EXPERT REVIEWS

DNA vaccines for biodefense

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Lesley C Dupuy and Connie S Schmaljohn†

†*Author for correspondence*
Headquarters, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD 21702, USA
Tel.: +1 301 619 4103
Fax: +1 301 619 2439
connie.schmaljohn@amedd.army.mil

An ideal biodefense vaccine platform would allow for the quick formulation of novel vaccines in response to emerging or engineered pathogens. The resultant vaccine should elicit protective immune responses in one to three doses and be unaffected by pre-existing immunity to vaccine components. In addition, it should be amenable to combination and multi-agent formulation, and should be safe for all populations and the environment. DNA vaccines can potentially meet all of these requirements; thus, this platform is being tested with several biodefense threats. Here, we provide a review of the current status of the development efforts for DNA vaccines against several relevant biodefense pathogens: *Bacillus anthracis*, Ebola and Marburg viruses, smallpox virus, and Venezuelan equine encephalitis virus.

Keywords: alphavirus • anthrax • *Bacillus anthracis* • biodefense • DNA vaccine • Ebola virus • electroporation • gene gun • Marburg virus • monkeypox virus • poxvirus • smallpox • vaccinia virus • variola virus • Venezuelan equine encephalitis virus

Concerns about the deliberate release of naturally occurring pathogens or of novel genetically engineered organisms has resulted in a flurry of biodefense research activities in the last decade. Vaccination is generally the best way to protect a population from the effects of an infectious organism; thus, much of the recent biodefense work has focused on developing and stockpiling vaccines that will be effective against such threats. DNA is a particularly attractive platform for biodefense vaccines in that DNA vaccines can be rapidly designed for both known and engineered organisms without the need to propagate a pathogen. In addition, manufacturing and quality control methods for producing DNA plasmids in bacteria are well-established, allowing for DNA vaccines to be both safe and cost-effective. Importantly, pre-existing immunity is not problematic for DNA vaccines due to the absence of a host immune response to the plasmid backbone, and delivery of mixtures of DNA vaccines offers the potential for multi-agent protection.

Despite these advantages, technical hurdles and immunogenicity problems have thus far prevented the routine clinical use of DNA vaccines. The biggest challenge to commonplace human use of DNA vaccines is the need for more effective mechanisms of delivery. Because the DNA must enter host cell nuclei for transcription to ensue, standard needle-based intramuscular injection is a very inefficient method of administration, as only a small amount of DNA is transported across cell membranes and expressed. Several different delivery devices and procedures have been developed to circumvent this issue. The three most effective delivery methods reported to date are:

- Complexing the DNA vaccines with cationic liposomes to facilitate transport across membranes (e.g., Vical's Vaxfectin™ reagent, CA, USA [1]);
- Particle-mediated epidermal delivery (PMED, or gene gun), which involves precipitating the DNA onto gold beads and using gas pressure to convey the DNA-coated gold into the skin [2];
- Electroporation, which involves injecting DNA into muscles or skin then rapidly applying very short pulses of electricity at the delivery site to temporarily disrupt cell membranes [3].

Although all of the methods have been tested in clinical studies, none of them has yet led to licensure of a human DNA vaccine.

In this review, we will summarize developments on DNA vaccines for five threats listed as priority biodefense pathogens by the US NIH and the US CDC: *Bacillus anthracis*, Ebola (EBOV) and Marburg (MARV) viruses, smallpox virus, and Venezuelan equine encephalitis virus (VEEV). We will also provide comment on future research that will be critical to the success of biodefense-related DNA vaccines.

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DNA vaccines for anthrax

Protective immunity & animal models

B. anthracis is the first category A agent listed on both the NIH priority pathogen and CDC bioterrorism registries. It is a Gram-positive, spore-forming bacterium. The vegetative form of the bacillus is not readily transmissible, but the spores can remain viable in the environment for many years and are easily transmissible. *B. anthracis* encodes its major virulence factors on two plasmids: pXO2, which produces a polyglutamic acid capsule that helps the bacteria evade phagocytosis, and pXO1, which produces three proteins that can form two binary toxins. The pXO1 protein protective antigen (PA) serves as a cell-binding component for both of the other proteins, lethal factor (LF) and edema factor (EF). Shortly after spore germination, the full-length 83-kDa PA (PA83) binds to mammalian host cell receptors and is subsequently cleaved by a host furin-like enzyme. The receptor-bound 63-kDa PA (PA63) fragment oligomerizes to form heptamers able to bind to and internalize LF and EF [4]. LF is a zinc-dependent metalloprotease that disrupts cell signaling [5] and EF is an adenylate cyclase that causes tissue edema [6].

Antibodies to PA are important for protection from the early stages of anthrax infection and PA alone, without other anthrax antigens, has been shown to be sufficient to elicit protective immunity in experimental animals [7–9]. Consequently, most vaccines, including DNA vaccines, have targeted PA. In authentic anthrax infections, the bacterium secretes PA; thus, it is presented as a soluble protein to immune cells where it probably enters the MHC class II pathway. Including a mammalian secretion signal on PA constructs, is therefore expected to provide an antigen that more closely resembles PA presentation to the immune system during infection. Although not all DNA vaccines tested have included a secretion signal, strong antibody responses to PA83 have not been observed without them. Generally, a tissue plasminogen activator (tPA) signal sequence has been used, but a limited amount of work has also been performed to compare signals that result in the secretion or direction and retention of PA in specific intracellular compartments. Other modifications to PA, such as codon optimization and fusion to other proteins, have also been tested [10].

A variety of small laboratory animal models have been used to evaluate anthrax vaccines, including mice, guinea pigs and rabbits. Of these, rabbits have been shown to be most valuable for predicting anthrax vaccine efficacy in humans. In addition, anti-PA antibody levels measured by ELISA and by toxin -neutralizing assays were shown to significantly correlate with protective immunity in rabbits [11]. Although mice are useful for assessing immune responses to vaccines, unlike many other animals, including humans, nontoxigenic pX01-cured anthrax strains are not completely attenuated for all strains of mice [12]. It appears that the main virulence factor for mice is the capsule; consequently, vaccination with PA-based vaccines often fails to protect mice from a challenge with spores. A/J strain mice are deficient in complement factor C5, which increases their susceptibility to toxigenic strains of *Bacillus anthracis* and can serve as a useful challenge model [13–16].

PA DNA vaccine constructs

The first report showing that PA DNA vaccination can protect from anthrax toxin challenge was performed in mice vaccinated intramuscularly three times at 3-week intervals with 50 µg of a plasmid expressing the 63-kDa form of PA with a tPA signal. The vaccinated mice developed anti-PA antibodies and seven out of eight of them were protected from intravenous injection of lethal toxin [17]. In another study, intramuscular injection of rats with approximately 175 µg of PA83 DNA in saline was compared with retroductal delivery to salivary glands of the same amount of DNA formulated with cationic lipid. After 16 weeks post-vaccination, rats were challenged by intravenous injection of a low dose of lethal toxin. All of the intramuscularly vaccinated rats died within 4 h, but four out of the six rats vaccinated by salivary gland perfusion survived for 24 h [18]. Note that only the vaccine delivered to the salivary gland was formulated with lipid, so it is not clear how much that formulation difference contributed to the improved survival. In addition, because only one vaccination was given, it is not possible to determine if a boost would have resulted in better protection by either regimen.

As indicated earlier, rabbits are the model of choice for testing anthrax vaccine efficacy. Protection of rabbits to subcutaneous challenge with 100 LD_{50} of Ames strain spores was obtained by gene-gun delivery of PA83 DNA with a tPA signal [19]. In that study, ten rabbits received approximately 20 µg of DNA three times at 4-week intervals. Control groups of rabbits were vaccinated with an irrelevant DNA vaccine, or with the human anthrax vaccine, anthrax vaccine adsorbed (AVA). All of the DNA-vaccinated rabbits developed antibody responses predictive of protective immunity. Overall levels of antibodies were the same for the groups vaccinated with DNA or with the AVA vaccine. Upon challenge, rabbits that received the irrelevant DNA vaccine died within 4 days, and three out of ten rabbits given AVA died. Only one of the PA DNA-vaccinated rabbits died, with a delayed time to death (8 days) as compared with the controls. Gene-gun vaccination appears, therefore, to be an effective delivery method for anthrax vaccines, and offers the advantage of eliciting protection with very low doses of DNA. However, gene-gun technology is quite labor-intensive and still faces developmental challenges for commercial use.

Electroporation delivery devices have also been tested with DNA vaccines for several biothreats, including anthrax. Large-animal studies have been or are being conducted with anthrax DNA vaccines; however, only one report of animal experiments using this technology has appeared to date [20]. In that study, delivery of a codon-optimized PA83 DNA construct with a tPA signal to mice, rats and rabbits was tested by intramuscular injection or intramuscular injection followed by electroporation. Mice and rats received 0.4–10 µg of plasmid and rabbits received 300 µg. In all three species, a single injection in conjunction with electroporation resulted in the rapid induction of high levels of IgG and neutralizing antibodies, whereas neutralizing antibodies were not detected in controls [20]. Although the animals were not challenged, the antibody levels detected in rabbits were predictive of protective immunity. This technology appears

to have much potential, but it too faces developmental challenges, particularly with respect to tolerability and acceptance for human populations.

In addition to the development of better delivery methods, several studies have focused on means for improving the DNA vaccine constructs themselves, for example by targeting PA to MHC class II compartments with an Immunoglobulin κ-chain leader sequence or with endoplasmic reticulum (ER) targeting and retention signals [21]. In mice, both targeted PA83 constructs performed well, while PA63 constructs did not. A follow-on study was performed in sheep with the PA83-targeted constructs and with a construct encoding only the host cell-binding portion (domain 4) of PA (PAD4), which was codon-optimized for mammalian expression and fused to the mouse invariant chain to improve MHC class II presentation [22]. The sheep received three intramuscular injections at weeks 0, 3 and 7 with the PA83 construct with the secretion signal in combination with either the ER-targeted PA83 or the PAD4 construct. Each sheep was given 1 mg of DNA per vaccination in phosphate-buffered saline (PBS) or complexed with a cationic lipid (Vaxfectin, Vical Inc., CA, USA). A control group was vaccinated with 25 µg of a protein vaccine (rPA83) adsorbed to an alhydrogel adjuvant. The antibody titers achieved with the protein vaccine were higher than those achieved with the DNA vaccines, but the DNA vaccines elicited longer lasting responses. The sheep that received the DNA with Vaxfectin had higher antibody titers than those that received the corresponding constructs in PBS, and the combination vaccine that included the PAD4 construct elicited higher titers than the combination with the ER-targeted gene product [22]. This study demonstrated that an anthrax DNA vaccine, when formulated with cationic lipid, could elicit reasonable antibody responses in large animals.

The PA83 construct with the immunoglobulin κ-chain leader sequence for secretion was also tested in mice after formulation with lysine-based molecules to enhance resistance to degradation followed by encapsulation in poly(lactide-co-glyolide-acid) (PLGA) nanoparticles [23]. PLGA particles with DNA not formulated with the lysine molecules were also tested. The antigenicity of the the nanoparticles, which were expected to result in time-release of the DNA vaccines, were evaluated by vaccinating mice intramuscularly three times at 3-week intervals. Antibody responses were detected by ELISA, but not by toxin-neutralizing assay, and were higher in mice given the particles with the lysine-complexed DNAs. Challenges were not conducted, so the potential of this methodology remains to be determined.

Targeting of PA63 to specific intracellular compartments associated with MHC class II or class I antigen presentation has also been tested as a means to improve the immunogenicity of DNA vaccines [24]. To enhance MHC class II presentation, chimeric PA63 constructs were generated to include tPA (for secretion), or lysosome-associated membrane protein (LAMP)1. LAMP1 and MHC class II molecules are found to colocalize in many antigen-presenting cell compartments. To enhance MHC class I presentation, a PA63-ubiquitin chimeric gene was constructed to direct proteasomal degradation. Mice were vaccinated by intramuscular injection of 100 µg of DNA in PBS and challenged by intravenous injection of lethal toxin. The highest antibody titers were obtained in mice vaccinated with the construct containing both tPA (N-terminal) and LAMP1 (C-terminal) targeting signals. Antibody isotype analysis demonstrated that mice given the tPA and/or LAMP1 constructs also displayed higher IgG_1:IgG_{2a} ratios than were obtained in mice vaccinated with PA63 without targeting signals, indicating a Th2 response. Moreover, the mice that were vaccinated with the tPA-PA63-LAMP1 construct developed higher levels of toxin-neutralizing antibodies than mice vaccinated with recombinant PA63 or PA83 (rPA) protein, and 90% survived challenge. By contrast, the ubiquitin construct did not elicit protective immunity when given with or without tPA and LAMP1 signals [24]. These results are consistent with the recognized importance of antibody responses to PA as a primary means of protecting against anthrax.

Combination DNA vaccines

In addition to DNA vaccines based on PA gene expression, a number of groups have investigated DNA vaccines expressing the *B. anthracis LF* gene, either alone or in combination with a PA DNA vaccine. In the first such study, mice vaccinated by gene gun with a combination of PA63 and LF DNA developed higher antibody titers and to both antigens than mice given either vaccine alone, although all of the vaccinated mice survived intravenous challenge with lethal toxin [25].

Another study to test PA and LF DNA constructs was conducted in a large number of rabbits [26]. The vaccines tested included PA83, which was codon-optimized and engineered to have a noncleavable furin site, and two truncated LF gene constructs. The vaccines were formulated with one of two cationic liposome adjuvants: Vaxfectin ([(±)-*N*-(3-aminopropyl)-*N*,*N*-dimethyl-2,3-bis(*cis*-9 tetradecenyloxy)-1-propanaminium bromide)] plus 1,2-diphytanoyl-*sn*-glycero-3-phosphoethanolamine) or DMRIE-DOPE ([(±)-*N*-(2-hydroxyethyl)-*N*,*N*-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide plus 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine), and 1 mg of DNA was given by intramuscular injection two or three times at 4-week intervals. All groups of the PA-, LF-, or PA plus LF DNA-vaccinated rabbits developed toxin-neutralizing antibodies, with the best titers observed in the rabbits that received three vaccinations with the PA83 DNA vaccine alone or in combination with LF DNA vaccines. Importantly, all rabbits (40/40) vaccinated with the PA DNA vaccine by itself or with the LF vaccine were protected from aerosol challenge with virulent *B. anthracis* spores and showed no rise in antibody titers after challenge. Five out of nine rabbits that received the LF DNA vaccine by itself were also protected, whereas none of the vector control or naive rabbits survived the challenge. An additional group of rabbits (n = 10) that had received the PA83 vaccine were not challenged until 7 months after vaccination. All of these rabbits also survived aerosol spore challenge, although they did have antibody rises after challenge, indicating that spore germination had occurred [26].

This study in the highly relevant rabbit model suggested that this vaccine formulation would be effective in humans. Consequently, the lipid-formulated PA83 vaccine was further

assessed in a preclinical study in rabbits for dose–escalation safety, in nonhuman primates (NHPs) for immunogenicity and efficacy against challenge with a lethal dose of *B. anthracis* spores, and in a Phase I, dose–escalating clinical trial in healthy adults [27,28]. NHPs that received a 0.6-mg dose three times at 2-week intervals developed low levels of antibodies detected by ELISA. Although toxin-neutralizing antibodies were not detected, three out of four NHPs survived challenge with *B. anthracis* spores.

In the clinical trial, volunteers were scheduled to receive three doses of 0.2, 0.6 or 2 mg of total PA83 DNA or saline placebo at 1-month intervals. After the first vaccination, the 0.2- and 0.6-mg dose levels were generally well tolerated; however, reactogenicity, including injection site pain and systemic reactions, was observed, so the remaining two injections in that group were reduced to 0.6 mg. Only one individual in each of the two lower dose groups developed anti-PA antibody responses. Overall, only 10% of the low-dose group developed antibodies to either PA or LF, while 33 and 80% of the two higher groups developed antibodies to at least one of the vaccine components, with most responses in the highest-dose group. Only one subject developed toxin-neutralizing antibodies [28]. Although the antibody responses in the NHPs and humans from these studies are disappointing, it is encouraging that the NHPs were protected from challenge. The results also point toward the need for a better understanding of what constitutes correlates of protective immunity for humans.

Except for PA and LF DNA vaccines, very little work has been reported with combination vaccines using other *B. anthracis* genes. A small study in mice examined the potential for combining a DNA vaccine expressing a *B. anthracis* exosporium gene, *bclA*, with a PA83 DNA vaccine [29]. Outbred mice were vaccinated three times at 2-week intervals by gene gun with the *bclA* DNA vaccine or with that vaccine in combination with the ER-targeted or secreted PA83 vaccines described earlier [21]. The *bclA* plasmid induced strong anti-BclA responses, while vaccination with a mixture of the two PA83 constructs induced strong PA-specific responses. Vaccination with the combination of the BclA and PA vaccines induced antibodies against both antigens, which were comparable to the responses of mice vaccinated against only one of the proteins. Upon challenge with Ames spores, five out of ten mice given the combination vaccine survived, whereas two out of ten given the BclA vaccine and three out of ten given PA vaccines survived. This small study is not sufficient to determine whether adding this exosporium component could improve DNA vaccine efficacy for anthrax.

Prime–boost approaches

A number of groups have evaluated PA or combination PA and LF DNA vaccines in mice by DNA priming followed by boosting with rPA (Table 1). A single mouse study using a recombinant adenovirus (Ad) boost was also reported [30]. Although the prime–boost approach may improve antibody responses compared with DNA vaccination alone, it should be considered as a last resort and only used when a DNA vaccine cannot be developed that offers protective immunity on its own, as the need to generate both DNA and protein vaccines obviates most of the advantages of the DNA platform.

Anthrax summary

There is a recognized need for a better vaccine than AVA, the only licensed anthrax vaccine for human use. This vaccine suffers from lot-to-lot variability, a need for extensive dosing, and reactogenicity. The leading candidate to replace AVA is rPA, which is in clinical testing. While AVA (now called Biothrax®) is being stockpiled by the US Government, it is expected that a licensed rPA vaccine will eventually replace it. Any anthrax vaccine that will be commercially successful will need to offer some advantage over both the AVA and rPA vaccines, such as quicker dosing regimens, improved cost–effectiveness and better tolerability. Although DNA vaccines show promise for the prevention of anthrax in animal models, to date they have not proven to be highly immunogenic in humans.

DNA vaccines for Ebola & Marburg hemorrhagic fever viruses

Characteristics of filoviruses

Ebola and Marburg viruses belong to the family *Filoviridae*, and cause severe hemorrhagic fevers in humans and NHPs. Recent evidence suggests that the natural hosts for the viruses are likely to be fruit bats [31,32], but the viruses are also transmissible among primates by close contact with infected individuals, or contact with infectious body fluids or even contaminated objects. Both filoviruses are recognized biological warfare threats, and MARV has been reported to have been weaponized [33]. The viruses have been categorized as priority class A pathogens by both the NIH and CDC owing to their virulence, ease of dissemination, lack of effective countermeasures to prevent or treat them, and their potential to cause public panic and social disruption.

Taxonomic classification of EBOV currently includes four viral species: Zaire (ZEBOV), Sudan (SEBOV), Cote d'Ivoire (COIEBOV) and Reston (REBOV) Ebola viruses. A new EBOV was identified in blood samples collected from patients with Ebola hemorrhagic fever in Uganda in 2007. This virus, which is distantly related to COIEBOV, has been tentatively named Bundibugyo EBOV [34]. MARV is considered a single species; however numerous, highly diverse MARVs have been isolated, thus it is likely that additional species will soon be taxonomically defined [35]. Reston EBOV has not been found to be pathogenic for humans, but all others are, with both EBOV and MARV having caused outbreaks with mortalities as high as 90% (reviewed in [36]).

Filoviruses have nonsegmented, negative-strand RNA genomes of approximately 19 kb in size, which encode seven viral structural proteins. The gene order and function of the resultant proteins are:

- The major nucleoprotein (NP)
- VP35, a component of the polymerase complex and involved in subverting innate immunity
- VP40, the matrix protein
- The major surface glycoprotein (GP)
- VP30, a minor nucleoprotein involved in EBOV transcription initiation

- VP24, a hydrophobic membrane-associated protein involved in transcription
- L, the major component of the RNA-dependent RNA polymerase complex

For EBOV, the primary gene product of the virus complementary sense messenger RNA is a soluble form of GP (sGP), which is not a structural protein. The structural protein form of GP is generated through transcriptional editing, which causes a shift in the gene's reading frame. The result is that sGP and GP share 295 N-terminal amino acids but then diverge, with sGP and GP terminating after an additional 69 or 381 amino acids, respectively. sGP has been suggested to serve as a decoy for antibody responses or as a means to reduce the virulence of EBOV [37]. Mature GP is a highly glycosylated type 1 membrane protein. It is generated by post-translational proteolytic cleavage of a precursor by a cellular furin-like enzyme. This cleavage results in a large N-terminal fragment (GP1) and a smaller C-terminal fragment (GP2) that reassociate by disulfide bonding. Trimers of GP1 and GP2 form the virion spikes, thus GP is the main target of antibody responses [38].

Table 1. DNA vaccines for anthrax (*Bacillus anthracis*) tested in animals or humans.

Genes	Delivery method(s)	Model	Ref.
PA63-tPA *PA83-tPA*	Im. injection	Mouse	[17,72]
PA83-tPA	Cationic lipid	Rat	[18]
PA83-tPA	Gene gun	Rabbit	[19]
PA83-tPA	Electroporation	Mouse, rat, rabbit	[20]
PA83-IgGκ *PA83-ER* *PAD4*	Gene gun, cationic liposome, PLGA	Mouse, sheep	[21–23]
PA83TPA, UB, LAMP1	Im. injection	Mouse	[24]
PA63+LF	Gene gun	Mouse	[25]
PA83+LF	Im., cationic lipid	Rabbit, nonhuman primate, human	[26,28]
PA83-IgGκ *+bclA*	Gene gun	Mouse	[29]
PA83	Im. injection/rPA boost	Mouse, rabbit	[72,73]
PA63, LF, PA63+LF	Gene gun, biojector im./ rPA boost	Rabbit	[74]
PA63	Topical, perflubron-microemulsion/rPA boost	Mouse	[75]
PAD4-IL-2	Im. injection/rPA boost	Mouse	[30]

Im.: Intramuscular; LAMP: Lysosome-associated membrane protein; PLGA: Poly(lactide-co-glyolide-acid); rPA: Recombinant protective antigen; tPA: Tissue plasminogen activator.

Rodent studies

A number of different vaccine approaches for EBOV and MARV have been tested in rodents, including DNA vaccines given alone or in prime–boost regimens. Although filoviruses are not naturally lethal to rodents, both EBOV and MARV can be adapted to kill guinea pigs after a few sequential passes. Lethal mouse models for both viruses have also been developed, although much more extensive viral passaging was needed [39,40].

In one study using the ZEBOV mouse model, gene-gun delivery of approximately 5 µg of plasmid DNA expressing the *GP* or *NP* genes of ZEBOV were shown to protect mice from challenge with EBOV with as few as two doses delivered at a 4-week interval [19]. Both vaccines were also found to elicit antibody and cytotoxic T lymphocyte (CTL) responses. In addition, the GP vaccine provided partial protection from ZEBOV challenge for at least 9 months after vaccination [41]. The GP DNA vaccine was also tested in guinea pigs, alone and in combination with DNA vaccines for MARV, VEEV and anthrax. In that study, guinea pigs received approximately 2.5 µg of each DNA three times at 4-week intervals and were challenged 3 weeks later. Partial protection to ZEBOV was observed when the vaccine was given alone or in combination with the other three vaccines [19].

In another study, three intramuscular injections of 50 µg of plasmid DNA expressing the ZEBOV *NP* or *GP* genes elicited non-neutralizing antibody responses in mice, although in this study CTL responses were only found to GP [42]. The mice were not challenged but, in the same study, guinea pigs vaccinated four times at 2-week intervals by intramuscular injection of 100 µg of the GP or NP DNA vaccines were protected from challenge for 10 days, at which time they were euthanized. A subsequent study in guinea pigs showed that intramuscular injection of the ZEBOV GP DNA (75 µg) in combination with ZEBOV NP DNA (25 µg) also resulted in protective immunity to ZEBOV challenge and did not change the antibody titers measured by ELISA against inactivated ZEBOV as compared with those from guinea pigs vaccinated with only GP DNA [43]. Similarly, guinea pigs vaccinated by intramuscular injection of 25 µg each of ZEBOV GP, ZEBOV NP, SEBOV GP and COIEBOV GP had similar ELISA titers to those vaccinated with the ZEBOV GP and were protected from challenge with ZEBOV [43].

DNA vaccines for MARV have also been evaluated in guinea pigs. Gene-gun delivery of three or four doses of approximately 2.5 µg of GP DNA vaccines derived from two distant Kenyan isolates of MARV, strains Musoke and Ravn, elicited antibody responses and protected all guinea pigs from challenge with homologous virus [19]. The Musoke MARV DNA vaccine was also partially protective to MARV challenge when it was given by gene gun in combination with DNA vaccines for EBOV, VEEV and anthrax [19].

NHPs & Phase I clinical trials

In two separate experiments with a gene gun-delivered GP DNA vaccine for MARV (strain Musoke), two out of three cynomolgus macaques survived challenge with homologous MARV [19]. All

of the monkeys developed fevers at some point after challenge, but whilst the control monkeys and the nonprotected monkeys in each experiment developed viremias and had elevated levels of liver enzymes, the protected monkeys remained aviremic and displayed normal liver enzyme profiles. These results indicate that DNA alone can offer protective immunity to NHPs from MARV challenge and suggest that modest improvements in the immunogenicity of the vaccines might offer complete protection.

To date, DNA vaccines alone have not been reported to protect NHPs from challenge with EBOV; however, a Phase I clinical study was conducted to evaluate the safety of a combination EBOV DNA vaccine, which was intended to facilitate future studies using them in prime–boost regimens [44]. The vaccine consisted of a mixture of ZEBOV GP, ZEBOV NP and SEBOV GP plasmids. Three groups of volunteers were vaccinated three times at approximately 4-week intervals by intramuscular injection of 2 mg (n = 5), 4 mg (n = 8) or 8 mg (n = 8) of the combination EBOV vaccine. Each group also included two volunteers who received the same volume of a PBS placebo control. Antibodies to at least one of the three antigens were measured by ELISA in samples from all vaccinees. $CD4^+$ or $CD8^+$ T-cell GP-specific responses were detected by intracellular cytokine staining in 20 out of 20 or six out of 20 vaccinees, respectively. Neutralizing antibodies could not be detected. This study demonstrates that EBOV DNA vaccines are safe and immunogenic in humans, thus opening the door for further development and improvement of these vaccines.

Prime–boost approaches

DNA vaccines for EBOV and MARV have thus far not proven as robust in protecting animals from challenge as either the recombinant Ad or vesicular stomatitis virus (VSV) systems [45–49]. Since the VSV system uses a live virus backbone, safety issues require additional study. The Ad vectors are replication defective, making safety less of an issue; however, Ad vectors face two other challenges, in that very high levels of particles are required to elicit protective responses and most humans have pre-existing immunity to the common Ad vectors used. Consequently, efforts are still underway to develop methods to improve the immunogenicity of DNA vaccines for filoviruses; for example, through electroporation delivery. In the interim, DNA vaccines are being tested in several prime–boost approaches, either as a way to prime a desired immune response, or as a way to overcome pre-existing vector immunity.

For EBOV, NHPs have been protected from challenge using DNA vaccine priming followed by boosting with recombinant Ad expressing ZEBOV GP [43]. In a subsequent study, these same investigators showed that a single vaccination with the Ad vector expressing ZEBOV GP was sufficient to protect macaques against EBOV challenge; thus, it is not clear how important the DNA vaccination was in the protection observed earlier [45]. A study in mice suggested that DNA priming might be important for overcoming pre-existing immunity to Ad or vaccinia virus (VACV) vectors in that animals receiving the DNA priming dose had developed much higher CTL and antibody responses than mice vaccinated with only the recombinant Ad or VACVs [50].

For MARV, a DNA prime followed by a baculovirus-derived protein boost proved more effective in protecting guinea pigs than a DNA vaccination by gene gun by itself, but this same strategy was not successful against EBOV in guinea pigs [51].

Filovirus summary

Ebola viruses and MARVs cause rapid and catastrophic infections. There are no vaccines currently available for their prevention, but there are several approaches that have shown promise in the laboratory. All approaches, including DNA vaccines, suffer from certain disadvantages. Although numerous studies have been performed to date (Table 2), DNA alone has not been shown to offer complete protective immunity to challenge with EBOV or MARV. Therefore, better delivery methods or improvements in the constructs themselves will be required to make the DNA platform competitive with other more effective vaccine technologies.

DNA vaccines for poxviruses

Status of smallpox vaccines

Variola virus (VARV) is a large complex DNA virus of the family *Poxviridae* that is the causative agent of smallpox. VARV infection in humans results in significant morbidity and mortality, and person-to-person spread is common. Worldwide vaccination against VARV using live VACV delivered by scarification with a bifurcated needle resulted in the eradication of smallpox disease in 1979. The cessation of regular vaccination after this eradication has left the world population increasingly susceptible to an accidental or deliberate release of VARV. Owing to the potential for major public health impact, VARV is identified as a category A select agent by the CDC. The potential threat of VARV or a genetically modified poxvirus has resulted in renewed interest in poxvirus vaccination. In addition, monkeypox virus (MPXV) continues to cause human epidemics in Africa, and a recent outbreak of human MPXV in the midwestern USA resulted from the accidental importation of MPXV-infected animals. Despite being highly protective, the historic live VACV vaccine Dryvax® (Wyeth Laboratories, NJ, USA) and the more recent plaque-purified and cell culture-derived live VACV vaccine ACAM2000™ (Acambis, Cambridge, UK) are associated with rare but life-threatening adverse events, including myocarditis, eczema vaccinatum and progressive vaccinia. As a result, these vaccines are contraindicated for large segments of the population, including those with pre-existing immunodeficiencies and dermatological conditions such as eczema. Although attenuated versions of the live VACV vaccine such as modified vaccinia virus Ankara (MVA) have been developed for improved safety, these attenuated viruses still contain genes that can code for immunomodulatory proteins or proteins with unknown function. The potential adverse events associated with the live and attenuated VACV vaccines have led to the development of subunit poxvirus DNA and/or protein vaccines.

Subunit DNA vaccine targets

The first reported evaluation of the immunogenicity and protective efficacy of poxvirus DNA vaccines involved plasmids expressing proteins present on the extracellular enveloped virion (EEV), one

of two major forms of infectious virus. In this study, mice were vaccinated four times at 2-week intervals, with each mouse receiving an intramuscular injection of 100 µg of a DNA construct expressing individual VACV *A33R*, *A34R*, *A36R* or *B5R* genes known to encode proteins present in the EEV outer envelope [52]. The mice were then challenged with intranasal administration of VACV and the observed levels of protection were 100% (*A33R*), approximately 20% (*A34R*), 50% (*A36R*) and approximately 80% (*B5R*). These results demonstrated that a poxvirus DNA vaccine could be protective in mice and also further identified the A33R and B5R EEV proteins as good antigenic targets for poxvirus vaccination.

Evaluation of individual and combined DNA vaccines expressing proteins present on the EEV and on the other major infectious form of poxviruses, the intracellular mature virion (IMV), has also been performed. In the first such study, mice were vaccinated three times at 3-week intervals by gene gun, with each mouse receiving 1 µg of a DNA plasmid expressing VACV *A33R* or the IMV membrane-associated protein *L1R*, or with 1 µg of each of the two plasmids delivered in combination [53]. Although both individual vaccines protected most mice from intraperitoneal VACV challenge, only the *L1R* vaccine elicited neutralizing antibodies. Mice receiving both vaccines were completely protected from challenge. In a second study, the plasmids encoding the EEV antigen B5R or the IMV antigen A27L were tested alone and in combination [54]. DNA vaccination with *A27L* alone resulted in only 10% protection against intraperitoneal VACV challenge in mice, despite the production of VACV-neutralizing antibodies detectable by plaque reduction neutralization test (PRNT). Although mice vaccinated with the *B5R* plasmid developed high levels of anti-B5R antibodies as measured by ELISA, only 40% of these mice survived the challenge. Complete protection was observed in mice receiving both the *B5R* and *A27L* plasmids. The results of these studies demonstrated that combinations of DNA vaccines targeting both the EEV and IMV infectious forms of VACV provided superior protection in mice compared with the individual constructs alone.

To further assess a combination approach, redundant targeting of both IMV and EEV membrane proteins was examined using a combination of all four individual plasmids expressing L1R, A33R, A27L and B5R (4pox) [54]. Mice vaccinated with 1 µg of each of these four constructs by gene gun on different gold developed strong VACV IMV-neutralizing antibody responses, and anti-A33R and anti-B5R antibody titers were detected. These mice were completely protected from intraperitoneal VACV challenge, and experienced less weight loss than scarified mice or mice vaccinated with combinations of two genes. This same combination was also tested in mice using a novel skin electroporation device to deliver the DNA vaccines [55]. Mice were vaccinated with the four DNA vaccines administered on separate microneedle arrays, each coated with 30 µg of plasmid, at weeks 0, 3 and 8. Analysis of sera obtained after the final vaccination revealed that they had similar levels of anti-VACV antibodies and of VACV IMV-neutralizing antibodies as seen in positive control mice scarified with live VACV. In addition, results from immunofluorescence assays (IFAs) revealed that these DNA-vaccinated mice developed antibody responses against all four VACV antigens and the mice were completely protected against intranasal VACV challenge.

Table 2. DNA vaccines for filoviruses tested in animals or humans.

Genes	Delivery method(s)	Model	Ref.
Ebola virus			
GP, NP	Gene gun	Mouse, guinea pig	[41]
GP, NP	Im. injection	Mouse, guinea pig	[42,43]
GP, NP	Im. injection (Biojector 2000)	Human	[44]
GP, NP	DNA prime, adenovirus boost	Mouse, NHP	[43,50]
GP	Gene gun DNA prime, Im. protein boost	Guinea pig	[76]
Marburg virus			
GP, GPa	Gene gun	Guinea pig, NHP	[19,51,76]
GP	Gene gun DNA prime, Im. protein boost	Guinea pig	[51]

GP: Glycoprotein; Im.: Intramuscular; NHP: Nonhuman primate; NP: Nucleoprotein.

Other VACV genes encoding EEV, IMV or intracellular enveloped virus (IEV) membrane proteins have been studied as potential DNA vaccine antigens for VACV, but to date no others have been useful in eliciting protective immunity in mice [56].

Modification of gene products

In addition to improving vaccine efficacy by targeting multiple genes, it has also been possible to improve the antigenicity of VACV gene products by adding signal sequences. In one study, DNA vaccines expressing either the wild-type VACV IMV protein gene *D8L* (*wtD8L*) or a modified *D8L* gene with the transmembrane and C-terminal regions removed and with a tPA leader sequence added to the N-terminus (*tPA-D8L*) were evaluated in mice [57]. The *tPA-D8L* had a higher level of expression in transiently transfected cells than the *wtD8L* and an increased amount of D8 antigen was secreted into the supernatant with the tPA-D8L construct. To examine the immunogenicity of the D8L DNA vaccines, mice were vaccinated four times at 2-week intervals with 12 µg of DNA per vaccination delivered by gene gun. Assays performed on sera obtained 2 weeks after the fourth vaccination revealed that the *tPA-D8L* DNA vaccine produced higher levels of anti-D8L antibodies and anti-VACV IgG antibodies and higher levels of VACV IMV-neutralizing antibodies than the *wtD8L* construct. In addition, vaccination with the tPA-D8L DNA vaccine resulted in similar levels of VACV-neutralizing antibodies as mice vaccinated with live VACV. Although complete protection was observed in mice vaccinated with both of the *D8L* DNA vaccines after

intraperitoneal VACV challenge, the *tPA-D8L* provided slightly better protection against transient weight loss postchallenge. Adding the *tPA-D8L* DNA vaccine to combinations of A27L and B5R constructs or to A27L, B5R, A33R and L1R, also led to improved protection in the mouse intraperitoneal challenge model. Furthermore, adding *tPA-D8L* DNA to the A27L, B5R, L1R and A33R combination of plasmids resulted in an increase in protection from 26 to 66% of mice challenged intranasally with VACV.

In another study, a DNA construct in which the VACV *L1R* coding sequence was inserted behind the tPA leader sequence was used to vaccinate mice twice at a 3-week interval with 1 µg of the plasmid delivered by gene gun [58]. Analysis of sera obtained after vaccination revealed that including the tPA leader sequence resulted in significantly improved anti-L1R and VACV IMV-neutralizing antibody titers. In addition, two vaccinations performed at a 3-week interval with the *tPA-L1R* DNA plasmid in combination with the other 4pox DNA vaccine constructs (*A33R*, *B5R* and *A27L*) resulted in significantly increased VACV neutralizing antibody responses as compared with the 4pox DNA vaccine with the unmodified *L1R* DNA included. Although complete protection was observed in mice vaccinated with both the previous 4pox DNA vaccine and the 4pox DNA vaccine containing *tPA-L1R* after intranasal VACV challenge, mice receiving the *tPA-L1R*-containing 4pox DNA vaccine exhibited significantly less transient weight loss after day 3 postchallenge than mice vaccinated with the previous 4pox combination. In a subsequent report, it was also demonstrated that including an N-terminal murine immunoglobulin κ-chain leader sequence in DNA vaccines expressing mammalian codon-optimized VACV *L1R* genes further modified to remove three potential glycosylation sites improved the anti-L1R and VACV neutralizing antibody responses in mice [59]. In this study, mice vaccinated four times at 2-week intervals with 3 µg of the optimized *L1R* DNA vaccines with and without the leader sequence, and with and without truncation of the C-terminal transmembrane region delivered by gene gun were protected from intranasal VACV challenge; however, no significant difference in the transient postchallenge weight loss was observed between these groups.

Recent structural studies provide some insight into the improved immunogenicity of *L1R* DNA vaccines containing N-terminal signal sequences. The potently neutralizing monoclonal antibody (mAb)-7D11 was found to bind a discontinuous epitope consisting of two loops of the L1R protein bound by a disulfide bond [60]. As the virus-encoded proteins required for disulfide bond formation are not present in cells transfected with the subunit poxvirus DNA vaccines, trafficking of the L1R protein through the ER using a leader signal sequence is required to produce the disulfide bond formation required for proper folding. These results, taken together with similar previous results for the VACV D8L antigen [57], demonstrate that modification of DNA constructs expressing certain poxvirus antigens to include an N-terminal signal sequence can improve the immunogenicity and protective efficacy of subunit poxvirus DNA vaccines.

NHP studies

The 4pox combination DNA vaccines were also evaluated in NHPs [54]. Four doses consisting of 2 µg of each of the *L1R*, *A33R*, *A27L* and *B5R* DNA constructs were delivered to each of six monkeys by gene gun, while six positive control monkeys were vaccinated with live VACV [54]. Five of the DNA-vaccinated monkeys developed anti-VACV virion antibodies, and three developed VACV neutralizing antibodies. In addition, sera from DNA-vaccinated monkeys contained antibodies that cross-reacted with orthologous proteins from MPXV in radioimmunoprecipitation assays and neutralized MPXV in PRNT. Following 1–2 years after receiving the 4pox DNA vaccine or the *L1R* DNA vaccine alone by gene gun delivery, monkeys were boosted with a single DNA vaccination as performed during the initial vaccination [61]. The ability of the DNA boost to generate a memory antibody response was demonstrated by a marked rise in the anti-VACV antibody titers and in the VACV neutralizing antibody titers in serum samples collected postboost as compared with those collected before the DNA boost. After the DNA boost, the monkeys were challenged intravenously with MPXV and then monitored for disease. Although all of the monkeys vaccinated and boosted with the *L1R* DNA vaccine alone survived the challenge, these animals all developed severe monkeypox disease. By contrast, all animals vaccinated and boosted with the 4pox DNA vaccine not only survived the challenge, but the disease severity was mild in two out of three of the monkeys and moderate in one out of three monkeys. All monkeys vaccinated with live VACV survived challenge and were completely protected from disease, and the lethality of the challenge was confirmed by the lack of survival of animals vaccinated with empty vector DNA. Taken together, results on the immunogenicity and protective efficacy of the 4pox DNA vaccine consisting of plasmids expressing the VACV *L1R*, *A33R*, *A27L* and *B5R* genes in mice and NHP provided compelling evidence regarding the feasibility of developing combination subunit DNA vaccines for the successful prevention of poxvirus infection.

Prime–boost approaches

Prime–boost approaches have been explored as a mechanism for increasing the immunogenicity and protective efficacy of poxvirus subunit vaccines. In one report, mice were vaccinated intramuscularly three times at 2-week intervals with 100 µg of DNA vaccines encoding VACV *A4L*, *A27L* or *H5R* [62]. After 1 week following the third DNA vaccination, the mice received a boost intraperitoneally with 5×10^6 plaque-forming units of live VACV. Both cellular and humoral responses were then evaluated for samples obtained 1 week after the third and final DNA vaccination or 26 days after the VACV boost. Analysis of antigen-specific IFN-γ responses induced in splenocytes by enzyme-linked immunospot (ELISPOT) assay revealed that, although there was only a very low IFN-γ response after DNA vaccination alone, there was a considerable increase in IFN-γ production after the VACV boost and the highest response was seen in mice primed with the *A4L* DNA vaccine. Similar assays performed after $CD8^+$ T-cell depletion further indicated that these cells were primarily responsible

for the IFN-γ responses detected in this study. Proliferation of $CD4^+$ T cells measured with carboxyfluorescein succinimidyl ester staining and flow cytometry of splenocytes was also increased in mice primed with the individual A4L, A27L or H5R DNA vaccine compared with mice primed with vector alone. Although the serum IgG antibody responses against recombinant *A4L*, *A27L* and *H5R* VACV proteins were low in mice receiving only the DNA-priming vaccinations, boosting with VACV resulted in a significant increase in these responses. Results from this study further indicated that antigen-specific $CD8^+$ T-cell IFN-γ responses were generated in human HLA-A2.1-transgenic mice after vaccination with the individual *A4L*, *A27L* and *H5R* DNA vaccines and with a combination of three plasmids. In another study, NHPs were vaccinated with DNA plasmids encoding the MPXV orthologs of the VACV A27L, A33R, B5R and L1R proteins (A29L, A35R, B6R and M1R, respectively), with the equivalent recombinant proteins produced in *Escherichia coli*, or with the DNA vaccines as a prime followed by boosting with the proteins [63]. DNA vaccinations consisted of intramuscular delivery of 3 mg of each DNA and intradermal delivery of 1 mg of each DNA (4 mg total of each of the four DNA plasmids) at weeks 0, 4 and 10. Protein vaccinations consisted of intramuscular delivery of 100 µg of each protein at weeks 19 and 23. DNA vaccination alone elicited only low levels of antibodies that could bind to the four antigens in ELISA, while protein boosting of DNA-primed animals resulted in antibody levels superior to those seen in animals vaccinated with protein alone against all antigens except M1R. While no neutralizing antibodies were present in the sera of animals vaccinated with DNA alone, all of the animals in the protein-only and DNA prime/protein boost groups developed VACV- and MPXV-neutralizing antibody titers. DNA prime/protein boost vaccination also produced significantly higher IFN-γ^+/TNF-α^+ $CD4^+$ T-cell responses than those in the DNA or protein-only groups. Following MPXV challenge 4–5 weeks after the final vaccination, animals that received only DNA developed innumerable skin lesions and did not survive, while animals vaccinated only with proteins developed moderate-to-severe disease but survived. By contrast, DNA-primed animals administered the protein boost experienced only mild disease that resolved within days. The results from the studies described previously highlight the efficacy of subunit DNA prime/live VACV boost and subunit DNA prime/subunit protein vaccination strategies in developing protective immunity against poxviruses.

VARV & MPXV DNA vaccines

While most of the existing reports on the development of poxvirus DNA vaccines describe plasmids expressing VACV antigens, a molecular subunit poxvirus vaccine must also provide protection against VARV to be considered an acceptable alternative to the current conventional poxvirus vaccines. Owing to the high degree of homology between the VACV proteins expressed by the currently tested DNA constructs with their MPXV and VARV orthologs, it is believed that DNA vaccines based on the VACV genes will provide considerable cross-protection against the other poxviruses and possibly represent attractive candidates for next-generation pan-poxvirus vaccines. This idea is supported by the ability of the 4pox DNA vaccine expressing VACV antigens to provide protection against MPXV in NHPs [61]. However, the evaluation of DNA vaccines expressing VARV genes has also recently been reported [64]. In this study, the individual VARV genes *A30L*, *B7R* and *F8L*, the orthologs of the VACV genes *A27L*, *B5R* and *D8L*, respectively, were codon optimized to increase the frequency of codons used in mammalian expression and inserted into plasmid vectors behind a tPA leader sequence, which had previously been shown to improve the expression and immunogenicity of the VACV D8L antigen [57]. Mice vaccinated three times at 3-week intervals with 12 µg of the individual *A30L*, *B7R* and *F8L* VARV DNA vaccines delivered by gene gun developed high levels of antigen-specific IgG antibodies, and there was no significant difference in the titers against VARV and VACV antigens. In addition, vaccination with the *F8L* and *A30L* DNA vaccines produced VACV IMV-neutralizing antibodies at levels similar to those elicited by their VACV *D8L* and *A27L* counterparts and superior to those elicited by intradermal inoculation with live VACV. Mice receiving the individual VARV *A30L*, *B7R* and *F8L* DNA vaccines were completely protected from intraperitoneal VACV challenge. Mice vaccinated with a polyvalent combination of all three VARV DNA vaccines also were protected from intraperitoneal VACV challenge, and these mice exhibited less transient postchallenge weight loss and regained weight sooner than mice vaccinated with the monovalent vaccines. In addition, the polyvalent VARV DNA vaccine and a polyvalent vaccine consisting of the VACV orthologs achieved similar protection against intraperitoneal VACV challenge in mice. The immunogenicity and protective efficacy of DNA vaccines expressing VARV antigens illustrate the potential for developing a VARV antigen-based molecular subunit vaccine that may provide increased protection against VARV.

Despite the high homology between the orthologs of the antigens expressed in the presently evaluated poxvirus DNA vaccines, the heterogeneity between these proteins can also impact the cross-reactivity and cross-protection of the antibody responses generated by certain antigens. In a recent report, it was demonstrated that a VACV protective mAb directed against the VACV EEV protein A33R, mAb-1G10, did not bind its MPXV ortholog A35R [65]. However, binding of mAb-1G10 to MPXV A35R was restored by substitution of amino acids 117, 188 and 120 with those present in VACV A33R. In addition, the ability of a DNA vaccine expressing MPXV A35R to protect against heterologous VACV challenge was evaluated. Mice were vaccinated three times at 3-week intervals with 1 µg of the MPXV A35R DNA vaccine or a DNA vaccine expressing the VACV A33R antigen administered by gene gun. While 80% of the mice vaccinated with the VACV A33R DNA were protected against intranasal VACV challenge, only 60% of the mice that received the MPXV A35R DNA vaccine survived. The surviving mice that received the VACV A33R plasmid also exhibited significantly less postchallenge transient weight loss than those vaccinated with the MPXV ortholog DNA. The results of this study demonstrated that a small degree of heterogeneity can impact the cross-protection of DNA vaccines

expressing certain poxvirus antigens and highlighted the importance of careful selection and redundant targeting of the antigens selected for use in a pan-poxvirus subunit vaccine.

Poxvirus summary

The immunogenicity and protective efficacy of DNA vaccines expressing numerous poxvirus antigens, including those from VACV, MPXV and VARV, have been demonstrated in mouse and NHP poxvirus models involving multiple routes of infection (Table 3). It has also been shown that combinations of DNA vaccines that redundantly target antigens present on both the IMV and EEV infectious forms of poxviruses will probably be required to produce a successful pan-poxvirus molecular subunit vaccine. The results of existing studies have also illustrated the potential efficacy of vaccination strategies incorporating DNA prime followed by live VACV or recombinant protein boost. Although the gene gun has been the predominant form of delivery of the poxvirus DNA vaccines in the existing reports, alternative delivery by electroporation has also shown promise for improving the efficacy of subunit molecular poxvirus vaccines. Finally, modification of the DNA vaccines expressing certain poxvirus antigens to include codon optimization, removal of potential sites of glycosylation, addition of N-terminal signal sequences and removal of C-terminal transmembrane regions has also improved the immunogenicity and protective efficacy of DNA-based subunit poxvirus vaccines. Therefore, although human clinical trial data are currently lacking, subunit poxvirus DNA vaccines represent an attractive alternative to existing conventional live and live-attenuated vaccines. After successful completion of clinical trials, the molecular subunit poxvirus vaccines could then be considered as a possible replacement for the existing licensed vaccine, as an alternative vaccine for those for which the live vaccine is contraindicated, or as a prime followed by boosting with conventional vaccines to reduce the potential for adverse effects.

Table 3. DNA vaccines for poxviruses tested in animals.

Genes	Delivery method(s)	Model	Ref.
Vaccinia virus			
A33R, A34R, A36R, B5R	Im. injection	Mouse	[52]
A33R, L1R, A33R+L1R	Gene gun	Mouse	[53]
A27L, B5R, A27L+B5R	Gene gun	Mouse	[54]
A27L+A33R+B5R+L1R	Gene gun	Mouse, NHP	[54,61]
A13L, A27L, A33R, A34R, A36R, A56R, B5R, D8L, H3L, L1R	Im. injection	Mouse	[56]
D8L, tPA-D8L, A27L+B5R, A27L+B5R+tPA-D8L, A27L+A33R+B5R+L1R, A27L+A33R+B5R+L1R+tPA-D8L	Gene gun	Mouse	[57]
A4L, A27L, H5R	Im. DNA prime, live VACV boost	Mouse	[62]
A4L, A27L, H5R, A4L+A27L+H5R	Im. injection	HLA-A2.1 transgenic Mouse	[62]
A27L+A33R+B5R+L1R	Skin electroporation	Mouse	[55]
tPA-L1R, tPA-L1R+A27L+A33R+B5R	Gene gun	Mouse	[58]
IgGκ-L1R	Gene gun	Mouse	[59]
Monkeypox virus			
A29L, A35R, B6R, M1R	Im./id. DNA prime, protein boost	NHP	[63]
A35R	Gene gun	Mouse	[65]
Variola virus			
tPA-A30L, tPA-B7R, tPA-F8L, tPA-A30L+tPA-B7R+tPA-F8L	Gene gun	Mouse	[64]

id.: Intradermal; Im.: Intramuscular; NHP: Nonhuman primate; tPA: Tissue plasminogen activator.

DNA vaccines for alphaviruses

Status of vaccines for equine encephalitis viruses

Venezuelan, eastern and western equine encephalitis viruses (VEEV, EEEV and WEEV, respectively) are arthropod-borne positive-stranded RNA viruses of the family *Togaviridae* that cause periodic epizootics in the Americas. These New World alphaviruses are recognized as potential agents of biowarfare or bioterrorism, largely owing to their associated morbidity and mortality in humans, ease of production, considerable stability and high infectivity in aerosols. As a result, these encephalitic alphaviruses are defined as category B bioterrorism agents by the CDC. Although no licensed human vaccines currently exist for these pathogens, conventional vaccines are available under investigational new drug status. A live-attenuated VEEV vaccine, TC-83, is generally safe and elicits long-lasting protective immunity; however, it causes fever, headache and malaise in approximately 25% of vaccinated individuals, while approximately 20% of vaccine recipients fail to develop neutralizing antibodies. Formalin-inactivated virus vaccines for VEEV, EEEV and WEEV are safe and well tolerated, but they require frequent boosting to elicit detectable immune responses and provide only poor protection against aerosol challenge in some rodent models. The reactogenicity of the live vaccine and the poor immunogenicity of the inactivated vaccines have prompted the development of improved vaccines for VEEV, EEEV and WEEV.

Rodent studies

The immunogenicity of a DNA vaccine encoding the E3, E2 and 6K structural proteins of VEEV strain TC-83 has been

examined in mice following intramuscular, intradermal or gene gun delivery [66]. In this study, mice were vaccinated on days 0 and 72 with 50 µg of the DNA vaccine by intramuscular or intradermal injection, or with 0.6- or 4-µg doses delivered by gene gun. Analysis of serum samples obtained on day 21 indicated that ten out of ten animals from all groups had responded to the vaccination as determined by anti-VEEV TC-83 ELISA and similar titers were seen for all groups at this time point. However, ELISA on serum samples taken on day 93 showed that mice given 4 µg of DNA by gene gun had higher anti-VEEV antibody levels than those of the other groups and these mice also had the highest titers of persisting antibody in terminal serum samples collected on day 173. Furthermore, western blotting demonstrated that antibodies present in these same terminal serum samples reacted to the VEEV E2 glycoprotein, which contains the majority of known VEEV-neutralizing epitopes. The results of this study indicated that a VEEV DNA vaccine expressing the E2 glycoprotein is immunogenic in mice, and that gene gun delivery resulted in superior anti-VEEV antibody responses as compared with intramuscular or intradermal injection, despite the use of one-twelfth the dose of the DNA construct.

Studies to evaluate the protective efficacy of a VEEV DNA vaccine have also previously been performed in both mice and guinea pigs [19]. Here, mice were vaccinated three times at 3-week intervals with approximately 3 µg of a plasmid DNA encoding the 26S structural subgenomic mRNA (C-E3-E2–6K-E1) of VEEV subtype IA/B administered by gene gun. The DNA-vaccinated mice developed strong anti-VEEV antibody responses as measured by ELISA, but only low levels of VEEV-neutralizing antibodies were detected by PRNT. After 3 weeks following the final vaccination, the mice were challenged with VEEV by either the subcutaneous or aerosol route. There was 100% survival in the VEEV DNA-vaccinated mice after subcutaneous challenge, 80% survival after aerosol challenge, and the uniform lethality of these challenges was confirmed by a complete lack of survival of negative control mice vaccinated with empty plasmid DNA. In another study, guinea pigs were vaccinated three times at 4-week intervals with 5 µg of the VEEV DNA vaccine or with 5 µg each of the VEEV, EBOV, MARV and anthrax DNA vaccines [19]. The guinea pigs vaccinated with the VEEV DNA alone had measurable antibody titers to VEEV and 100% survival was observed after a uniformly lethal VEEV subcutaneous challenge performed 21 weeks after the initial vaccination. Furthermore, animals vaccinated with all four DNA vaccines had only slightly reduced VEEV antibody titers and slightly reduced protection (~80%) as compared with animals receiving the VEEV DNA alone. The results of these studies provided the first evidence that a VEEV DNA vaccine is capable of eliciting high levels of protection against VEEV challenge in multiple animal models and against multiple routes of infection. In addition, the results obtained with the combined VEEV, EBOV, MARV and anthrax DNA vaccines highlight the potential for developing successful multi-agent biodefense vaccines using this approach.

The efficacy of a DNA vaccine against WEEV has also been evaluated in mice [67]. Mice were vaccinated four times at 2-week intervals with 5 µg of a DNA vaccine expressing the 26S structural genes of WEEV strain 71V-1658 by gene gun. After 2 weeks following the final vaccination, the mice were challenged intranasally with WEEV and survival rates were 100% against WEEV strain 71V-1658, 62% against strain Fleming and 50% against strain CBA87. Although the authors indicated that no anti-WEEV antibodies were detected in the vaccinated mice, cellular responses were observed by CTL-proliferation assays. The results of this work suggest that a DNA vaccine approach also represents a promising strategy for WEEV vaccination and further illustrates the potential for achieving some cross-protection against multiple WEEV strains with this vaccine.

Recently, a VEEV DNA vaccine with improved immunogenicity and protective efficacy has been developed [68]. In an attempt to improve the immunogenicity and cross-reactivity of encephalitic alphavirus envelope glycoproteins, the DNA encoding the E1 and E2 glycoproteins of VEEV subtypes IA/B and IE, Mucambo virus (MUCV), EEEV (strain PE6) and WEEV (strain CBA87) were recombined *in vitro* to generate libraries of chimeric genes expressing variant envelope glycoproteins. The variants were then administered as DNA vaccines in mice, and the resulting sera were screened against the parent viruses by ELISA. Variants from a library in which the *E1* gene from VEEV IA/B was held constant and the *E2* genes of the five parent viruses were recombined were found to elicit improved anti-VEEV IgG antibody responses compared with the parental antigens. Mice were vaccinated three times at 3-week intervals with 10 µg of either of two representative variants from this library or with the parental VEEV DNA vaccine by gene gun. Analysis of sera obtained 3 weeks after the final vaccination revealed that the selected recombined variants induced significantly higher levels of VEEV-neutralizing antibodies. In addition, vaccination with one of the variant DNA vaccines resulted in 100% protection of the mice against aerosol VEEV challenge and this protection correlated with the potent virus-neutralizing ability. This represents the first report of a DNA vaccine that offers complete protection against aerosol VEEV challenge in mice.

Prime–boost approaches

In addition to DNA-only approaches, the evaluation of prime–boost strategies for vaccination against encephalitic alphaviruses has also been performed. In one such study, mice were vaccinated with three 1-µg doses of a DNA vaccine expressing the VEEV E3-E2–6K structural proteins at 2-week intervals by the gene gun followed by intranasal boosting with a replication-deficient human Ad type 5 expressing the same VEEV E3-E2–6K proteins 2 weeks later [69]. Additional mice received either only the three DNA vaccinations or only a single dose of the Ad-based VEEV vaccine. Boosting the DNA-vaccinated mice with the Ad-based vaccine resulted in a significant increase in VEEV-specific IgG antibodies compared with the groups receiving only DNA or the Ad-based vaccine alone. The mice boosted with the Ad-based vaccine also developed increased VEEV-neutralizing antibodies as compared with the other groups. Importantly, boosting with the Ad-based vaccine also significantly increased the protective efficacy against aerosol challenge with VEEV. Survival of mice

receiving the Ad-based boost was 83% compared with 25% survival with DNA only and 42% after the single dose of the Ad-based vaccine alone. In a separate report, a DNA prime, orally delivered protein boost vaccination strategy against viral encephalitis using the prototype alphavirus Sindbis virus (SINV) was detailed [70]. Two 50-µg doses of a DNA vaccine expressing the SINV E2 glycoprotein were administered intramuscularly to mice on days 0 and 14 followed by oral administration of doses of a recombinant SINV E2-maltose-binding protein fusion (MBP-E2) on days 21, 28 and 35. This vaccination strategy resulted in 50% protection against an intranasal challenge on day 49 with a virulent, murine-adapted strain of SINV, while no survival was observed in groups of mice that received only DNA or only protein vaccination. The results from these two studies demonstrate the utility of prime–boost vaccination strategies in the prevention of viral encephalitis.

Alphavirus summary

In summary, the studies described previously demonstrate the immunogenicity and protective efficacy of DNA vaccines developed to provide protection from encephalitic alphavirus infection (Table 4). The utility of prime–boost strategies for encephalitic alphavirus vaccination has also been illustrated. To date, the ability of DNA vaccines to provide protection against multiple routes of VEEV infection and in multiple small animal species has been established, including the ability of DNA vaccines to provide complete protection against aerosol challenge with VEEV and WEEV in mice. The gene gun has been the chosen method for delivery of encephalitic alphavirus DNA vaccines in the majority of published studies. However, as described for some of the other DNA vaccines in this review, intramuscular electroporation has recently been reported to represent a potent means of administering VEEV, EEEV and WEEV DNA vaccines [71]. Studies involving the evaluation of encephalitic alphavirus DNA vaccines in NHP models of infection are still lacking in the current literature; however, results presented at a recent conference indicate the continued development of candidate alphavirus DNA vaccines and that such publications should be forthcoming [71]. Demonstrated immunogenicity and protective efficacy in NHPs should then provide the basis for the advancement of encephalitic alphavirus DNA vaccines into clinical trials in humans.

Table 4. DNA vaccines for alphaviruses tested in animals.

Genes	Delivery method(s)	Model	Ref.
Venezuelan equine encephalitis virus			
E3-E2–6K	Gene gun	Mouse	[66]
C-E3-E2–6K-E1	Gene gun	Mouse, guinea pig	[19]
E3-E2–6K	Gene gun DNA prime, adenovirus boost	Mouse	[69]
Variant *E3-E2–6K-E1*	Gene gun	Mouse	[68]
Western equine encephalitis virus			
C-E3-E2–6K-E1	Gene gun	Mouse	[67]
Sindbis virus			
E2	Im. DNA prime, oral protein boost	Mouse	[70]

Im.: Intramuscular.

Conclusion

DNA vaccines for biodefense pathogens have shown promise in both preclinical and clinical studies. Safety issues, including concerns about DNA integration into host chromosomes, have thus far proven unfounded in the numerous toxicology studies that have been presented to the FDA in investigational new drug submissions. Manufacturing methods for DNA plasmids have been greatly improved and are now well established and regulated. Commercial DNA vaccines are already available for veterinary use in large animals; thus, there is great hope and anticipation that we will soon see a licensed DNA vaccine for human use.

Expert commentary

The ability to quickly generate vaccines against an existing, emerging or novel genetically engineered pathogen is critical for biodefense purposes. A vaccine platform that is amenable to mixed, combination vaccines is also highly desirable. In theory, DNA vaccines are ideal for meeting these goals, although in practice, they have not yet realized their full potential. A number of recent noteworthy advances suggest that DNA vaccines might soon become a viable alternative to other recombinant DNA or conventional approaches for biodefense vaccines. Progress in the delivery methods and in construct optimization are among the most important of these advances.

With respect to delivery methods, it is now clear that simple muscle injection of plasmids does not result in a sufficient immune response in humans, especially for eliciting neutralizing antibodies. Gene gun delivery has been used with success in NHPs; however, there are still no reported studies in which this method has been highly successful in humans. Although gene gun delivery is very attractive for biodefense vaccines, in that the DNA can be delivered by needle-free hand-held disposable devices, the technology involved in making the devices is still very labor-intensive and dosing regimens have generally required numerous administrations at several time points. Consequently, it is likely that improvements are still going to be required before gene gun delivery is the method of choice for biodefense DNA vaccines.

Another technology that shows promise for DNA vaccine delivery is electroporation. Currently, both skin- and muscle-delivery devices are being tested in animals and humans, although no large clinical studies have yet been reported. Assuming that humans reflect results seen in large animals and in certain Phase I trials, this

delivery method may prove to be the most efficacious of any yet developed. Nevertheless, intramuscular delivery of DNA using current electroporation devices may suffer from issues of human tolerability and acceptance, and there are no reports of efficacious intradermal delivery by electroporation to date.

Significant improvement in constructs has been demonstrated by modifying codons to reflect those most commonly seen in mammalian cells. This, along with the removal of cryptic splice sites and other regions that interfere with message stability, can dramatically improve the expression of certain genes. In addition to codon optimization, the addition of various targeting and secretion signals can improve the presentation of the gene products to immune systems. Additional work to define how such changes influence specific antigens produced from DNA vaccines will be required, as many of the results are antigen dependent.

Five-year view

Over the next 5 years, we expect to see at least one DNA vaccine licensed for human use. Research priorities over this time will continue to focus on increasing the immunogenicity of DNA vaccines by improving delivery methods, modifying the constructs or including genetic adjuvants, such as cytokine genes, to elicit a desired immune response. Possible new approaches for increasing the effectiveness DNA vaccines might be dendritic cell targeting, facilitating the nuclear transport of the DNA, or timed release of plasmids to extend gene expression. In addition, we expect that better manufacturing methods and novel purification procedures will be developed, which should make DNA vaccines even more cost effective.

Finally, although prime–boost regimens have been found to be effective for some pathogens, this technology is not ideal for biodefense purposes owing to the cost associated with producing two types of vaccines and the extended dosing requirements. Therefore, it is unlikely that this strategy would be favored for any individual vaccine unless there is no other alternative. However, this strategy could have great utility if it could be used for a combination approach in which a cocktail of DNA vaccines would provide priming for downstream vaccination with either established inactivated vaccines, or with specific DNA or other types of recombinant vaccines.

Disclaimer

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Key issues

- DNA vaccines offer several advantages over traditional biodefense vaccine approaches, such as a simple and cost-effective technology, ability for rapid design, safety in all populations and the environment, and the absence of pre-existing immunity to a vaccine component.
- Protective immunity has been demonstrated in animals vaccinated with DNA vaccines for several key biodefense pathogens, including *Bacillus anthracis*, filoviruses, poxviruses and equine encephalitis viruses. Combinations of DNA vaccines were found to have similar antigenicity and immunogenicity in animals as the individual vaccines in some studies, but every combination will need to be evaluated for interference and efficacy.
- Methods to augment immunogenicity of DNA vaccines that have proven successful include new delivery methods, such as electroporation, and modification of constructs by codon optimization and/or inclusion of cellular targeting signals.
- Although no DNA vaccine has yet been licensed for human use, several clinical trials have been or are being conducted.
- Further work to correlate animal responses to those of humans will be critical for licensing biodefense DNA vaccines, especially those for which proof-of-efficacy will solely be derived from animal studies.

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Affiliations

- Lesley C Dupuy, PhD
Headquarters, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD 21702, USA
Tel.: +1 301 619 4103
Fax: +1 301 619 2439
lesley.dupuy@amedd.army.mil
- Connie S Schmaljohn, PhD
Virology Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD 21702, USA
Tel.: +1 301 619 4103
Fax: +1 301 619 2439
connie.schmaljohn@amedd.army.mil