

Research

Open Access

Improved Efficacy of a Gene Optimised Adenovirus-based Vaccine for Venezuelan Equine Encephalitis Virus

Amanda J Williams, Lyn M O'Brien, Robert J Phillpotts and Stuart D Perkins*

Address: Biomedical Sciences Department, Defence Science and Technology Laboratory, Porton Down, Salisbury, Wiltshire, SP4 OJQ, UK

Email: Amanda J Williams - ajwilliams@dstl.gov.uk; Lyn M O'Brien - lmobrien@dstl.gov.uk; Robert J Phillpotts - bjphillpotts@dstl.gov.uk; Stuart D Perkins* - sdperkins@dstl.gov.uk

* Corresponding author

Published: 31 July 2009

Received: 30 March 2009

Virology Journal 2009, **6**:118 doi:10.1186/1743-422X-6-118

Accepted: 31 July 2009

This article is available from: <http://www.virologyj.com/content/6/1/118>

© 2009 Crown Copyright, Dstl

Abstract

Background: Optimisation of genes has been shown to be beneficial for expression of proteins in a range of applications. Optimisation has increased protein expression levels through improved codon usage of the genes and an increase in levels of messenger RNA. We have applied this to an adenovirus (ad)-based vaccine encoding structural proteins (E3-E2-6K) of Venezuelan equine encephalitis virus (VEEV).

Results: Following administration of this vaccine to Balb/c mice, an approximately ten-fold increase in antibody response was elicited and increased protective efficacy compared to an ad-based vaccine containing non-optimised genes was observed after challenge.

Conclusion: This study, in which the utility of optimising genes encoding the structural proteins of VEEV is demonstrated for the first time, informs us that including optimised genes in gene-based vaccines for VEEV is essential to obtain maximum immunogenicity and protective efficacy.

Background

Venezuelan equine encephalitis virus (VEEV) is a positive-stranded, enveloped, RNA virus of the genus *Alphavirus* in the family *Togaviridae*. VEEV causes a disease in humans characterized by fever, headache, and occasionally encephalitis. It is the cause of recent outbreaks in South America [1] and is considered to be a potential biological weapon [2-6].

There is a complex variety of different serogroups of VEEV. Only serogroup I varieties A/B and C have caused major outbreaks involving hundreds of thousands of equine and human cases [1]. Serogroups II through VI and serogroup I varieties D, E and F are enzootic strains, relatively avirulent in equines and not usually associated with major equine outbreaks, although they do cause human illness which can be fatal [7].

There is currently no vaccine licensed for human use to protect against infection with VEEV, although two vaccines have been used under Investigational New Drug status in humans. TC-83, a live-attenuated vaccine, and C-84, a formalin-inactivated version of TC-83, are not considered suitable for use because of poor immunogenicity and safety [8]. A further live-attenuated vaccine, V3526, derived by site-directed mutagenesis from a virulent clone of the IA/B Trinidad Donkey (TrD) strain of VEEV has recently been developed. V3526 has been shown to be effective in protecting rodent and nonhuman primates against virulent challenge [9-11] but demonstrated a high level of adverse events in phase I clinical trials [12].

We have previously developed adenovirus (ad)-based vaccines which encode the structural proteins of VEEV. The structural proteins of VEEV (core, E3, E2, 6K and E1) are

initially translated from a 26S subgenomic RNA as a single polyprotein. Following proteolytic cleavage, individual proteins are produced that are incorporated into the mature virion [13]. The most potent immunogen, E2, when co-expressed with E3 and 6K by the adenoviral vector, is able to confer protective efficacy in mice against lethal aerosol challenge [14]. For protection against VEEV, the antibody response is the principal correlate of protection [15]. An ad-based vaccine approach is additionally advantageous because of the ability to administer the vaccine by a mucosal route, eliciting immunity important for protection against aerosol challenge [16]. Our previously constructed recombinant adenovirus expressing E3-E2-6K genes from VEEV serotype IA/B (RA/VEEV#3) was able to confer 90–100% protection against 100LD₅₀ of strains IA/B, ID and IE of VEEV. However, it was less protective against higher challenge doses and requires three intranasal doses. Therefore, we have examined methods for improving the immunogenicity of this vaccine candidate.

Methods for optimising genes are sophisticated and becoming increasingly established for a variety of applications such as expression in prokaryotes, yeast, plants and mammalian cells [17]. Codon usage adaptation is one method of increasing the immunogenicity of epitope-based vaccines as it can enhance translational efficiency. Codon bias is observed in all species and the use of selective codons in genes often correlates with gene expression efficiency. Optimal codons are those that are recognised by abundant transfer RNAs (tRNAs) with tRNAs expressed in lower levels being avoided in highly expressed genes. A prominent example of successful codon adaptation for increased mammalian expression is green fluorescent protein from the jellyfish *Aequorea victoria* [18]. However, as well as influencing translation efficiency through more appropriate codon usage, the levels of messenger RNA (mRNA) available can also have a significant impact on the expression level. Increasing the RNA levels by methods such as optimisation of GC content, and removal of cis-acting RNA elements that negatively influence expression can also be achieved through the rational design of genes. Because alteration of these parameters is a multi-task problem and cannot be achieved as effectively through linear optimisation, we used multi-parameter optimization software (GeneOptimizer™, Genart GmbH, Regensburg) which allows different weighting of the constraints and evaluates the quality of codon combinations concurrently.

This is the first demonstration of the optimisation of structural genes of the VEEV. We have both codon adapted and gene optimised the E3-E2-6K genes for expression in mammalian cells from an ad-based vaccine. We show that this process can improve antibody levels by up to ten-fold following administration of the vaccine to mice and that

this confers increased protection from virus challenge. This study provides important information to inform the design of vaccines for VEEV, which may be applied to pre-clinical VEEV vaccines such as ad-based vaccine [14], DNA vaccines [19–21], and sindbis virus-based vaccine vectors [22].

Results

Optimisation of genes expressing E3-E2-6K of VEEV

The genes encoding the structural proteins, E3-E2-6K, were optimised using GeneOptimizer™ (Genart GmbH, Regensburg). This included codon usage adaptation, optimal for mammalian expression. One measure of codon quality is the Codon Adaptation Index (CAI), a measurement for the relative adaptiveness of the codon usage of a gene towards the codon usage of highly expressed genes. The CAI scores of the wildtype and optimised genes were 0.75 and 0.98 respectively. Additionally, the optimised gene had an increased GC content of 61% (compared to 52%) and 4 prokaryotic inhibitory motifs, 3 cryptic splice donor sites and 3 RNA instability motifs (ARE) were removed. The new gene sequence (VEEV#3-CO) is aligned with the wild-type sequence in figure 1.

RA/VEEV#3-CO virus expresses VEEV antigen

An adenovirus construct was prepared which expresses the optimised gene sequence (RA/VEEV#3-CO). Staining of fixed HEK 293 cells infected with RA/VEEV#3-CO with mouse polyclonal anti-VEEV antibody produced a strong fluorescence absent from uninfected cells (not shown) or cells infected with empty adenovirus (RA) (Figure 2). This confirms the expression of VEEV antigen. RA/VEEV#3-CO also gave strong fluorescence in infected HEK 293 cells stained with VEEV E2-specific monoclonal antibodies 1A3A-9, 1A4A-1 and 1A3B-7, confirming expression of the E2 structural protein (data not shown). None of the monoclonal antibodies reacted with RA infected cells. Antigen expression levels were quantitatively analysed by ELISA using three different detection antibodies, which indicated that the gene optimised adenovirus construct expressed increased levels of antigen compared to the non-gene optimised adenovirus construct (Figure 3).

Optimised ad-based vaccine elicits an increased anti-VEEV immune response compared to non-optimised

It was reasoned that the increased antigen expression of the codon-optimised vaccine would lead to an increased VEEV-specific immune response. Mice were immunised on days 0 and 7 with 10⁶ pfu and on day 21 with 10³ pfu of ad-based vaccines and sera were analysed after 2 doses (day 16) and after 3 doses (day 23). The suboptimal dosing regimen as compared to that used previously [14] was designed to allow effective demonstration of improved vaccine efficacy in our animal challenge model. At both timepoints, the ad-based vaccine with optimised genes

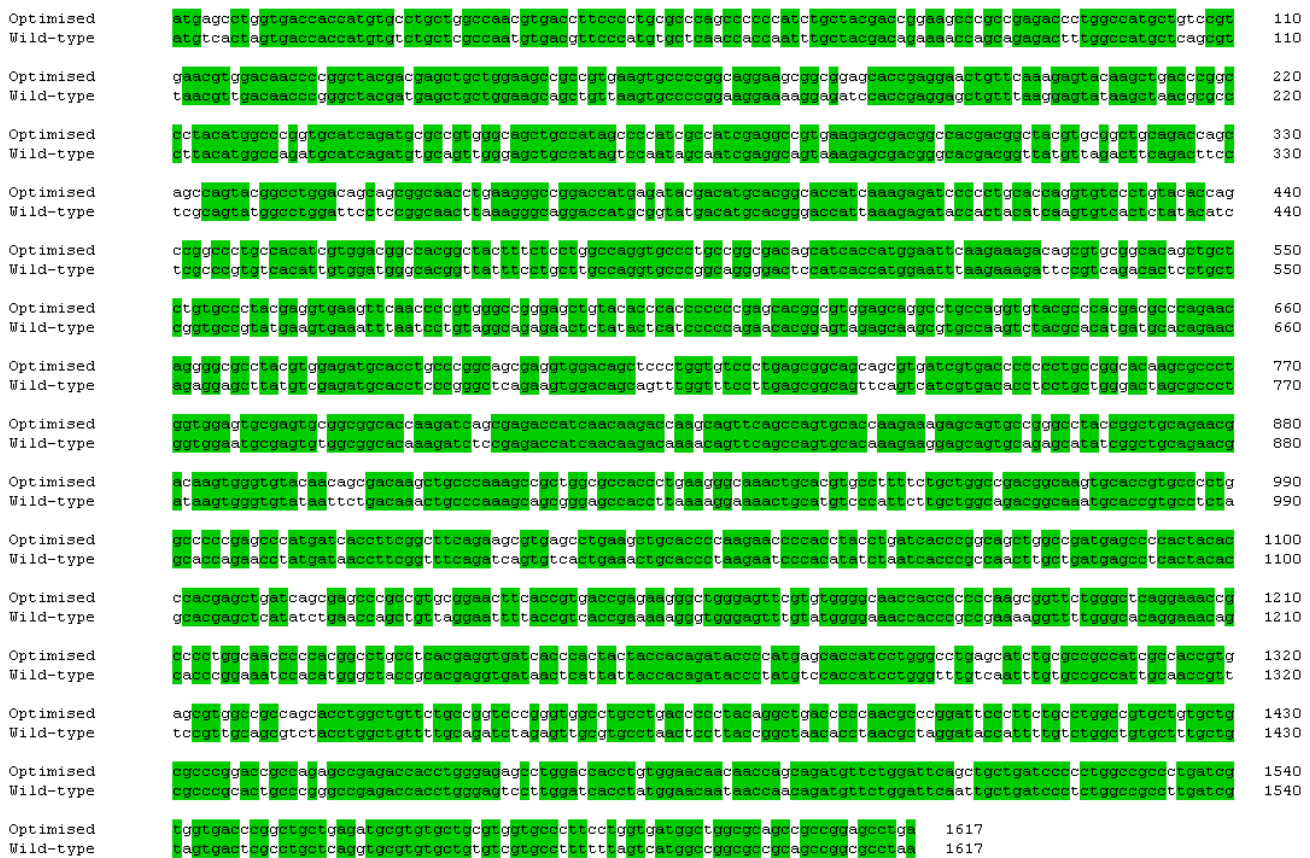


Figure 1
Optimised sequence of VEEV structural genes. The VEEV structural genes E3-E2-6K were optimised for expression by the addition of Kozak sequences, adaptation to optimal codon usage and removal of negative cis-acting sites using GeneOptimizer™. The optimised and wildtype sequence were aligned using Clone Manager 9. Areas highlighted in green indicate areas of identical sequences.

induced approximately 10-fold more VEEV-specific antibody than the non-optimised equivalent (Figure 4). This effect was statistically significant ($p < 0.0001$, Two way ANOVA).

Optimised ad-based vaccine confers protection in mice against homologous VEEV challenge

Groups of 10 mice were challenged by the aerosol route with VEEV (serogroup IA/B). Optimised vaccine significantly protected more mice against homologous virus challenge (90% survival) than either non-optimised vaccine (20% survival) or empty adenovirus (0% survival) ($p = 0.001$ and $p = 0.0001$ respectively, Mantel-Haenszel Logrank) (Figure 5).

Discussion

Previous efforts to improve the immunogenicity of an ad-based vaccine for use against VEEV have been unrewarding. Adjuvants such as CpG and interferon alpha, have not

only failed to improve immune responses but have increased the vector-specific response, potentially removing the possibility of repeated booster doses [23,24]. We have also shown that although a DNA vaccine can effectively prime the immune response prior to an ad-based vaccine, heterologous prime-boost appeared to offer little advantage over homologous adenovirus boosting [20]. We therefore reasoned that further optimisation of the components of the ad-based vaccine may improve immune responses.

Gene optimisation has been shown to be effective for a number of treatment applications where a protein is synthesised in vivo following gene delivery and is becoming routinely used for a range of applications [25-27]. For example, codon optimisation of the gene for the Respiratory syncytial virus F protein expressed from a DNA vaccine improved the performance relative to wild-type. Stronger antibody responses and better control of virus

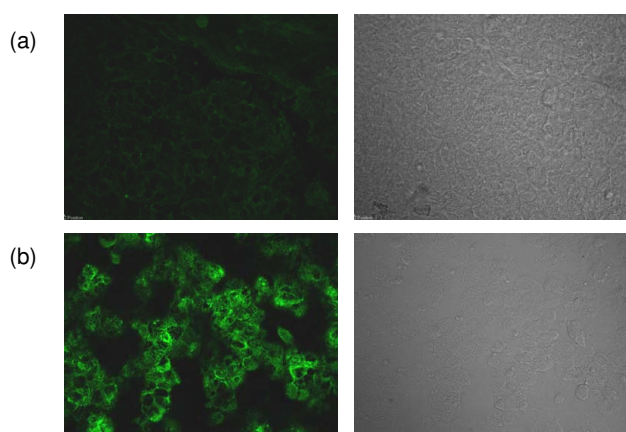


Figure 2
Expression of VEEV proteins from recombinant adenoviruses. HEK 293 cells were infected with RAd (a) or RAdVEEV#3-CO (b) and stained with polyclonal anti-VEEV followed by anti-mouse whole molecule IgG conjugated to FITC.

replication after challenge was observed in the Balb/c mouse model [28]. Codon optimisation of the Ag85B gene which encodes the secretory antigen of *Mycobacterium tuberculosis* has also proved beneficial [29]. A stronger Th1-like and cytotoxic T cell immune response in Balb/c mice resulted in an increased protective efficacy in an aerosol infection model. Codon usage adaptation of the gag protein of HIV delivered by a DNA vaccine increased gene expression by 10-fold compared to wild-type. A substantially increased humoral and cellular immune response in Balb/c mice was elicited, which was independent of the route of administration [30]. Similarly, optimisation of the Pr55gag genes in a DNA vaccine substantially increased gene expression, largely due to increased mRNA stability of the optimised transcripts [31]. Gene optimised HIV genes are currently encoded in DNA vaccine constructs undergoing human clinical trials [32,33]

Genes delivered by other platforms have also been optimised. For example, vaccinia viral vectors encoding the optimised HIV genes Gag-Pol-Nef are effective in small animal models and humans [32,34,35]. Finally, it has been demonstrated that the benefits of gene optimisation may be particularly acute where two of these approaches are combined in a prime-boost immunisation regimen [32,33].

In this study, we have focused our efforts on ad-based vaccines. Because ad-based vaccines allow *in vivo* synthesis of the antigen, a wide range of immune responses can be elicited. We have included a gene optimised version of the major antigenic determinant for VEEV, E2, along with the

chaperone proteins E3 and 6K within our ad-based vaccine. Delivery of this antigen by the ad-based vaccine is able to elicit the principle correlate of protection, a VEEV-specific antibody response [36-40]. CD4⁺ T cells [41], $\alpha\beta$ TCR-bearing T cells [42], cytokine responses and mucosal immunity following intranasal delivery [16] may also be initiated, though these mechanisms are believed to be of minor importance relative to antibody responses.

There are relatively few published methods for significantly enhancing the performance of ad-based vaccines. Some success has recently been achieved with a complement-based molecular adjuvant (mC4 bp). However, successful application of this to malaria vaccines has yet to prove universally applicable [43]. Gene optimisation has shown promise for a number of infectious diseases. For example, ad-based malaria vaccines have been developed containing malarial antigens optimised for expression in mammalian cells. Codon adaptation significantly increased the expression level of *Plasmodium* antigen in mammalian cells [44]. In another study developing an ad-based avian influenza (AI) vaccine, it was found that a synthetic AI H5 gene with codons optimised to match the chicken tRNA pool was more immunogenic than its counterpart without codon-optimisation [45]. Furthermore, an ad-based vaccine expressing gene optimised SIV mac239 gag gene was chosen to demonstrate the potential utility of ad-vectors derived from rare serotypes to elicit immune responses in the presence of pre-existing anti-Ad5 immunity [46].

Conclusion

In the current study, we are able to reproduce beneficial effects on vaccination efficacy of gene optimisation, for the first time with structural genes from the *Alphavirus*, VEEV. This is significant because while previous attempts to improve the protective efficacy of ad-based vaccines for this infectious disease have proven unsuccessful [20,23,24], we have increased both the immune response and protective efficacy of this vaccine through gene optimisation. An ad-based vaccine for VEEV may be particularly attractive given the increased inherent safety of this approach compared to live-attenuated vaccines and the potential of ad-based vaccines to be multivalent, potentially including genes from other alphaviruses such as western and eastern equine encephalitis viruses and chikungunya.

Methods

Plasmids, cells and viruses

Plasmid pVEEV#3 was previously constructed [14]. It contains the E3-E2-6K structural genes from the TC-83 strain of VEEV (attenuated TrD strain) with three mutations changing the sequence to that found in the virulent TrD strain. This gene sequence was replaced by the optimised

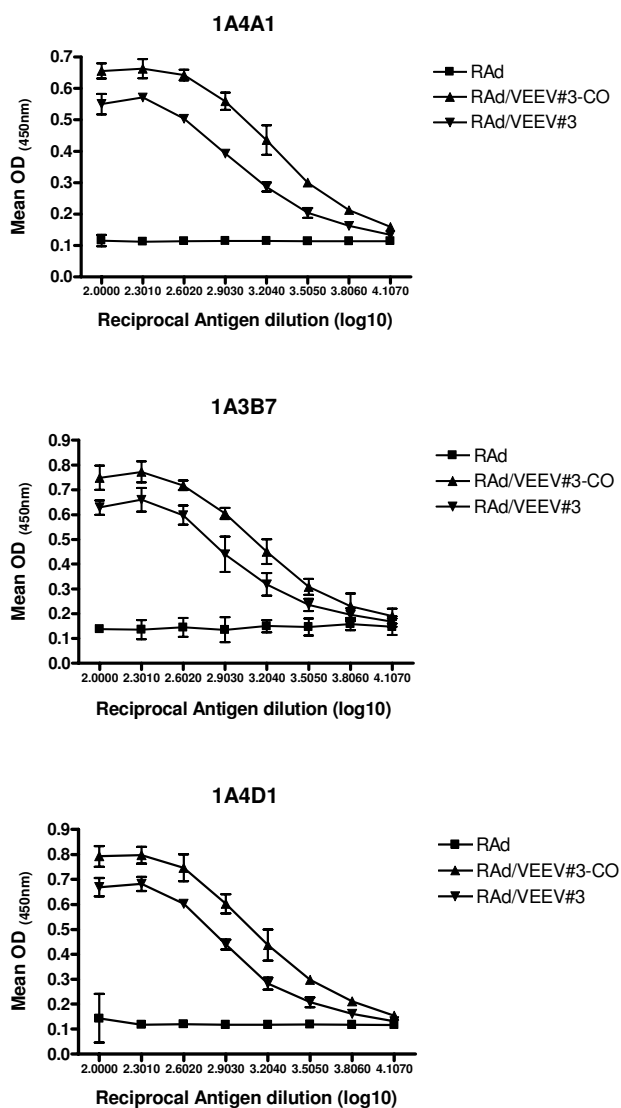


Figure 3
Antigen production by RAAd/VEEV#3 and RAAd-VEEV#3-CO. A549 cells were infected with adenovirus constructs, harvested 48 hours later and the cell pellets detergent extracted. Extracted antigens were tested by ELISA against VEEV monoclonal antibodies, 1A4A1, 1A3B7 and 1A4D1. Error bars represent the 95% CI of the assay.

gene sequence to produce the plasmid pVEEV#3-CO. The E3-E2-6K gene sequence was optimised and synthesised by Genart GmbH (Regensburg, Germany) and then cloned into the pVEEV#3 backbone using the Bam HI sites to create the plasmid pVEEV#3-CO. Recombinant adenovirus (RAAd/VEEV#3-CO) was constructed and purified as described previously for RAAd/VEEV#3 [14]. The optimised gene sequence in the recombinant ad was characterised by sequencing. The viral DNA of RAAd/VEEV#3-CO was

extracted using the QiaAmp DNA blood mini kit (Qiagen) and the E3-E2-6K genes were PCR amplified. This was then cloned into pCR[®]4-TOPO[®] (Invitrogen) for sequencing (Lark Technologies, Inc). Empty adenovirus containing no VEEV genes is designated RAAd [14].

HEK 293 and A549 cell lines (European Collection of Animal Cell Cultures, UK) were propagated by standard methods using the recommended culture media. VEEV serogroup IA/B (Trinidad donkey; TrD) was kindly supplied by Dr. B. Shope (Yale Arbovirus Research Unit, University of Texas, Austin, Texas, USA). Virulent virus stocks were prepared and titred as previously described [14].

Immunofluorescence

Recombinant adenoviruses were tested for expression of VEEV proteins by immunofluorescence. HEK 293 cell monolayers in T25 flasks were infected with the recombinant ads or empty ad vector (RAAd) for 48 hours at an MOI of 1. Cells were then harvested, washed and resuspended in PBS. The suspension (5 µl) was spotted onto glass slides which were then air dried and fixed in acetone at -20 °C for 15 minutes. The slides were reacted for 1 hour at 37 °C with a 1/400 dilution of mouse polyclonal anti-VEEV antibody in PBS/1% FCS or 10 µg/ml of the E2-specific monoclonal antibodies 1A3A-9, 1A4A-1 and 1A3B-7 in PBS/1% FCS. Mouse polyclonal anti-VEEV antibody was a kind gift from Dr. B. Shope of the Yale Arbovirus Research Unit, University of Texas, Austin, Texas, USA and E2-specific monoclonal antibodies were a kind gift of Dr. J.T. Roehrig, Division of Vector-Borne Infectious Diseases, CDC, Fort Collins, Colorado, USA. After three washes in PBS, cells were stained for 1 hour at 37 °C with FITC-labelled anti-mouse whole molecule IgG (Sigma) diluted 1/800 in PBS/1%FCS. The slides were washed a further four times in PBS before being mounted in 50% glycerol and examined using a UV microscope.

ELISA

Mouse sera, harvested from the marginal tail vein or by cardiac puncture, were assayed for VEEV-specific antibodies using sucrose density gradient-purified, β-propiolactone-inactivated antigen from strain TC-83 [14]. Immunoglobulin concentrations were estimated by comparison of the absorbance values generated by diluted serum samples (three replicates) with a standard curve prepared from dilutions of mouse IgG (Sigma, U.K.). To examine the expression of VEEV structural proteins, confluent monolayers of A549 cells in T25 flasks were infected with RAAd60, RAAd/VEEV#3 or RAAd/VEEV#3-CO (m.o.i. 1000) and incubated for 48 hours. Antigen was then prepared from cells by detergent extraction [14] and used to coat ELISA plates (starting dilution of 1/100, diluted 1/2 in coating buffer until 1/12800). VEEV E2 protein was detected using 10 µg/ml 1A4A1, 1A3B7 or

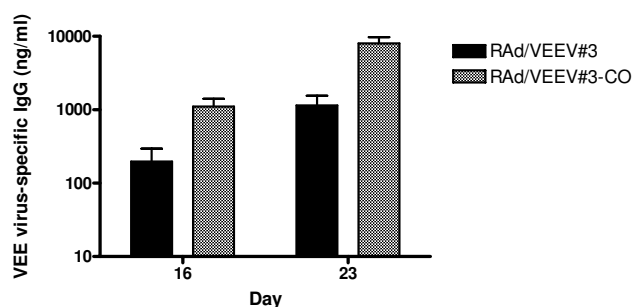


Figure 4
VEEV-specific total IgG responses in mice. Groups of 10 Balb/c mice were immunized with either RAAd/VEEV#3 (solid bars) or RAAd/VEEV#3-CO (checked bars) on days 0, 7 and 21. Sera were collected on days 16 and 23 and assayed for anti-VEEV total IgG. Error bars represent 95% CI.

1A4D1 followed by a 1/4000 dilution of HRP-labelled anti-mouse whole molecule IgG (Immunologicals Direct).

Animals, immunisation and challenge with virulent VEEV

Groups of 10 Balb/c mice, 6–8 weeks old (Charles River Laboratories, UK) were immunised intranasally under halothane anaesthesia on days 0 and 7 with 10^6 pfu and on day 21 with 10^3 pfu of RAAd/VEEV#3, RAAd/VEEV#3-CO or RAAd in 50 μ l PBS. Seven days after the final immunisation, the animals were challenged via the airborne route by exposure for 20 min to a polydisperse aerosol generated by a Collision nebuliser [47]. Mice were contained loose within a closed box during airborne challenge. The virus dose (100 LD₅₀) was calculated by sampling the air in the box and assuming a respiratory minute volume for mice of 1.25 ml/g [48]. After challenge, mice were observed twice daily for clinical signs of infection (pilo-erection, hunching, inactivity, excitability and paralysis) by an observer who was unaware of treatment allocations. In accordance with UK Home Office requirements and as previously described, humane endpoints were used [49]. These experiments therefore record the occurrence of severe disease rather than mortality. Even though it is rare for animals infected with virulent VEEV and showing signs of severe illness to survive, our use of humane endpoints should be considered when interpreting any virus dose expressed here as 50% lethal doses (LD₅₀).

Statistical methods

Statistical analysis was performed using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, CA, USA, <http://www.graphpad.com>). All data was normalised using a log transformation. Two-way ANOVA with Bonferroni's Multiple Comparison Test and statisti-

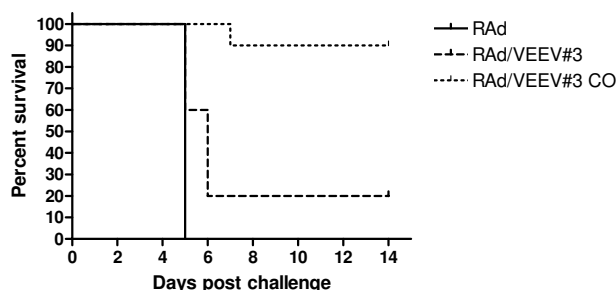


Figure 5
Protection against aerosolized VEEV challenge. Immunized mice (n = 10) were challenged on day 42 with 100 LD₅₀ of virulent airborne VEEV serotype IA/B and monitored for 14 days. Mice were immunized with RAAd, RAAd/VEEV#3 or RAAd/VEEV#3-CO as indicated.

cal analysis of survival using the Mantel-Haenszel logrank test were performed as detailed in the Results section.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AJW, LMOB and SDP carried out the study. RJP participated in the design of the study. AJW and SDP drafted the manuscript. All authors read, contributed to and approved the manuscript.

Acknowledgements

The authors would like to thank Amanda Gates, Amanda Phelps and Lin Eastaugh for their valuable contributions to this work. This work was funded by the UK Ministry of Defence.

References

- Weaver SC, Ferro C, Barrera R, Boshell J, Navarro JC: **Venezuelan equine encephalitis.** *Annual Review of Entomology* 2004, **49**:141-174.
- Klietmann WF, Ruoff KL: **Bioreterrorism: implications for the clinical microbiologist.** *Clin Microbiol Rev* 2001, **14**:364-81.
- Alibek K: **The Emerging BW Threat: Lessons from the Russian Offensive BW R&D Program, 1945 to 1992.** Maxwell AFB, Alabama.
- Stockholm International Peace Research Institute: **The problem of chemical and biological warfare.** In *CB Weapons Today Volume 2.* Almqvist and Wiksell, Stockholm; 1973.
- Hawley RJ, Eitzen EM Jr: **Biological weapons – a primer for microbiologists.** *Annu Rev Microbiol* 2001, **55**:235-53.
- Hilleman MR: **Overview: cause and prevention in biowarfare and bioterrorism.** *Vaccine* 2002, **20**:3055-67.
- Johnson KM, Martin DH: **Venezuelan equine encephalitis.** *Adv Vet Sci Comp Med* 1974, **18**:79-116.
- Pittman PR, Makuch RS, Mangiafico JA, Cannon TL, Gibbs PH, Peters CJ: **Long-term duration of detectable neutralizing antibodies after administration of live-attenuated VEE vaccine and following booster vaccination with inactivated VEE vaccine.** *Vaccine* 1996, **14**:337-343.
- Fine DL, Roberts BA, Terpening SJ, Mott J, Vasconcelos D, House RV: **Neurovirulence evaluation of Venezuelan equine encephalitis (VEE) vaccine candidate V3526 in nonhuman primates.** *Vaccine* 2008, **26**:3497-3506.

10. Reed DS, Lind CM, Lackemeyer MG, Sullivan LJ, Pratt WD, Parker MD: **Genetically engineered, live, attenuated vaccines protect nonhuman primates against aerosol challenge with a virulent IE strain of Venezuelan equine encephalitis virus.** *Vaccine* 2005, **23**:3139-3147.
11. Pratt WD, Davis NL, Johnston RE, Smith JF: **Genetically engineered, live attenuated vaccines for Venezuelan equine encephalitis: testing in animal models.** *Vaccine* 2003, **21**:3854-62.
12. Gale S: **Interscience Conference on Antimicrobial Agents and Chemotherapy – 48th Annual Meeting and Infectious Diseases Society of America – 46th Annual Meeting Updates on therapeutic research – Part 1.** *Drugs* 2009, **12**:1-2.
13. Strauss JH, Strauss EG: **The alphaviruses: gene expression, replication, and evolution.** *Microbiol Mol Biol Rev* 1994, **58**:491-562.
14. Phillpotts RJ, O'Brien L, Appleton RE, Carr S, Bennett A: **Intranasal immunisation with defective adenovirus serotype 5 expressing the Venezuelan equine encephalitis virus E2 glycoprotein protects against airborne challenge with virulent virus.** *Vaccine* 2005, **23**:1615-23.
15. Griffin D, Levine B, Tyor W, Ubol S, Despres P: **The role of antibody in recovery from alphavirus encephalitis.** *Immunological Reviews* 1997, **159**:155-161.
16. Elvin SJ, Bennett AM, Phillpotts RJ: **Role for mucosal immune responses and cell-mediated immune functions in protection from airborne challenge with Venezuelan equine encephalitis virus.** *J Med Virol* 2002, **67**:384-93.
17. Graf M, Schoedel T, Wagner R: **Rationals of Gene Design and de novo Gene Construction.** In *Systems Biology and Synthetic Biology* Edited by: Fu P, Latterich M, Panke S. Wiley; 2009.
18. Zolotukhin S, Potter M, Hauswirth WW, Guy J, Muzyczka N: **A "humanized" green fluorescent protein cDNA adapted for high-level expression in mammalian cells.** *J Virol* 1996, **70**:4646-4654.
19. Riemenschneider J, Garrison A, Geisbert J, Jahrling P, Hevey M, Negley D, Schmaljohn A, Lee J, Hart MK, Vanderzanden L, et al.: **Comparison of individual and combination DNA vaccines for B. anthracis, Ebola virus, Marburg virus and Venezuelan equine encephalitis virus.** *Vaccine* 2003, **21**:4071-80.
20. Perkins SD, O'Brien LM, Phillpotts RJ: **Boosting with an adenovirus-based vaccine improves protective efficacy against Venezuelan equine encephalitis virus following DNA vaccination.** *Vaccine* 2006, **24**:3440-3445.
21. Bennett AM, Phillpotts RJ, Perkins SD, Jacobs SC, Williamson ED: **Gene gun mediated vaccination is superior to manual delivery for immunisation with DNA vaccines expressing protective antigens from Yersinia pestis or Venezuelan Equine Encephalitis virus.** *Vaccine* 1999, **18**:588-96.
22. Paessler S, Ni H, Petrakova O, Fayzuln RZ, Yun N, Anishchenko M, Weaver SC, Frolov I: **Replication and Clearance of Venezuelan Equine Encephalitis Virus from the Brains of Animals Vaccinated with Chimeric SIN/VEE Viruses.** *J Virol* 2006, **80**:2784-2796.
23. Perkins SD, Williams AJ, O'Brien LM, Laws TR, Phillpotts RJ: **CpG Used as an Adjuvant for an Adenovirus-Based Venezuelan Equine Encephalitis Virus Vaccine Increases the Immune Response to the Vector, But Not to the Transgene Product.** *Viral Immunology* 2008, **21**:451.
24. O'Brien L, Perkins S, Williams A, Eastaugh L, Phelps A, Wu J, Phillpotts R: **Alpha interferon as an adenovirus-vectored vaccine adjuvant and antiviral in Venezuelan equine encephalitis virus infection.** *Journal of General Virology* 2009, **90**:874-882.
25. Capone S, Zampaglione I, Vitelli A, Pezzanera M, Kierstead L, Burns J, Ruggeri L, Arcuri M, Cappelletti M, Meola A, et al.: **Modulation of the Immune Response Induced by Gene Electransfer of a Hepatitis C Virus DNA Vaccine in Nonhuman Primates.** *The Journal of Immunology* 2006, **177**:7462-7471.
26. Zeng M, Xu Q, Elias M, Pichichero ME, Simpson LL, Smith LA: **Protective immunity against botulism provided by a single dose vaccination with an adenovirus-vectored vaccine.** *Vaccine* 2007, **25**:7540-7548.
27. Aurisicchio L, Mennuni C, Giannetti P, Calvaruso F, Nuzzo M, Cipriani B, Palombo F, Monaci P, Ciliberto G, La Monica N: **Immunogenicity and safety of a DNA prime/adenovirus boost vaccine against rhesus CEA in nonhuman primates.** *International Journal of Cancer* 2007, **120**:2290-2300.
28. Ternette N, Tippler B, Berla K, Grunwald T: **Immunogenicity and efficacy of codon optimized DNA vaccines encoding the F-protein of respiratory syncytial virus.** *Vaccine* 2007, **25**:7271-7279.
29. Ko HJ, Ko SY, Kim YJ, Lee EG, Cho SN, Kang CY: **Optimization of Codon Usage Enhances the Immunogenicity of a DNA Vaccine Encoding Mycobacterial Antigen Ag85B.** *Infect Immun* 2005, **73**:5666-5674.
30. Deml L, Bojak A, Steck S, Graf M, Wild J, Schirmbeck R, Wolf H, Wagner R: **Multiple Effects of Codon Usage Optimization on Expression and Immunogenicity of DNA Candidate Vaccines Encoding the Human Immunodeficiency Virus Type 1 Gag Protein.** *J Virol* 2001, **75**:10991-11001.
31. Graf M, Deml L, Wagner R: **Codon-optimized genes that enable increased heterologous expression in mammalian cells and elicit efficient immune responses in mice after vaccination of naked DNA.** *Methods Mol Med* 2004, **94**:197-210.
32. Harari A, Bart PA, Stohr W, Tapia G, Garcia M, Medjitna-Rais E, Burnett S, Cellerai C, Erlwein O, Barber T, et al.: **An HIV-1 clade C DNA prime, NYVAC boost vaccine regimen induces reliable, polyfunctional, and long-lasting T cell responses.** *J Exp Med* 2008, **205**:63-77.
33. McCormack S, Stohr W, Barber T, Bart PA, Harari A, Moog C, Ciuffreda D, Cellerai C, Cowen M, Gamboni R, et al.: **EV02: A Phase I trial to compare the safety and immunogenicity of HIV DNA-C prime-NYVAC-C boost to NYVAC-C alone.** *Vaccine* 2008, **26**:3162-3174.
34. Gomez CE, Najera JL, Jimenez V, Bieler K, Wild J, Kostic L, Heidari S, Chen H, Frachette MJ, Pantaleo G, et al.: **Generation and immunogenicity of novel HIV/AIDS vaccine candidates targeting HIV-1 Env/Gag-Pol-Nef antigens of clade C.** *Vaccine* 2007, **25**:1969-1992.
35. Didierlaurent A, Ramirez JC, Gherardi M, Zimmerli SC, Graf M, Orbea HA, Pantaleo G, Wagner R, Esteban M, Kraehenbuhl JP, et al.: **Attenuated poxviruses expressing a synthetic HIV protein stimulate HLA-A2-restricted cytotoxic T-cell responses.** *Vaccine* 2004, **22**:3395-3403.
36. Bennett AM, Elvin SJ, Wright AJ, Jones SM, Phillpotts RJ: **An immunological profile of Balb/c mice protected from airborne challenge following vaccination with a live attenuated Venezuelan equine encephalitis virus vaccine.** *Vaccine* 2000, **19**:337-47.
37. Hart MK, Lind C, Bakken R, Robertson M, Tammariello R, Ludwig GV: **Onset and duration of protective immunity to IA/IB and IE strains of Venezuelan equine encephalitis virus in vaccinated mice.** *Vaccine* 2001, **20**:616-622.
38. Phillpotts RJ, Wright A: **TC-83 vaccine protects against airborne or subcutaneous challenge with heterologous mouse-virulent strains of Venezuelan equine encephalitis virus.** *Vaccine* 1999, **17**:982-8.
39. Phillpotts RJ: **Venezuelan equine encephalitis virus complex-specific monoclonal antibody provides broad protection, in murine models, against airborne challenge with viruses from serogroups I, II and III.** *Virus Research* 2006, **120**:107-112.
40. Phillpotts RJ, Jones LD, Howard SC: **Monoclonal antibody protects mice against infection and disease when given either before or up to 24 h after airborne challenge with virulent Venezuelan equine encephalitis virus.** *Vaccine* 2002, **20**:1497-504.
41. Yun NE, Peng BH, Bertke AS, Borisevich V, Smith JK, Smith JN, Pousard AL, Salazar M, Judy BM, Zacks MA, et al.: **CD4+ T cells provide protection against acute lethal encephalitis caused by Venezuelan equine encephalitis virus.** *Vaccine* 2009, **27**:4064-4073.
42. Paessler S, Yun NE, Judy BM, Dziuba N, Zacks MA, Grund AH, Frolov I, Campbell GA, Weaver SC, Estes DM: **Alpha-beta T cells provide protection against lethal encephalitis in the murine model of VEEV infection.** *Virology* 2007, **367**:307-323.
43. Draper SJ, Moore AC, Goodman AL, Long CA, Holder AA, Gilbert SC, Hill F, Hill AVS: **Effective induction of high-titer antibodies by viral vector vaccines.** *Nat Med* 2008, **14**:819-821.
44. Li S, Locke E, Bruder J, Clarke D, Doolan DL, Havenga MJE, Hill AVS, Liljestrom P, Monath TP, Naim HY, et al.: **Viral vectors for malaria vaccine development.** *Vaccine* 2007, **25**:2567-2574.
45. Toro H, Tang Dc, Suarez DL, Zhang J, Shi Z: **Protection of chickens against avian influenza with non-replicating adenovirus-vectored vaccine.** *Vaccine* 2008, **26**:2640-2646.

46. Barouch DH, Pau MG, Custers JHHV, Koudstaal W, Kostense S, Havenga MJE, Truitt DM, Sumida SM, Kishko MG, Arthur JC, et al.: **Immunogenicity of recombinant adenovirus serotype 35 vaccine in the presence of pre-existing anti-Ad5 immunity.** *Journal of Immunology* 2004, **172**:6290-6297.
47. Phillipotts RJ, Brooks TJ, Cox CS: **A simple device for the exposure of animals to infectious microorganisms by the airborne route.** *Epidemiol Infect* 1997, **118**:71-5.
48. Guyton AC: **Measurement of the respiratory volume of laboratory animals.** *Am J Physiol* 1947, **150**:70-77.
49. Wright AJ, Phillipotts RJ: **Humane endpoints are an objective measure of morbidity in Venezuelan encephalomyelitis virus infection of mice.** *Arch Virol* 1998, **143**:1155-62.

Publish with **BioMed Central** and every scientist can read your work free of charge

"BioMed Central will be the most significant development for disseminating the results of biomedical research in our lifetime."

Sir Paul Nurse, Cancer Research UK

Your research papers will be:

- available free of charge to the entire biomedical community
- peer reviewed and published immediately upon acceptance
- cited in PubMed and archived on PubMed Central
- yours — you keep the copyright

Submit your manuscript here:
http://www.biomedcentral.com/info/publishing_adv.asp

