Kruskal-Wallis ANOVA on rank-transformed data. For parametric data, if the ANOVA indicated statistical significance among experimental groups, then the Dunnett *t*-test was used to delineate which groups (if any) differed from the control. For nonparametric data, if the ANOVA indicated statistical significance among experimental groups, then the Dunn test was used to delineate which groups (if any) differed from the control. The probability value of less than 0.05 (two tailed) was used as a critical level of significance for all tests, and references to statistical significance refer to values ≤ 0.05. Statistical analysis in these studies was conducted using SigmaStat Statistical Software, Version 1 (Jandel Scientific, San Rafael, CA).

Immunogenicity

Tests were performed to verify that an active dose of vaccine was delivered in studies V, VI, and VII; specifically, ELISAs were performed to demonstrate the development of antibodies against one or more plasmid gene-inserts. For studies V and VI, optimized concentrations of purified recombinant antigens were coated onto Immunol-2 HB microtiter plates (Thermo Labsystems, Milford, MA) overnight at 4°C.

As detailed in Table 6, the SARS and Ebola antigens were generated in VRC laboratories. The VRC plasmids were expressed in 293 cells and purified for the major protein product. Plates were washed and blocked (20% FBS/1% BSA–buffered solution) for 1 h at 37°C. Duplicate wells of the rabbit sera dilutions were incubated for 2 h at 37°C followed by biotin-labeled anti-rabbit IgG/IgA/IgM (KPL, Gaithersburg, MD) (1 h, 37°C), streptavidin-HRPO (30 min, room temperature) (KPL), and TMB substrate (30 min, room temperature) (KPL). Color development was stopped by the addition of sulfuric acid, and plates were read within 30 min at 450 nm.

A minimum of two dilutions of the prevaccination and end of study samples were run for each animal and antigen in duplicate wells. Mean ODs for each dilution were compared to that of the same dilution of the preimmunization sample. Sera were considered responsive if the postvaccination OD was > 0.2 than the prevaccination sample of the same animal.

For study VII, The Focus Technologies (Cypress, CA) WNV ELISA IgG Assay (Product EL0300G) was revised to be used for analysis of rabbit sera. ELISA plates precoated with PreM and E proteins were warmed to room temperature, 150 μl of 1× Wash Buffer were added to each well and incubated at room temperature for 5 min, followed by aspiration and blotting dry. Duplicate wells of 1:100, 1:1000, and 1:10,000 dilutions of the rabbit sera were incubated for 2 h at 37°C followed by biotin-labeled anti-rabbit IgG/IgA/IgM (KPL) (1 h, 37°C), streptavidin-HRPO (30 min, room temperature) (KPL), and TMB substrate (30 min, room temperature) (KPL). Color development was stopped by the addition of sulfuric acid, and plates were read within 30 min at 450 nm, with reported results based on the average of duplicate wells, corrected for the average of the same rabbit and dilution-matched prevaccination sample. Samples with corrected OD > 0.2 were considered seroreactive.

RESULTS

As a general pattern, treatment-related toxicity was seen only at the sites of inoculation (Draize scoring and gross and/or histopathology) and was primarily attributable to the delivery device but, in some circumstances, could also be attributed to inflammation due to the intended immune response to the vaccine. These findings were recoverable and were predominately mild to moderate.

Any protocol deviations were noted in the final report for each study. Except where noted, all were minor and considered unlikely to impact the study outcome. These included small short-term deviations in room temperature, clinical observations not made on one animal on one day, rare coagulated blood or tissue specimens that could not be analyzed, animals identified with an ear tattoo instead of an ear tag, animals caged in aluminum rather than polycarbonate cages, animals slightly heavier or a week older at the initiation of the study than called for in the protocol, and so on. No protocol deviations were noted that impacted the quality or integrity of the studies.

Study I: Repeated-Dose Toxicology in Rabbits with HIV Vaccine Candidate—Product 1

All animals survived to scheduled termination. Clinical observations included a treated female with a small stationary tissue mass near the left dose site in the week prior to termination and a control female who was limping in the last week of the study.

Draize scoring of the injection sites revealed the following: three treated females had minimal edema in the week following the second dose, three treated females had moderate edema in the week following the second dose, one control male had severe edema in the week following the second dose, one treated male had minimal erythema following the first dose, one control male and two treated females had minimal erythema in the week following the second dose, two treated females had mild erythema in the week following the second dose, and one control male and two treated females had moderate erythema in the week following the second dose.

There were no clear treatment-related effects on clinical pathology parameters, although there were a number of statistically significant differences (data not shown). Many of these differences were of a magnitude or direction that was not clinically significant or that remained within the normal values established for the gender, laboratory, and species. Other than chronic inflammation at the injection sites attributed to recovery of trauma due to injection, there were no treatment-related effects on histopathology, and any histopathological findings were considered incidental. There were no treatment-related effects on body weights, food consumption, ophthalmology, organ weights or ratios, or gross pathology.

Study II: Repeated-Dose Toxicology in Rabbits with HIV Vaccine Candidates—Products 2 and 3

This study had a protocol deviation that could have impacted the study outcome. Due to insufficient test article supply, the fourth dose site of a female receiving Product 2 (HIV-1) was inoculated with only 0.4 ml instead of 0.5 ml (for a total dose of 7.6 mg instead of 8 mg) on SD29. On SD57, four females receiving Product 2 (HIV-1) were inoculated in only three sites (for a total of 6 mg instead of 8 mg), and the final female in that dose group received 0.4 ml in the fourth injection site (for a total dose of 7.6 mg instead of 8 mg). Finally, a female receiving Product 3 (HIV-1) was inoculated with only 0.25 ml in the fourth dose site (for a total of 7 mg instead of 8 mg) on SD57. Immunology was not performed in this study to verify that active doses were delivered.

Product 2—All animals survived to scheduled termination. While treated males gained weight during the study, their weights were statistically significantly lower than control males on SD22, 29, 36, 43, 50, and 71 (i.e., approximately half of the time points). The differences in the males' body weights were between 4 and 7% less than control males. There was no treatment-related effect on females' body weights.

Draize scoring of the injection sites revealed an increased frequency of irritation in the treated animals compared to control animals, but without increased severity. There was no treatment-related clinical pathology for treated males; however, treated females had statistically

significantly increased total protein (6.5 g/dl, SD 0.24, vs. 6.06 g/dl, SD 0.21) and globulin concentrations (4.02 g/dl, SD 0.23, vs. 3.74 g/dl, SD 0.18). These remained within the historical normal range for the species and laboratory and were not considered to be biologically significant and did not correlate with any histopathology.

There were no treatment-related effects on organ weights in treated females; however, treated males had statistically significantly increased lung-to-body weight ratio (but not statistically significantly different absolute lung weights). There were no treatment-related effects on clinical observations, ophthalmology, gross pathology, and histopathology.

Product 3—All animals survived to scheduled termination. Clinical observations included stained coat in one control male on SD71 and lethargy, ruffled fur, stained coat, and diarrhea in one control female at various times between SD4 and SD71. There were no treatment-related clinical observations for treated females; however, treated males had diarrhea (one animal on SD29 and another on SD70), a stained coat (one male on SD67−71), and cuts on the body and a stained coat (one male on SD49−56 and on SD48−49 and 51−52, respectively).

Draize scoring of the injection sites revealed an increased frequency, but not severity, of irritation in the treated animals when compared to control animals. There were no treatment-related effects on clinical chemistries in either gender of treated animals or on hematology values in treated females. There was a statistically significant decreased platelet count in treated males (322 Th/mcl, SD 46.2, vs. 408.6 Th/mcl, SD 50.8); however, the mean remained within the historical range for the species and laboratory.

There was a statistically significant increase in spleen-to-body weight ratios in treated females but no treatment-related effects on organ weights in treated males. Increases in relative spleen weight may be a reflection of the intended immune response to vaccination. There were no treatment-related effects on body weights, ophthalmology, gross pathology, and histopathology.

Study III: Repeated-Dose Toxicology in Rabbits with HIV Vaccine Candidates—Product 6 and Product 2 Adjuvanted with Product 4

One animal was mis-sexed at the outset of this study, so six males and four females received Product 2 (HIV-1) adjuvanted with Product 4 (cytokine adjuvant) instead of five of each gender. All animals survived to scheduled termination. Draize observations included minimal edema and erythema in a few of the placebo recipients and minimal to mild edema and erythema in all treated animals.

There were no treatment-related effects on the body weights of the treated male animals or the female animals receiving the unadjuvanted vaccine, but there were statistically significantly lower mean body weights (absolute decreases of 11−16%) on SD50−99 of females receiving the adjuvanted vaccine. Mean body weight gains were statistically significantly decreased (i.e., lesser gains in weight, not loss of weight) on SD57−64 and SD71−78 in males receiving the adjuvanted vaccine and on SD64−71 in females receiving the adjuvanted vaccine.

There were a few incidences of statistically significant differences in levels of food consumption during the study as follows: decrease on SD86−87 in males receiving adjuvanted vaccine; decreases on SD92−94 and 95−96 in males receiving the unadjuvanted vaccine; decreases on total (across the entire study) and on SD44−45, 55−56, 58−60, 76−77, 79−80, 81−82, 84−85, 87−90, and 91−92 in females receiving the adjuvanted vaccine; and increases on SD5−6, 12−13, 15−16, and 19−21 and decreases on SD58−59, 65−66, 67−69, 72−73, and 88−89 in females receiving the unadjuvanted vaccine. Although the females receiving the adjuvanted vaccine had statistically significantly less total food consumption (15,002 g, SD

641) than the control females (17,212 g, SD 947.5; and the females receiving the unadjuvanted vaccine, 17,013 g, SD 1507.9, although this group was not the basis for comparison), their total food consumption for the study was quite comparable to the treated (15,568 g, SD 1329.9, for males receiving adjuvanted vaccine, 15,472 g, SD 1819.5, for males receiving unadjuvanted vaccine) and control males (15,737 g, SD 1333.2). In addition, this amount of food consumption is comparable to the females (treated or control) in other studies (e.g., study V described below), when normalized for the length of the study. Thus, the biological significance of these statistically signifi-cant differences is difficult to discern, if there is any.

Among the hematology parameters, pre-dosing mean platelet volume was statistically significantly increased (6.26 fl, SD 0.42, vs. 5.74 fl, SD 0.19; normal range 4.9−10.2 fl) in the females subsequently receiving the unadjuvanted vaccine in comparison to control females. This difference was not seen at termination. However, the mean corpuscular volume (68.8 fl, SD 0.8, vs. 65.6 fl, SD 2.5; normal range 48.7−68.8) was statistically significantly increased at SD99 in the females receiving the unadjuvanted vaccine. Absolute monocytes (0.803 Th/mcl, SD 0.302, vs. 0.388 Th/mcl, SD 0.159; normal range 0.05−0.59) were statistically significantly increased at SD99 in males receiving the adjuvanted vaccine. Given that the direction of the changes was not indicative, in the absence of other symptoms or pathological findings, of any biological significance and given the lack of pattern (correlation between genders or treatments), these differences were not considered to be treatment related or clinically relevant. In addition, with the exception of the increase in absolute monocytes in males, the other differences remained within the normal range for the laboratory and species. Monocytosis could be an indication of the intended immune response or could be secondary to muscle damage at the site of injection (i.e., an indication of inflammation and repair). However, the mean value for absolute monocytes in the males receiving the adjuvanted vaccine seemed to be driven primarily by one outlier that had the highest absolute monocyte count of all animals in the study at that time point (1.25 Th/mcl; this animal had neutrophil and lymphocyte counts which remained within normal ranges and a total WBC count which was at the upper limit of the normal range).

There were no treatment-related effects on organ weights or weight ratios for the males receiving either the adjuvanted or unadjuvanted vaccines or for the females receiving the unadjuvanted vaccine. Females receiving the adjuvanted vaccine had statistically significantly increased brain-to-body weight ratios and decreased absolute and relative ratios to the brain weights for the heart and liver. These differences were considered incidental because they did not correlate with any gross or histopathological findings and may be related to the increased food consumption and terminal weights of the control females in relation to the females receiving the adjuvanted vaccine rather than to toxicity.

There were no treatment-related effects on clinical or cage-side observations, ophthalmology, bone marrow, chemistries, or gross pathology except for one male receiving the adjuvanted vaccine which had a “thickened” injection site.

The primary findings of treatment-related histopathology were seen at the sites of injection. An increased incidence of chronic inflammation in the deep dermis and subcutaneous tissues was seen in the males receiving the adjuvanted vaccine and to a lesser degree, depending on the inoculation site, the males receiving the unadjuvanted vaccine. The females demonstrated less difference between groups and sites. Chronic inflammation was scored on the basis of minimal numbers of mononuclear cells situated perivascularly, although, in the muscle, macrophages were also noted in a single myofiber. Several animals had regenerative changes and/or fibrosis in the deeper muscle, which was attributed to receipt of an injection rather than the test article injected.

An increased incidence of minimal chronic inflammation was observed in livers of treated male animals compared to control males. There also was an increased incidence of hyperplasia of the prostate in treated male animals (2/5 receiving unadjuvanted vaccine, 3/6 receiving adjuvanted vaccine) compared to control males (0/5). Hyperplasia in the prostate, as well as in the vesicular gland (evaluated as an incidental tissue included on the slide with prostate), was characterized by increased crowding of basal nuclei, indicating an increased number of cells within some of the acini. These findings did not correlate with any gross pathology. Males receiving the unadjuvanted vaccine had an increased incidence of subacute interstitial inflammation in the lung compared to males receiving the adjuvanted vaccine or the control. Subacute interstitial inflammation in the lung was characterized by multifocal, minimal to slight/mild infiltrates of foamy macrophages, rare heterophils, and thickening of alveolar septa. All these histopathological changes in the liver, prostate, and lung of males are of uncertain significance as related to treatment since there were no detected correlating clinical pathology or gross pathology findings. No differences in severity were noted among the affected groups.

Gender differences were noted in the incidence of certain lesions, such as regeneration and vacuolation of tubular epithelium in the kidney and lymphoid depletion in the thymus. Generally, female rabbits had more regeneration and vacuo-lation of tubular epithelium in the kidney and a decreased incidence and severity of lymphoid depletion in the thymus compared to the males. Lymphoid depletion, in this study, most likely represented physiological involution of the thymus with increasing age. One control male had slight/mild lymphoid depletion correlated to a gross observation of severe reduction in size of the thymus. There were no other significant lesions in this rabbit that would suggest stress of disease as a cause for the reduction in size of the thymus.

Finally, the majority of the gross observations had no microscopic correlations. Cysts observed along the oviduct or uterus were correlated to periovarian cysts and were considered incidental findings. One female receiving the unadjuvanted vaccine had discolored cystic ovaries which were correlated to the presence of multiple corpora hemorrhagica. The significance of this finding was uncertain.

Study IV: Repeated-Dose Toxicology in Rabbits with Ebola Vaccine Candidate—Product 7—and HIV-1 Vaccine Candidate—VRC-3900

Ebola candidate—Product 7—All animals survived to scheduled termination. Clinical signs noted during the study included alopecia (one male from SD46 to 56 except SD50), stained coat (one male on SD21), diarrhea (one male on SD57), and vocalization (one female on SD46−48) in the controls and stained coat (three males on SD57, 64, and 71, on SD22, and on SD50 and 71, respectively, and four females on SD8, 15, and 19, on SD7−8, on SD34−67 and 69−71, and on SD58, respectively), diarrhea (one male on SD36−37, 41, 43, and 71 and one female on SD70), erythema (one male on SD29 and 36), and vocalization (one female on SD31, 45−47, 50−67, and 69−71) for the treated animals.

Draize scoring of the injection sites revealed an increased frequency, but not severity, of irritation in the treated animals compared to control animals. There were no treatment-related differences in body weights, ophthalmology, clinical pathology parameters (hematology or chemistries), organ weights, or gross pathological findings; however, two treated animals—one male and one female—were found to have yellow-pigmented skin in the prepuce area on necropsy.

On histopathology, one treated female was found to have evidence of muscle regeneration at an injection site, which was attributed to the normal reparative process following injection. Two treated males were found to have minimal to mild hemorrhage of the adrenal cortex, which was not accompanied by any cellular change and the cortex-to-medulla ratio was not altered.

This was not seen in treated females; however, one treated and one control female had nodular hyperplasia of the cortex of the adrenal glands.

VRC-3900—All animals survived to scheduled termination. Clinical signs noted during the study included alopecia (one male from SD46 to 56 except SD50), stained coat (one male on SD21), diarrhea (one male on SD57), and vocalization (one female on SD46−48) in the controls and ruffled fur (one male on SD20 and 36), thin appearance (one female on SD36), stained coat (three males on SD12, 14−33, 35−36, 40−60, 64, and 70−71; on SD51−53; and on SD20, 30−32, 36−39, and 47−48 and four females on SD27; on SD30−32 and 43−49; on SD23−26, 40−48, and 50−55; and on SD22−28, 30−33, 40−42, 44−49, 51−56, and 58−63), hyperactivity/excitability (one female on SD51−53), diarrhea (three males on SD2, 5, 9−11, 14−15, 18−27, 29−30, 35, 40−66, and 68−71; SD40; and SD70 and three females on SD2, 44−49, and 69; on SD41−48, 51−53, and 69−70; and on SD9−11, 13, 19, 22−35, 40−66, and 68−71), and erythema (two males both on SD36) in treated animals.

Clinical pathology analyses revealed several statistically significant differences, all of which remained within historical normal limits (data not shown). One hematology parameter that was statistically significantly different and outside the historical normal range was increased lymphocytes in treated males (71.8%, SD 5.2, vs. 56.4%, SD 7.7); however, this was not observed in females. This increase may be a reflection of the intended immune response to vaccination.

Draize scoring of the injection sites revealed an increased frequency, but not severity, of irritation in the treated animals compared to control animals. There were no treatment-related differences in body weights, ophthalmology, histopathology, or gross pathological findings; however, one treated male was found to have yellow-pigmented skin in the prepuce area on necropsy. There were no differences in organ weights for treated males versus controls; however, treated females had statistically significantly increased heart weight and heart-to-body weight ratio (12 g, SD 1.7, vs. 8.8 g, SD 1.4, and 0.3 ± 0.04 vs. 0.2 ± 0.04). This did not correlate with any histo-pathology, and there is no clear relationship to hematological or clinical chemistry parameters.

Study V: Repeated-Dose Toxicology in Rabbits with Ebola Vaccine Candidate—Product 12

All animals survived to scheduled termination. There was no effect on clinical or cageside observations with only one treated male appearing thin on SD66. Minimal to moderate edema and minimal to mild erythema were observed in control animals by Draize scoring and likely represent the effects of injection by Biojector. The incidence of these findings was increased after the third and fourth inoculations in the treated group, suggesting a treatment effect; however, the effect was reversible over time.

There were increased body weight gains in the treated animals (males between SD36−43 and SD71−78, females from SD43 to 50), which were considered incidental as they did not correlate with differences in body weights, there was no consistent pattern, and because of the direction of the difference (increased weight gain in treated animals vs. controls). Likewise, there was no treatment-related effect on food consumption, although there were statistically significant differences in treated males as follows (decrease on SD13−14, increase on SD42−43, and increase on SD76−77).

There were no clear treatment-related effects on clinical pathology parameters, although there were a number of statistically significant differences (data not shown). Many of these differences were of a magnitude or direction that was not clinically significant or that remained within the normal values established for the gender, laboratory, and species.

Treatment with the Ebola product in this study had no effect on ophthalmology, body weights, bone marrow, or absolute organ weights or weight ratios. One statistically significant finding was a decrease in the adrenal gland-to-brain weight ratio in treated females at SD66, but this did not correlate with differences in absolute adrenal gland weights and was observed only in one gender.

There was also no treatment-related effect on gross pathology except at the injection sites. At SD66, one or two animals/gender/group had discoloration at the injection site with apparent recovery by SD78. These findings were likely related to the injection method rather than the vaccine.

There were no clear treatment-related effects on histo-pathology except at the injection sites. There was minimal to mild infiltration or inflammation and accompanying apparent hemorrhage at each of the injection sites in most animals (treated and controls) with increased frequency of findings of infiltration/inflammation in the treated animals. This was interpreted to indicate a role of both the injection method (Biojector) and the treatment to inflammation at the injection sites. Renal changes identified as nephropathies and mineralization were more predominant in treated females than controls but decreased in treated males compared to controls, so as there was no clear pattern and as this is a common spontaneous finding in laboratory rabbits, this finding was considered to be incidental. Also, there was no correlation to organ weight differences, gross pathology, or clinical pathology.

Immunogenicity of each of the Ebola antigens was demonstrated by seroconversion (measured by ELISA) in the vaccinated animals and not the placebo recipients verifying delivery of an active dose of product in this toxicology study. The objective of these investigations was only to assure that the vaccine induced an immune response, i.e., that an active dose was delivered. As such, the samples were not run over a wide enough dilution range to definitively determine end-point dilutions. As summarized in Table 7, in all cases the vaccinated animal group had end-point antibody titers that exceeded the highest dilution run.

Study VI: Repeated-Dose Toxicology in Rabbits with SARS Vaccine Candidate—Product 15

All animals survived to scheduled termination. One treated female was observed as being thin on SD64 and 66. There were no ophthalmologic findings throughout the study, although one treated female had a corneal inclusion at the start of the study (prior to treatment). Draize observations were found after inoculation in both groups, attributable to the injection device. However, these observations were slightly more frequent and slightly more severe in the treated animals, likely a reflection of inflammation due to the intended immune response of the vaccine.

There were no treatment-related clinical or cageside observations or differences in body weights and body weight changes, although weights of treated males were statistically lower at SD71 and 78 due to the unintentional effect of removal of the heavier treated males terminated at SD66 and the impact of this on the group means—given that this finding did not correlate with differences in food consumption during this time period or body weight changes. Treated males had statistically significantly lower food consumption than control males on SD3−7, 39−40, 42−43, 44−45, 50−51, 55−57, 61−66, and 68−70; yet, these differences did not result in differences in body weights or weight changes. Treated females had statistically significantly lower food consumption than control females on SD24−45, 29−30, and 67−68, which did not correlate with differences in body weights or weight changes. The magnitude of these differences was only on the order of 15−20% less; thus, these differences were considered incidental.

There were no clear treatment-related effects on clinical pathology parameters, although there were a number of statistically significant differences (data not shown). Many of these differences were of a magnitude or direction that was not clinically significant or that remained within the normal values established for the gender, laboratory, and species with the exception of prothrombin time (PT) differences (7.74, SD 0.05, vs. 7.79, SD 0.07; normal range 8.17 −8.72). The PT values for both treated and control animals tended to be shorter than the normal range, which is not biologically meaningful.

While statistically significant organ weight differences were noted, they did not correlate between genders and between termination time points (e.g., seen at recovery termination when they were not noted at immediate termination), had no correlation with gross or histopathological findings, or did not correlate in absolute organ weight differences (only in relative ratios); thus, they were all taken as incidental.

Only gross pathological findings at the injection site correlated with histopathological findings. Dark discoloration at one or more injection sites was observed in 3/5 control males, 1/5 control females, and 3/5 treated females on SD66. Reversibility was demonstrated at the recovery termination, and no animals had these gross lesions on SD78.

Several histopathological findings were made which are often found in rabbits and which were of equivalent frequency and/or severity in control and treated animals and on each or either termination time point, thus considered to be incidental. In contrast, histopathological findings at the injection site were considered related to the repeated injections even as they were found at equivalent frequency and/or severity between controls and treated animals and correlated with the gross pathology. These included minimal mononuclear cell infiltrates, minimal or mild subacute inflammation, and focal muscle fiber degeneration/regeneration. While likely related to the injection procedure, there did not appear to be a clear pattern of an increase in frequency or severity in the treated group over the control group.

Immunogenicity was demonstrated by seroconversion (by ELISA) to the SARS S protein in the vaccinated animals and not the placebo recipients, verifying delivery of an active dose of the product in this toxicology study. The objective of these investigations was primarily to assure that the vaccine induced an immune response, i.e., that an active dose was delivered. As such, the samples were not run over a wide enough dilution range to definitively determine end-point dilutions. As summarized in Table 7, in all cases the vaccinated animal group had end-point antibody titers that exceeded the highest dilution run.

Study VII: Repeated-Dose Toxicology in Rabbits with WNV Vaccine Candidate—Product 17

All animals survived to scheduled termination. Draize observations were found after inoculation in both groups, attributable to the injection device. However, these observations were slightly more frequent and slightly more severe in the treated animals, likely a reflection of inflammation due to the intended immune response of the vaccine. These Draize findings recovered with time, demonstrating reversibility, but recurred with later inoculations.

There were no treatment-related differences in body weights, body weight changes, or food consumption. While treated males had lower food consumption on SD44−45, 63−64, 67−68, and 69−70, except for the first of these, these days correspond to a period during which they had higher body weight changes (i.e., gained more weight than controls), so these findings were considered incidental. Higher food consumption was observed in treated males on SD1 −2 and 5−6 and on nine different days for treated females. These differences (most of which are increases of low magnitude) are more likely the result of biological variability than treatment-related toxicity (which would generally result in decreases).

There were also no treatment-related clinical or cageside observations, ophthalmology, clinical pathology, or effects on organ weights or organ weight ratios, except in higher absolute spleen weights, spleen-to-body weight ratio, and spleen-to-brain weight ratio in treated males at SD66. This may be attributable to the intended immune stimulation of the vaccine. However, this was not observed in treated females at this time point. By SD78, these findings were no longer seen. In addition, the thyroid-to-brain weight ratio was higher in treated females on SD66 and the kidney-to-body weight, kidney-to-brain weight, and liver-to-body weight ratios were higher at SD78. These findings did not correlate to differences in absolute organ weights for either gender or in differences in organ weight ratios in males. Also, they did not correlate between time points or to any gross or histopathological findings. Thus, they were considered incidental.

The only treatment-related gross pathological findings were at the sites of injection and were related to the injection method rather than the actual treatment (i.e., they were not vaccine related). At SD66, four of five control females and five of five treated females had injection site discoloration at the most recent injection sites. Also at SD66, only one treated male and no controls had this same finding. No animal was observed with injection site discoloration at the SD78 necropsy, demonstrating the reversibility of this injection site reactogenicity. In addition, one treated female on SD66 had a dark discoloration on the mucosal stomach, which was considered incidental.

Likewise, treatment-related effects in histopathology correlated with the injection method, although there may have been some worsening of these effects in the vaccinated animals. These findings included inflammatory cell infiltrates, hemorrhage, and myofiber degeneration/necrosis of the skeletal muscle at the sites of injection and, occasionally, fibrosis in the subcutis or skeletal muscle at the sites of injection. The infiltrates consisted generally of neutrophils (heterophils) and mononuclear cells focally distributed within the subcutis, dermis, or interstitial tissue of the muscle. In general, the more recent injection sites were more significantly involved than those used more remotely in time. Although these observations were noted in both control and treated animals, the findings tended to be of greater frequency or severity in the treated animals. After the recovery period, inflammation, myofiber degeneration/necrosis, and, more prominently, fibrosis were noted with higher frequency and severity in the vaccinated animals versus the controls.

Immunogenicity was demonstrated by seroconversion (by ELISA) to the WNV PreM and E proteins in the vaccinated animals and not the placebo recipients, verifying delivery of an active dose of the product in this toxicology study. The objective of these investigations was to assure that the vaccine induced an immune response, i.e., that an active dose was delivered. As such, the samples were not run over a wide enough dilution range to definitively determine end-point dilutions. As summarized in Table 7, in all cases the vaccinated animal group had end-point antibody titers that exceeded the highest dilution run.

DISCUSSION

For intramuscular inoculation by needle and syringe or by Biojector of VRC DNA vaccine candidates (to prevent disease from HIV/AIDS, Ebola virus, WNV, or SARS virus) at doses up to 8 mg per immunization, the primary “toxicological” findings have been at the sites of injection as observed by Draize scores, gross pathology, and histopathology. No other “target” organs for toxicity have been discovered through the conduct of repeated-dose toxicology studies or the corresponding biodistribution studies described in the companion article in this issue. The reactogenicity observed at the injection sites is likely the result of inflammation due to both the delivery device and the intended immune stimulation of the vaccine. All animals survived to scheduled termination. Any clinical or cageside observations (such as abrasions, stained coat, or soft feces) have been found to be unrelated to vaccination due to their sporadic

nature, equivalent occurrence in controls as in treated animals, lack of correlation between genders, or attribution to other causes (such as lesions due to ear tags). There were also no ophthalmologic findings with our DNA vaccine candidates, including the human cytokine adjuvant—unlike observations with certain chemical adjuvants (Allison and Byars, 1991; Li *et al.*, 1993; Petty *et al.*, 1989).

Differences in body weight, body weight change, and food consumption appear to be poor indicators of toxicity for DNA plasmid vaccines. Frequently, statistically significant differences were observed, but these failed to correlate with other findings and were generally of a magnitude and/or direction that was clinically irrelevant. Thus, for DNA vaccine candidates, statistical analysis of body weights, body weight changes, and food consumption data has proven to be of limited utility. It is important to note that adjustments were not made for the multiplicity of analyses being done, and thus by random chance, some of these analyses are likely to appear significant even when there was no true difference. These adjustments were not made as a more stringent examination of safety parameters. If these parameters are to be statistically analyzed in future studies, then they should probably be subjected to adjustments of the *p* values for the multiplicity of analyses. Alternatively, a more stringent *p* value—such as 0.01 instead of 0.05 might provide a better indicator of true, relevant differences.

Likewise, a myriad of “differences” were seen in clinical pathology parameters in various studies (data not shown), yet they rarely correlated with the same observation at other time points during the study, with the other gender, with any gross or histopathological findings, or with clinical findings. The vast majority of these statistically significant differences remained within the normal laboratory ranges for the gender, species, and laboratory. Many of the findings were of a magnitude or direction that is clinically irrelevant, even if statistically significant. Measurement of these parameters has thus far also proven to be of limited utility in assessing true toxicological signals from DNA plasmid vaccines and most often represents a confusing and likely irrelevant “signal,” i.e., noise.

Overall, plasmid DNA vaccine candidates—despite differing backbone/promoters and vastly different gene-inserts to prevent diseases from Ebola virus, HIV/AIDS, WNV, or SARS—when delivered intramuscularly by needle and syringe or by Biojector in doses up to 8 mg per immunization result in toxicity only at the site of injection. This local reactogenicity is self-limiting and reversible and is generally of a mild-to-moderate nature. However, these toxicology studies have permitted an evaluation to be made that the candidates were safe to enter human clinical trials. Importantly, thus far, the lack of significant toxicities observed in preclinical testing has been borne out in the human clinical trials conducted to date (Martin *et al.*, 2005). With time, as these candidates reach expanded phases of clinical development, their safety in humans will be established and a retrospective analysis can be performed to determine if the repeated-dose toxicology studies proved of utility to signal potential intrinsic or immunotoxicities.

Numerous similar repeated-dose toxicology studies on DNA vaccines expressing a variety of vaccine immunogens and a human cytokine adjuvant have been performed to support the development of several vaccine candidates. This has permitted refinements of preclinical safety protocols over time, as well as eventual efficiencies in preparing regulatory submissions by providing more data on the safety of various candidate vaccines. These similarities are observed even with vaccines for differing disease indications, built on similar, although not identical, plasmid vaccine platforms. Protocols V, VI, and VII represent the most refined protocols and are the models which will likely be followed for single-modality regimens, when future repeated-dose toxicology studies are necessary for new candidate vaccines. When small changes are made to vaccine constructs, preclinical safety data and phase 1 clinical data from the similar constructs have been submitted for regulatory review to support clinical use of the

newer, slightly modified constructs. For an example of this, see the discussion in the companion article on biodistribution studies with these candidate vaccines, in this issue. These regulatory efficiencies save months in the clinical development pathway of new candidate vaccines. Given the urgency of public health crises, such as the HIV/AIDS epidemic with 14,000 new infections daily, and the threat of global spread of newly emerging viral infections like SARS, this shortened development time is crucial.

ACKNOWLEDGMENTS

The authors would like to acknowledge the sage statistical advice of Martha Nason and the insightful comments on study reports by John Jessop. We would also like to acknowledge the staff of BioReliance who conducted the early studies, studies II and IV, and three studies undertaken in mice (data not reported) and the staff of GeneLogic for their conduct of the rest of the studies. Further, we would like to acknowledge Rick Stout (Bioject, Tualatin, Oregon) for providing the Biojector devices and the staff of Vical, Inc. (San Diego, CA), for manufacturing all clinical trial materials under contract to NIAID. This research was supported by the Intramural Research Program of the NIH, Vaccine Research Center, NIAID.

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TABLE 1

Study Designs

Study designation	Species	Product[a]	Dose (mg)	Dosing schedule	Blood draw schedule	Necropsy schedule
I	Rabbits	1 (HIV-1)	4	SD1, 28, 56	Necropsy	SD70/71
II	Rabbits	2, 3 (HIV-1)	8	SD1, 29, 57	Necropsy	SD71
III	Rabbits	6 (HIV-1), 2 (HIV-1) + 4 (cytokine adjuvant)	8	SD1[b], 29, 57, 85	PTID, necropsy[c]	SD99
IV	Rabbits	7 (Ebola), VRC-3900 (HIV-1)	4	SD1, 29, 57	Necropsy	SD71
V	Rabbits	12 (Ebola)	8	SD1, 22, 43, 64	PTID[c], SD3, 28, 59, necropsy	SD66, SD78
VI	Rabbits	15 (SARS)	4	SD1, 22, 43, 64	PTID[c], SD3, 28, 59, necropsy	SD66, SD78
VII	Rabbits	17 (WNV)	4	SD1, 22, 43, 64	PTID[c], SD4, 28, 59, necropsy	SD66, SD78

[a] Product number designations match the product descriptions given in the Materials and Methods of the companion article on biodistribution in this issue.

[b] Product 6 (HIV-1) was not dosed on SD1, only on SD29, 57, and 85; Product 2 (HIV-1) adjuvanted with Product 4 (cytokine adjuvant) was dosed on all four SDs.

[c] PTID, prior to initial dose.

TABLE 2

Clinical Pathology Parameters

Chemistries		
A/G ratio	Alanine aminotransferase	Albumin
Alkaline phosphatase	Aspartate aminotransferase	Calcium
Carbon dioxide (CO_2)[a]	Cholesterol	Chloride
Creatinine	Creatinine kinase	Gamma Glutamyl transpeptidase (GGT)[b]
Globulin	Glucose	Lactate dehydrogenase[b]
Phosphorase	Potassium	Sodium
Total bilirubin	Total protein	Triglycerides
Urea nitrogen		
Hematology		
Cellular morphology[c]	Erythrocyte count	Hematocrit
Hemoglobin	Leukocytes	Leukocyte differential
Mean cell hemoglobin	Mean cell hemoglobin concentration	Mean cell volume
Mean platelet volume[d]	Platelet count	Reticulocytes[e]
Coagulation		
Activated partial thromboplastin time[a]	Prothrombin time[a]	

[a] In studies V, VI, and VII, not assessed in studies I, II, III, and IV.

[b] Only in study III.

[c] In studies I, III, V, VI, and VII, not assessed in studies II and IV.

[d] In all studies except study II.

[e] In studies II and V (counts). In studies V, VI, and VII, slides were to be evaluated only if toxicity was indicated; not assessed in study I and III.

TABLE 3

Organs Collected and Weighed

Adrenal glands[a]	Brain	Esophagus[b]
Eyes[b]	Gonads	Heart
Kidneys	Liver (with drained gallbladder)	Lungs with main stem bronchii
Parathyroids/thyroids[a]	Pituitary,[ac]	Prostate[b]
Spleen	Thymus[c]	Uterus

[a] In studies III, V, VI, and VII, not weighed in studies I, II, or IV.

[b] In study I only.

[c] In all studies except study I.

TABLE 4

Organs Examined for Gross Pathology

External surface of body	Injection sites	Orifices
Cranial cavity and contents	Thoracic cavity and contents	Abdominal cavity and contents

TABLE 5

Organs Examined Histopathologically

Adrenals	Aorta (thoracic)	Brain
Cecum	Cervix[a]	Colon
Duodenum	Epididymes	Esophagus
Eyes	Femur	Gallbladder
Gonads	Harderian glands[b]	Heart
Injection sites (including underlying muscle and overlying subcutis)	Ileum	Jejunum
Kidneys[c]	Lacrimal glands	Liver
Lung with main stem bronchii	Mandibular or submandibular lymph nodes	Mandibular salivary glands
Mesenteric lymph nodes	Mammary glands (♀, ♂)	Optic nerves[d]
Pancreas	Parathyroids	Pituitary
Prostate	Rectum	Seminal vesicles
Sciatic nerve	Skeletal muscle[d]	Skin
Spinal cord (cervical, midthoracic, lumbar)	Spleen	Sternum[e]
Stomach	Thymus	Thyroids
Trachea	Tongue	Urinary bladder
Uterus	Vagina	Gross lesions
Animal ID[f]		

[a] In studies I, III, V, VI, and VII, not analyzed in studies II and IV.

[b] In all studies except study II.

[c] Left kidney snap-frozen for analysis of immunotoxicity if indicated, in studies V, VI, and VII.

[d] Not analyzed in studies I, II, III, and IV.

[e] Includes bone with marrow.

[f] Not analyzed in studies II, III, and IV.

TABLE 6

Recombinant Antigens Used in Immunogenicity Analysis for Studies V and VI

Antigen	Source
SARS Spike	VRC Plasmid 8318
Ebola NP	VRC Plasmid 6402
Ebola GP-Zaire	VRC Plasmid 6605

TABLE 7

Summary of Antibody ELISA Analysis in Studies V, VI, and VII

Study	Agent	VRC vaccine	Antigen	Titer
Study V	Ebola virus	Product 12	GP-Zaire	≥ 6400
			NP	≥ 6400
Study VI	SARS virus	Product 15	Spike	≥ 10,000
Study VII	West Nile virus	Product 17	PreM and E	≥ 10,000