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Comparison of individual and combination DNA vaccines for *B. anthracis*, Ebola virus, Marburg virus and Venezuelan equine encephalitis virus

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Abstract

Multiagent DNA vaccines for highly pathogenic organisms offer an attractive approach for preventing naturally occurring or deliberately introduced diseases. Few animal studies have compared the feasibility of combining unrelated gene vaccines. Here, we demonstrate that DNA vaccines to four dissimilar pathogens that are known biowarfare agents, *Bacillus anthracis*, Ebola (EBOV), Marburg (MARV), and Venezuelan equine encephalitis virus (VEEV), can elicit protective immunity in relevant animal models. In addition, a combination of all four vaccines is shown to be equally as effective as the individual vaccines for eliciting immune responses in a single animal species. These results demonstrate for the first time the potential of combined DNA vaccines for these agents and point to a possible method of rapid development of multiagent vaccines for disparate pathogens such as those that might be encountered in a biological attack.

Keywords: *Bacillus anthracis*; VEEV; EBOV and MARV; DNA vaccines

1. Introduction

Multivalent vaccines are highly desirable for routine use against infectious diseases and also as a safeguard against pathogens introduced deliberately through bioterrorism acts. Among the challenges in developing multiple conventional vaccines for highly pathogenic organisms are: (1) the expense and risk involved in producing and inactivating the pathogens so that they are safe for humans; (2) the need for repeated injections of several vaccines delaying the onset of protective immunity; (3) interference with immune responses by sequential vaccination to related pathogens; and (4) requirements for adjuvants in some vaccine formulations. DNA vaccines offer potential solutions to most of these problems. DNA vaccines can be produced in bacteria inexpensively and quickly at low biocontainment levels, do not require inactivation, can be combined readily, and can be delivered by a number of methods, including painless cutaneous inoculations. DNA vaccines also have an advantage over other recombinant DNA vaccines introduced with viral vectors (e.g. vaccinia virus or adenovirus) in that they have no protein carrier, so preexisting host immunity is not a barrier to their efficacy. Although DNA vaccine technology still has many technical hurdles to overcome before it will be used routinely for preventing human diseases, results in clinical studies demonstrate that it is a viable approach [1,2]. To date, few studies of combination DNA vaccines have been reported, with most focusing on multiple genes of a single agent, for example those of the malaria parasite [3], *Mycobacterium tuberculosis* [4], or hepatitis B

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virus [5]. No studies have yet been reported on combination vaccines for multiple disparate high-hazard pathogens. To test DNA vaccines as a strategy for preventing disease caused by a combination of very dissimilar pathogens, all of which are potential biowarfare agents, we constructed candidate vaccine plasmids encoding the protective antigen (PA) of *Bacillus anthracis*, the envelope glycoproteins (GP) of Ebola (EBOV) or Marburg (MARV) viruses, or the capsid and envelope glycoproteins of Venezuelan equine encephalitis virus (VEEV).

The vaccine that we constructed for anthrax encodes the 83 kDa protective antigen of *B. anthracis*. PA is the receptor-binding subunit of both of the two active toxins produced by *B. anthracis*, lethal toxin and edema toxin [6]. During anthrax infection, the 83 kDa PA binds to mammalian host cell receptors and is subsequently cleaved by a host furin-like enzyme. The receptor bound 63 kDa fragment oligomerizes to form heptamers able to bind to and internalize lethal factor (LF) and edema factor (EF) [7]. Antibodies to PA are important for protection from the early stages of anthrax infection [8] and PA alone, without other anthrax antigens, has been shown to be sufficient to elicit protective immunity in experimental animals [9]. In addition to the toxins, another virulence factor of *B. anthracis* is a capsule that inhibits phagocytosis [10]. The human vaccine for anthrax licensed in the United States, anthrax vaccine adsorbed (AVA), is a PA-containing sterile culture filtrate of a nonencapsulated strain of *B. anthracis* adsorbed onto aluminum hydroxide. Although AVA was licensed for human use many years ago, problems with reactogenicity and the need for multiple immunizations have resulted in efforts to produce an improved vaccine.

Like *B. anthracis*, the filoviruses EBOV and MARV, are recognized biological warfare threats. Both viruses were included in the biological weapons arsenal of the former Soviet Union and MARV was weaponized [11]. More recently, suspicion of an attempt to use EBOV for bioterrorism emerged from reports that members of the same Japanese cult responsible for a chemical attack with sarin nerve gas in the Tokyo subway system, traveled to Africa during an EBOV outbreak, possibly to collect samples of EBOV for their biological weapons arsenal [12]. With mortality rates as high as 90%, filoviruses are among the most deadly of known human pathogens but there is much that is still unknown about these viruses, including their natural reservoir, and the basis of protective immunity. The filoviruses are single-strand, negative-sense RNA viruses that encode seven or eight proteins [13]. Virions have one surface envelope glycoprotein, and one major nucleocapsid protein, NP, and vaccination with both of these proteins has been demonstrated to offer protective immunity in some animal models (reviewed in [14,15]).

The alphavirus, VEEV causes a disease in humans characterized by fever, headache, and occasionally encephalitis. VEEV is usually transmitted by mosquito bite, but is also highly transmissible by aerosol. Of the six subtypes of VEEV (I–VI), strains of subtype I have caused most of the known equine epizootics and human epidemics in North, Central and South America. Other subtypes are enzootic and are typically less virulent for horses than the epizootic strains, although they retain pathogenicity for humans (reviewed in [16]). Although a licensed human vaccine for VEE is not available, two conventional vaccines for VEE have been used under IND status in humans. A live-attenuated vaccine, TC-83, was developed in 1961 by 83 serial passages of the virulent Trinidad donkey strain in guinea pig heart cell cultures [17]. Although TC-83 is generally safe and elicits long-lasting protective immunity, it causes fever, headache, and malaise in approximately 23% of vaccinated people while 18% of vaccine recipients fail to develop neutralizing antibodies [18]. A formalin-inactivated TC-83 vaccine, C84, is safe and well tolerated; however, it provides incomplete mucosal protection as reflected in its inability to protect against aerosol challenge in some rodent models [19]. Neither vaccine is currently being pursued for licensure.

As a first step toward evaluating the potential of multiagent DNA vaccines for highly pathogenic organisms, we tested individual DNA vaccines for *B. anthracis*, MARV, EBOV and VEEV in established animal models of disease, and then compared a combination of the four vaccines to the individual vaccines in a single animal species. For these studies, we delivered the DNA plasmids by gene gun to the skin of the test animal [20]. These studies demonstrate for the first time the utility of this approach for four high-hazard pathogens.

2. Materials and methods

2.1. DNA vaccine construction

All genes were inserted into expression plasmids behind a cytomegalovirus immediate early promoter. The DNA vaccine for anthrax was produced by PCR-amplifying the PA gene from a previously described construct [21]. Primers were designed such that PCR resulted in a DNA that could be cloned in frame with a tissue plasminogen activator (TPA) signal sequence of the plasmid pWRG7079. This plasmid differs from pWRG7077 [20] only in the inclusion of the TPA sequence at an *Nhe*I cloning site. Primers used were: 5′-GTCAGCTAGCGAGGTGATTCAGGCAGAAGTT-3′ and 5′-CAGTGCTAGCTCCTATCTCATAGCC-3′. The DNA vaccines for EBOV, MARV and VEEV were produced without additional signal sequences, by using plasmid pWRG7077 [20]. The cloning of the EBOV GP and NP genes and construction of the EBOV DNA vaccines were described previously [22]. The Musoke and Ravn strains of MARV were recovered from experimentally infected monkeys, then passed one time (Musoke) or three times (Ravn) in Vero cells. Cloning of the MARV Ravn GP was performed as described previously for MARV Musoke GP [23], using PCR primers: 5′-GACATGAAGACCATATA-3′

and 5′-CTTTATGTCATCCAATG-3′. The VEEV 26S gene was kindly provided by Smith and co-workers, USAMRIID, in plasmids described earlier [24]. The 26S insert was PCR-amplified using primers: 5′-GCCATGCGGCCGCATGTTCCCGTTCCAGCCAATG-3′ and 5′-CGGATGGATCCCTATGTAAGCAGCTTGCCAATTGC-3′, and the amplicons were cloned into the *Not*1 and *Bam*HI sites of pWRG7077 [20].

2.2. Immunization, serological assays and challenge of animals

Preparation of gene cartridges was as described earlier [20]. All vaccinations were performed by using the XR-1 delivery device (Powderject Vaccines Inc., Madison, WI) as described previously [20]. Dose and number of vaccinations for each construct are described in the text. For anthrax rabbit studies, New Zealand white rabbits (Charles River Laboratories, Wilmington, MA) were vaccinated by gene gun at weeks 0, 4, 8, and 21. Control animals were vaccinated by intramuscular injection of the AVA licensed human vaccine (BioPort, Lansing, MI), at weeks 0, 4, 8, and 12 and then challenged at week 24 by subcutaneous injection of virulent, heat-activated *B. anthracis* spores prepared as described earlier [8]. For detecting antibodies to anthrax, ELISA using purified PA antigen were performed as described earlier [25,26]. Toxin-neutralizing antibody tests (TNA) were performed as previously described [26,27]. DNA vaccination of 6–8-week old BALB/c mice with EBOV GP or NP DNA vaccines and challenge with a mouse-adapted strain of EBOV [28] were as described earlier [22], except that only two vaccinations were performed. IgG antibody ELISA was performed using purified, irradiated EBOV antigen [22]. For MARV studies, inbred Strain 13 guinea pigs were obtained from an in-house breeding colony. Outbred Hartley guinea pigs were purchased from Charles River Laboratories. Guinea pigs were vaccinated and challenged as described earlier [23]. Antibody responses were assessed by IgG antibody ELISA using cobalt-irradiated purified MARV, strains Ravn or Musoke [29]. For assessing the MARV vaccine in nonhuman primates, cynomolgus macaques (*Macaca fascicularis*) were anesthetized, bled and treated for signs of illness as described [30]. A detailed clinical evaluation, serum for viremia determination, and blood chemistries were obtained from anesthetized animals at times indicated in the text. Viremia was measured by plaque assay on Vero E6 cells. Vaccination of 6–8-week old BALB/c mice with the VEEV DNA vaccine was as described in the text. Mice were challenged by aerosol or by subcutaneous inoculation of VEEV, subtype 1A/B as described earlier [31]. Antibody ELISA, using irradiated VEEV antigen, and plaque reduction neutralization tests (PRNT) were performed as described [24]. For multiagent vaccine studies, Strain 13 guinea pigs were used for animals destined to be challenged with EBOV and Hartley guinea pigs were used for all other groups.

3. Results

3.1. Protection of rabbits with an anthrax DNA vaccine

A variety of small laboratory animals experience lethal infections when infected with *B. anthracis*, including mice, guinea pigs and rabbits. Of these, rabbits have been shown to be the animal model of choice for predicting anthrax vaccine efficacy in humans. In addition, anti-PA antibody levels in rabbits, as measured by ELISA and toxin-neutralizing antibody assay were found to be a significant correlate of protective immunity [26]. To evaluate our DNA vaccine expressing anthrax PA we vaccinated groups of 10 rabbits three times at 4-week intervals by gene gun inoculation. Each DNA vaccination consisted of a total of ∼20 μg of DNA distributed over eight sites on the abdomen of each rabbit. Control groups received the same number of gene gun vaccinations with a plasmid with no insert, or the AVA human vaccine, which was given at the human dose (0.5 ml) four times at 4-week intervals by intramuscular injection. Evaluating antibody responses by ELISA and TNA revealed that all rabbits vaccinated with the DNA vaccine or with AVA vaccine developed antibody responses predictive of protective immunity to anthrax challenge (Table 1) [26]. Slight decreases were noted in antibody levels by week 21 (12 weeks after the third vaccination) (Table 1). After a fourth vaccination, titers rebounded and the rabbits were challenged by subcutaneous injection of 100 LD_{50} of *B. anthracis* Ames strain heat-shocked spores. All rabbits that received the control plasmid vaccine died within 4 days of challenge, while 9/10 rabbits given the PA DNA vaccine and 7/10 given the AVA human vaccine survived (Table 1). The PA DNA-vaccinated rabbit that succumbed to infection died at day 8, and the AVA vaccinated rabbits died at days 4, 5 or 8 after challenge.

Post-challenge ELISA using sera of surviving rabbits showed a small increase in mean antibody titers (Table 1); however, for some animals, titers decreased after challenge, suggesting sterile immunity (data not shown). Thus, this study demonstrates that the PA-expressing DNA vaccine elicits protective immunity in an animal model that is relevant to human disease prevention.

3.2. DNA vaccines for MARV elicit protective immunity to rodents or nonhuman primates

To evaluate the DNA vaccine approach for MARV, we cloned and expressed the GP genes of two distant Kenyan isolates, strains Musoke [32] and Ravn [33]. The GP of these strains are predicted to have 77% amino acid homology, with most differences occurring in a central highly variable region. Although primary isolates of EBOV and MARV cause nonfatal illness in guinea pigs, a small number of passages in guinea pigs results in selection of variants able to cause fatal disease with pathological features similar to those seen in filovirus-infected primates [34]. For our studies, we gene gun-vaccinated inbred (Strain 13) or outbred (Hartley)

Table 1
Antibody responses of vaccinated rabbits and protection from challenge with virulent anthrax spores

Vaccine[a]	Survivors/total	Mean day of death	$\log_{10}$ GMT anti-PA ELISA[b]					TNA[c]	
			Wk 4	Wk 8	Wk 12	Wk 21	Wk 24	Wk 28[d]	Wk 24
PA DNA	9/10	8	3.83	4.97	5.79	5.26	5.86	6.06	2.82
AVA vaccine	7/10	6	5.14	6.19	5.28	5.04	4.86	5.40	2.69
Control DNA	0/9	3	<2.0	<2.0	<2.0	<2.0	<2.0	NA[e]	<2.0

[a] New Zealand white rabbits were vaccinated with DNA by gene gun at weeks (Wk) 0, 4, 8, and 21 or with the AVA vaccine at Wk 0, 4, 8, and 12 and then challenged at WK 24 by subcutaneous injection of virulent *B. anthracis* spores.

[b] Reciprocal geometric mean titers (GMT) against *B. anthracis* protective antigen (PA) at indicated number of weeks after the first vaccination were determined by ELISA using purified PA antigen as described earlier [25,26].

[c] Toxin-neutralizing antibody test (TNA) values represent the reciprocal of the dilution that protected 50% of J774A.1 cells from cytolysis by lethal toxin.

[d] Wk 28 GMT were calculated from sera taken from survivors at 28 days after challenge.

[e] NA: not applicable.

guinea pigs three (Musoke) or four (Ravn) times at 4-week intervals with approximately 2.5 μg of the MARV GP DNA to each of four sites on their shaved abdomens (approximately 10 μg per guinea pig at each time point). Control guinea pigs were vaccinated with the parent plasmid with no inserted gene. Antibody levels were measured by ELISA against purified irradiated MARV 4 weeks after each vaccination [29]. All of the MARV GP DNA-vaccinated guinea pigs developed antibodies to MARV (Table 2). Guinea pigs were challenged by subcutaneous injection of 1000 plaque forming units (pfu) of homologous virus 4 weeks after the final vaccination. All of the guinea pigs vaccinated with control DNA were viremic at day 7 post-challenge, as measured by plaque assay, and all succumbed to infection by day 9. In contrast, all of the guinea pigs vaccinated with the GP DNA vaccines were aviremic at day 7 and all appeared healthy throughout the 30-day observation period (Table 2).

Table 2
ELISA antibody titers, viremia and survival of guinea pigs vaccinated with GP DNA of Marburg virus, strains Musoke or Ravn, and challenged with ~1000 pfu of homologous virus[a]

Guinea pig strain	DNA vaccine	Viremia[b] $\log_{10}$ GMT	Survivors/ total	ELISA[c] $\log_{10}$ GMT
Strain 13	Musoke GP	<1.0	5/5	2.84
	Control	3.48	0/4	NA
Hartley	Ravn GP	<1.5	6/6	2.07
	Control	4.41	0/6	NA[d]

[a] Guinea pigs were vaccinated at 4-week intervals three (Musoke) or four (Ravn) times by gene gun administration of approximately 2.5 μg of DNA to each of four sites on their shaved abdomens (approximately 10 μg total DNA per vaccination).

[b] Guinea pigs were bled 7 days after challenge and viremia was measured by plaque assay on Vero E6 cells.

[c] ELISA geometric mean titers (GMT) of IgG antibody assays were measured for serum collected 3–4 weeks after the final vaccination using cobalt-irradiated, purified, MARV, strains Musoke or Ravn, as antigen [29].

[d] NA: not applicable. The average OD of sera from control guinea pig sera at each dilution was subtracted from the OD of cognate dilutions of GP DNA-vaccinated guinea pig sera.

Nonhuman primates are thought to be the best model for human disease caused by filoviruses; consequently, we also tested the DNA vaccine for MARV, strain Musoke, in cynomolgus macaques (*M. fascicularis*). In each of two separate studies, three monkeys were vaccinated three times at 4-week intervals with approximately 20 μg of GP DNA delivered to eight sites on the abdomen. A control monkey for the first experiment was vaccinated with the parent plasmid, and a control for the second experiment was not vaccinated with DNA. The use of only one control monkey per study was deemed sufficient based on results for other studies conducted with the same challenge virus at this institute in which 10 of 10 cynomolgus monkeys infected with MARV died ([30] and unpublished information).

Antibody responses of the monkeys were measured by ELISA against purified irradiated MARV before each vaccination and were found to be similar for all of the DNA-vaccinated monkeys (Table 3). Monkeys were challenged by subcutaneous injection of 1000 pfu MARV, strain Musoke (approximately 1000 LD_{50}), and examined daily for symptoms of disease. Temperatures were taken and blood samples collected for measuring viremia on days 3, 5, 7 and 10 after challenge. Control monkeys developed fevers >102 °F (normal range 98–102 °F) by day 5 after infection. All monkeys had fevers on days 7 and 10 after challenge indicating active infections (Table 3). Viremia was assessed by plaque assay on Vero E6 cells. In both studies, two of three GP DNA-vaccinated monkeys were aviremic on the days assayed, and survived challenge, while one monkey in each study developed viremia levels similar to those of control monkeys and died. In the second study, the vaccinated monkey that died had a slightly delayed time to death (Table 3).

During MARV infections of humans or monkeys, increased levels of liver enzymes are typical. In our studies, both control monkeys, and both of the DNA-vaccinated monkeys that died showed drastic rises in aspartate aminotransferase (AST) alanine aminotransferase (ALT) and alkaline phosphatase (ALP) levels (data not shown). In contrast, the DNA-vaccinated monkeys that survived had normal

Table 3
ELISA antibody titers, viremia and survival of cynomolgus macaques vaccinated with GP DNA of Marburg virus, strain Musoke, and challenged with ~1000 pfu of homologous virus

Monkey ID	Vaccine	ELISA[a] $\log_{10}$ GMT	Viremia[b] $\log_{10}$ GMT	Fever >102 °F (day after challenge)	Survival (day of death)
93414	GP	2.89	<1.5	+(7, 10)	+
215Q	GP	2.43	<1.5	+(7, 10)	+
21Q	GP	2.39	8.61	+(7)	−(9)
93262	Control	<1.5	8.17	+(5, 7)	−(8)
57-345	GP	2.69	<1.5	+(7, 10)	+
67-104	GP	2.37	<1.5	+(7, 10	+
126-348	GP	2.29	7.65	+(7, 10)	−(11)
66-141	Control	<1.5	8.26	+(5, 7)	−(9)

[a] ELISA geometric mean titers (GMT) of IgG antibody assays were measured for serum collected 3 weeks after the final vaccination.
[b] Virus in blood samples was measured by plaque assay on Vero E6 cells at days 5 and 8 (study 1), or days 3, 5, 7, 10 (study 2). The highest viremia detected for any sample is listed.

levels of all three liver enzymes except for one monkey that showed a slight rise in AST level 10 days after challenge (AST level 126, normal range 20–99). These results indicate that the DNA vaccine alone is able to offer immunity to nonhuman primates, but suggest that the protective effect is near the threshold of vaccine efficacy.

3.3. Two doses of DNA vaccines expressing EBOV GP or NP provide protective immunity to mice

For EBOV, both guinea pig and mouse models of disease have been developed using virus variants selected by animal passage [28,34]. Mice infected with a mouse-adapted virus develop disease characterized by high levels of virus in target organs and pathologic changes in livers and spleens akin to those found in EBOV-infected primates [28]. Using the mouse model, we previously demonstrated that vaccination with DNA expressing either the EBOV GP or NP gene could protect mice from EBOV challenge [22]. We further demonstrated CTL responses in mice given either vaccine. In those studies, we found that we needed numerous vaccinations to elicit protective immunity; however, we have since revised methods for evaluating the quality of the DNA/gold cartridges, which has led to improved protection. In three separate mouse studies, we found that two vaccinations with either the EBOV GP or NP DNA elicited consistently high antibody responses and conferred complete protection from EBOV challenge (Fig. 1). Our data indicate that DNA vaccination is very effective in the EBOV mouse model. Because the mouse model has not been shown to predict efficacy in primates [14], further studies are needed to determine the suitability of the DNA/gene gun approach for developing human vaccines for EBOV.

3.4. A DNA vaccine for Venezuelan equine encephalitis virus protects mice from aerosol and peripheral challenge

To evaluate the DNA/gene gun vaccine approach for VEEV, we constructed a plasmid containing cDNA representing the subgenomic (26S) mRNA of VEEV, subtype I/AB [24]. Expression of the 26S alphavirus mRNA yields a polyprotein that is further processed into the viral capsid protein (C), a precursor of the envelope glycoproteins E2 and E3 (PE2), and the glycoprotein E1. During viral morphogenesis, PE2 is cleaved in the Golgi to yield the mature E2 protein, which forms heterodimers with E1 on the viral envelope [35,36]. Both neutralizing anti-E2 Mabs, and non-neutralizing Mabs to either E1 or E2 of VEEV have been demonstrated to passively protect mice from challenge [37–39]. For our study, we vaccinated groups of 20 BALB/c mice three times at 3-week intervals with ~3 μg of the DNA vaccine delivered to two sites on the abdomen of each mouse. Control groups of mice were vaccinated with a plasmid with no insert or by intramuscular injection of 0.2 ml TC-83 human vaccine. At each vaccination and 2 weeks after the last vaccination, blood was collected and antibody

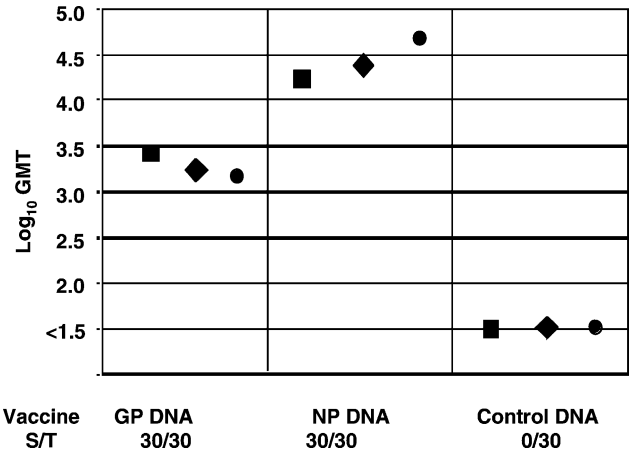

Fig. 1. Vaccination of mice with EBOV GP or NP DNA vaccines and challenge with EBOV. Mice were vaccinated by gene gun at weeks 0 and 4 with ~5 μg of DNA delivered to two sites on the abdomen and then challenged at week 8 by intraperitoneal injection of 1000 pfu of EBOV. IgG antibody ELISA were performed using purified, irradiated EBOV antigen [22]. The ELISA geometric mean titers (GMT) are shown for each of three separate experiments: (■) Experiment 1; (◆) Experiment 2; (●) Experiment 3 (S/T: survivors/total).

Table 4
Antibody responses, IgG isotypes and survival of mice ($n = 10$ per group) vaccinated with Venezuelan equine encephalitis virus structural protein (26S) DNA vaccine and challenged with 10^4 plaque forming units of homologous virus

Vaccine	Challenge route	$PRNT_{50\%}$ $\log_{10}$ GMT range	Survivors/total	Antibody isotype[a] ELISA $\log_{10}$ GMT		
				Total IgG	IgG1	IgG2
26S DNA	Subcutaneous	<1.6–2.5	10/10	3.44	2.30	<1.65
	Aerosol	<1.6–1.6	8/10			
Control DNA	Subcutaneous	<1.6	0/10	ND[b]	ND	ND
	Aerosol	<1.6	0/10			
TC-83	Subcutaneous	<1.6 >3.1	8/10	4.18	<1.65	3.88
	Aerosol	>3.1	9/10			

[a] VEEV-specific total IgG, IgG1 and IgG2 were determined by ELISA for serum samples collected 3–4 weeks after the final vaccination [23] and are shown as geometric mean titers (GMT).
[b] ND: not determined.

responses were measured by ELISA using irradiated VEEV antigen and by plaque reduction neutralization test [24]. DNA-vaccinated mice developed strong antibody responses, as measured by ELISA, but had low levels of neutralizing antibodies (Table 4). In contrast, mice that received the TC-83 vaccine developed very high neutralizing antibody responses, except for two mice that did not respond to vaccination. Three weeks after the final vaccination, half of the mice were challenged with VEEV by subcutaneous infection and the other half by aerosol infection with $\sim 10^4$ pfu of VEEV [31]. All mice that received the negative control DNA vaccine died upon VEEV challenge by either route of infection. All VEEV DNA-vaccinated mice survived the subcutaneous challenge, as did all of the TC-83-vaccinated mice that responded to the vaccine (Table 4). Two VEEV DNA-vaccinated mice and one TC-83-vaccinated mouse died after aerosol challenge.

To further investigate the differences in the immune responses generated by the DNA and the live-attenuated VEEV vaccines, we compared the antibody isotype profiles of vaccinated mice. We found that the gene gun-vaccinated mice had predominantly IgG1 antibody responses, indicative of a TH2-type response. In contrast, the TC-83-vaccinated mice had IgG2 responses, indicative of a TH1-type response. Although these data do not help to explain why the mice had such low neutralizing antibody responses, yet were protected from challenge, they are consistent with earlier studies indicating that gene gun vaccination generally elicits a TH2-type response in BALB/c mice [40].

3.5. Vaccination of guinea pigs with four distinct DNA vaccines results in immune responses to each pathogen

Our data indicate that DNA vaccines for *B. anthracis*, EBOV, MARV, and VEEV can induce protective immunity in animal models for each disease. To assess the DNA vaccine strategy for multiagent vaccines for these pathogens, we performed an additional study in which groups of guinea pigs were vaccinated with the individual vaccines or with a combination of all four vaccines. The guinea pig adapted EBOV that we used in these studies has previously been characterized only in inbred guinea pig models; therefore, for groups that were to be challenged with EBOV, we used inbred Strain 13 guinea pigs. For all other groups we used outbred Hartley guinea pigs. Guinea pigs receiving the individual vaccines were given approximately 5 μg of DNA at two sites on the abdomen. Guinea pigs receiving all four vaccines were given 5 μg of each vaccine by gene gun at two sites on the abdomen (total of 20 μg delivered to eight sites per guinea pig). The guinea pigs were vaccinated at weeks 0, 4 and 8, and challenged at week 21 by subcutaneous infection with EBOV, MARV or VEEV. Anthrax challenges were not performed because of results of a previous study in which we found that neither the DNA vaccine nor the AVA human vaccine was able to completely protect guinea pigs from spore challenge (data not shown).

Blood samples collected 3 weeks after the final vaccination were compared by ELISA to the appropriate pathogen's antigens (Table 5). Geometric mean titers (GMT) for groups receiving the individual or multiagent vaccines were nearly the same. Additional samples collected at weeks 17 and 21 after vaccination also showed nearly equivalent mean titers in the single and multiagent groups (data not shown). In all groups, antibody levels were low, which is likely a reflection of the small immunizing dose. In our earlier single agent experiments we generally delivered ~5 μg of DNA to each of 4–8 sites per animal as compared to only two sites per animal in the single agent or multiagent groups in this study. Despite these relatively low titers, protection was observed in all of the challenge groups, with no obvious differences between groups receiving the single and the multiagent vaccines.

All but one of the guinea pigs challenged with VEEV survived (Table 5). The guinea pig that died was in the multiagent group and had no antibody response to VEEV detected by ELISA or by PRNT (data not shown). That particular guinea pig responded poorly to all of the DNA vaccines, with no detectable ELISA titer to anthrax PA, and barely detectable responses to MARV and EBOV. All other guinea pigs had antibodies to VEEV detected by ELISA, but three

Table 5
Comparison of single agent and multiagent DNA vaccines in guinea pigs[a,b]

Vaccine	Guinea pig strain	ELISA[c] $\log_{10}$ GMT	Viremic/total[d]	Survivors/total[e]	Mean day of death (range)
EBOV DNA	Strain 13	2.5	4/6	4/6	9 (8–9)
Multi DNA	Strain 13	2.7	2/5[f]	3/5	16 (10–22)
Control DNA	Strain 13	<1.6	6/6	0/6	11 (9–14)
MARV GP DNA	Hartley	3.0	2/6	3/6	10 (8–11)
Multi DNA	Hartley	2.5	1/6	4/6	12 (10–13)
Control DNA	Hartley	<1.5	6/6	0/6	12 (9–13)
VEEV 26S DNA	Hartley	2.7	1/6	6/6	NA[g]
Multi DNA	Hartley	2.3	2/6	5/6	6 (NA)
Control DNA	Hartley	<2.0	6/6	0/6	6 (5–7)
TC-83	Hartley	>5.0	0/6	6/6	NA
Anthrax DNA	Hartley	2.5	NA	NA	NA
Multi DNA	Hartley	2.6	NA	NA	NA
Control DNA	Hartley	<1.7	NA	NA	NA
AVA	Hartley	>4.5	NA	NA	NA

[a] Groups of six guinea pigs were vaccinated at weeks 0, 4 and 8 with approximately 2.5 μg of DNA per gene gun administration. At each time point, guinea pigs receiving the individual vaccines were given two administrations on shaved abdomens (5 μg total DNA) and animals receiving all four vaccines were given eight administrations (5 μg of each DNA, 20 μg total).
[b] Guinea pigs were challenged at week 21 by subcutaneous infection with EBOV, MARV or VEEV.
[c] Geometric mean ELISA titer (GMT) was determined 3 weeks after the third vaccination.
[d] Viremia was assessed 3 days after challenge for VEEV and 7 days after challenge for EBOV and MARV by plaque assay of serum samples.
[e] Survival was monitored for at least 30 days after challenge.
[f] One guinea pig died during bleeding, reducing the group size to five animals.
[g] NA: not applicable.

others, two in the multiagent group and one in the single agent group, had no detectable neutralizing antibodies to VEEV (data not shown). The remainder of the guinea pigs had neutralizing antibody titers to VEEV ranging from 1:20 to 1:640. Blood samples collected 3 days after the VEEV challenge were also examined for infectious virus by plaque assay. Very low levels of viremia were detected in only one of six animal in the single agent group (titer 1.2×10^2 pfu/ml) and two of six animals in the multiagent group (0.2 and 1.3×10^2 pfu/ml). In contrast, all of the guinea pigs receiving the control DNA displayed viremias $>10^6$ pfu/ml. The viremic guinea pigs in the multiagent group had no detectable neutralizing antibodies to VEEV and the viremic guinea pig in the single agent group had a $PRNT_{50\%}$ titer of 1:20. One additional guinea pig in each group had an undetectable neutralizing antibody titer to VEEV, but they were not viremic. These results are similar to those that we obtained in BALB/c mice, in that the guinea pigs were protected from challenge with VEEV despite having low or no detectable neutralizing antibody responses.

Approximately half of all guinea pigs survived the filovirus challenges. All of the guinea pigs had detectable ELISA antibody titers before challenge, with no obvious quantitative differences between the titers of those that survived and those that died. In other studies, we have not seen a correlation of neutralizing antibody responses with protective immunity to EBOV or MARV in guinea pigs (unpublished information); therefore, we did not measure neutralizing antibody responses in this study. The lesser survival of MARV-challenged guinea pigs in this study as opposed to the single agent study may be the result of a lower immunizing dose in the multiagent vaccinated guinea pigs.

For the EBOV challenge groups, viremia measured at days 7 and 14 (of the survivors) by plaque assay revealed that two of the four survivors in the single agent group and one of the three survivors in the multiagent group were viremic at day 7 but recovered and were aviremic by day 14 (data not shown). One of the guinea pigs that died in the multiagent group had a delayed time of death (day 22) as compared to the controls, all of which died between nine and 14 days after challenge. For the MARV vaccine groups, all three guinea pigs that showed viremia 7 days after challenge died, with no delay to death noted as compared to controls.

4. Discussion

Our data suggest that multiagent DNA vaccines may be feasible for use against dissimilar, highly pathogenic organisms. We were able to vaccinate guinea pigs by gene gun with microgram quantities of four different DNA vaccines and elicit immune responses to each pathogen that were equivalent to responses obtained with individual vaccines. Although we did not compare other delivery methods, we have found that the gene gun is an efficient means to elicit humoral—and where measured—cell-mediated immune responses to a number of pathogens [20,22,23,41–43].

The success of the gene gun DNA vaccine approach is due in large part to the abundance of antigen presenting cells

found in the target site, skin [44]. However, because the DNA can only be transcribed in host cell nuclei, and because most of the gold bullets coated with DNA are deposited in the cell cytoplasm, only a small fraction of the cells receiving the DNA vaccine actually express the gene of interest. The gene gun methodology suffers, therefore, from the need to vaccinate multiple sites to achieve expression in an adequate number of host cells.

In the studies reported here, each vaccine was delivered to two separate sites on the abdomens of guinea pigs in both the individual vaccine groups and the multiagent vaccine groups. We chose this method, rather than combining the DNAs before delivery, in order to more directly compare the multiagent and single agent vaccines. This regimen resulted in the guinea pigs in the multiagent groups receiving eight separate gene gun administrations per dose, whereas those in the single agent groups received only two per dose. Although we do not believe that this difference influenced the results either positively or negatively, we cannot rule out either possibility until further experiments are conducted. In future studies it will also be important to determine if mixed DNAs given to fewer sites per animal are equally as efficacious as individual vaccines delivered to multiple sites. In addition, because the quantity of DNA that can be delivered in each gene gun administration is limited to approximately 3 μg, it will be necessary to determine the lower limit of each DNA required for immunogenicity, or alternatively, to test other methods that allow delivery of more DNA. Other skin delivery methods, such as electroporation, microneedle injectors, abrasive devices and chemicals that facilitate transport of DNA across the stratum corneum, have shown promising results in test systems [44,45], but it remains to be seen whether any of these methods is superior to the gene gun in eliciting immune responses to DNA vaccines.

For three of the four pathogens that we investigated, we were able to demonstrate that not only were immune responses similar in single agent and multiagent groups of guinea pigs, but protection was similar as well. For our anthrax studies, we chose not to challenge vaccinated guinea pigs because we and others previously found that not only are DNA vaccines not protective in this model, but that the AVA human vaccine also confers only partial protection to peripheral challenge with virulent spores and is poorly protective to aerosol challenge [46,47]. In rabbits, however, a statistical correlation between survival and levels if anti-PA antibody detected by ELISA or TNA was demonstrated [26]. In those studies, 2 weeks after a second dose of AVA vaccine was given, 9/10 rabbits with TNA titers equivalent to those we measured in this study ($\log_{10}$ GMT = 2.8) were protected from aerosol challenge with approximately 100 LD_{50} of spores [26]. We would predict, therefore, that our DNA vaccine could also protect rabbits from aerosol challenge, and we will be testing this and primate protection in future studies.

In guinea pigs, we tested vaccines against two of the most distantly-related isolates of MARV, the Musoke and Ravn strains. In data not reported here, we observed incomplete cross-protection to heterologous challenge of guinea pigs given either vaccine (unpublished information). Thus if DNA vaccination were to be pursued for use against MARV, it is possible that two or more GP genes from various isolates would need to be included in the combination vaccine.

Our results are the first reported showing efficacy of DNA vaccination for MARV challenge in nonhuman primates. In the two separate studies we performed in cynomolgus monkeys, two of three monkeys survived challenge. The monkeys that died had very similar antibody titers by ELISA to those that survived; suggesting that the antibody response measured with this assay cannot predict protective immunity, or that the protective response elicited with the DNA vaccines was on the threshold of efficacy. If so, it may be possible to improve the immune response to cross that threshold by increasing the number of vaccinations or delivering adjuvants with the vaccines. For example, recent studies demonstrated that plasmids encoding the alpha and beta subunits of cholera toxin or the heat-labile enterotoxin from *E. coli* are potent genetic adjuvants for DNA vaccines delivered by gene gun [48].

For EBOV, although our DNA vaccine was very efficacious in mice, it was poorly protective in guinea pigs in both the single agent and multiagent groups. Likewise, in a study by others, intramuscular injection of large quantities of an EBOV GP DNA vaccine resulted in protection of some, but not all guinea pigs [49]. Although an EBOV GP DNA vaccine alone may not be sufficient to protect guinea pigs from EBOV challenge, DNA vaccination might still be of use in a prime-boost regimen. In one report, monkeys vaccinated with an EBOV GP DNA vaccine followed by an adenovirus-vectored GP DNA vaccine survived a low dose challenge with EBOV [50]. The DNA alone and the adenovirus vector alone were not compared to the prime-boost in that monkey study, so it is not clear what contribution either made to the protective efficacy seen. Another recent report suggests that preexisting immunity to adenovirus or vaccinia virus vectors can be partially overcome by priming mice with a GP DNA vaccine to EBOV before vaccinating with the recombinant adenovirus or vaccinia viruses [51]. It has not yet been determined if this same result could be obtained in guinea pigs or in primates.

We investigated our EBOV GP DNA vaccine in a prime-boost regimen with baculovirus-expressed EBOV GP. Although in similar studies with MARV in guinea pigs we found this regimen to improve protective efficacy compared to DNA alone [23], we observed no better protection with the EBOV GP DNA prime-baculovirus-derived protein boost than we did with the DNA vaccine alone [52]. Thus, at present we have no information to indicate that our DNA vaccine improves protective efficacy by subsequent, heterologous vaccines.

For VEEV, our DNA vaccine protected both mice and guinea pigs from challenge in the absence of high levels of neutralizing antibodies. Although this might implicate

cell-mediated immune responses in protection, many lines of evidence indicate that protection from alphavirus diseases is most closely associated with humoral immunity. Moreover, it is known that protective neutralizing antibodies of alphaviruses are directed primarily to E2 but non-neutralizing antibody responses to both the E1 and E2 can protect (reviewed in [16]). Studies are currently underway in our laboratory to further define the mechanism of protection by this DNA vaccine.

In summary, it is clear that both technological and logistical problems need to be solved before DNA vaccines for high-hazard pathogens are used routinely. Among these is the formidable task of demonstrating their efficacy against low-incidence diseases. Nevertheless, our studies offer encouragement for the further development of combination DNA vaccines for high-hazard pathogens. It is likely that a variety of approaches and experimental conditions will need to be tested with each DNA vaccine or combination of vaccines to determine the best regimen for protection.

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