Future vaccines against emerging encephalitides

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Summary. The vaccine strategies available for control of emerging encephalitides range in a continuum from traditional approaches to those utilizing new technologies. In this report, we explore the use of live attenuated vaccines where the attenuating mutations have been selected in a rational way to improve attenuation without sacrificing effectiveness. A strategy for paired lethal and resuscitating mutations is presented that will greatly reduce the possibility of reversion to virulence. Finally, we describe an example of a vaccine vector system that could be rapidly adapted for use against these virus diseases as they emerge.

Introduction

Developing vaccines against existing and emerging encephalitis viruses is a challenging task. Outbreaks of these viruses are unpredictable and usually affect relatively small populations, or occur in poorer areas of the world. The market aspects of both circumstances make these diseases relatively unattractive targets for vaccine development by commercial enterprises. However, the scientific possibilities available for vaccine development for emerging diseases include a rich variety of new technologies as well as old approaches augmented with new technology. In this chapter, we will explore three of these approaches using an alphavirus, Venezuelan equine encephalitis virus (VEEV), as both a specific example and a model for other diseases.

Live attenuated vaccines

Traditional live attenuated vaccines have provided the most successful and cost effective interventions for the prevention of human disease. Most often, these have been derived by blind passaging in cell culture followed by empirical testing for attenuation and immunogenicity. Such vaccines can suffer from insufficient attenuation leading to moderate or severe reactions in vaccine recipients, or conversely they may be overattenuated leading to poor immunization. With the advent of molecular technologies and the careful explication of viral pathogenesis in animal

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models, it is possible to design live attenuated vaccines to avoid these problems and retain the low cost and long term efficacy advantages associated with this vaccine approach.

Informed selection of attenuating point mutations

VEEV causes encephalitis in mice, in horses and in a small proportion of human infections. Examination of the molecular genetics of VEEV and its pathogenesis in the mouse model suggest several principles upon which to base new live virus vaccines.

Optimal live virus vaccines are not simply viruses less fit for replication

The pathogenetic effects of virus replication are manifest through a series of complex interactions within the infected animal or human. In many ways, each differentiated tissue or organ system with which the virus interacts constitutes a different host, especially at the level of individual infected cells. Thus, a given virus is genetically optimized to replicate in a series of very different host cell types within the body during the course of disease. In contrast, passage of viruses in cell culture for the derivation of attenuated viruses amounts to a selection for virus mutants optimized for replication in a single type of cultured cell. It is not too surprising then, that many such cell-adapted mutants are attenuated, being less able to accomplish the intricate series of interactions with multiple cell types required to cause disease *in vivo*.

This understanding suggests that rather than blind passage in cell culture, one should actually select for viruses that can grow more rapidly in cell culture [4, 5]. Sindbis virus, the prototype alphavirus, was passaged in baby hamster kidney (BHK) cells under selection for rapid growth, and this led to the selection of highly attenuated mutants within fewer than 10 passages. Comparison of over a hundred attenuated isolates from this passage series revealed that all the attenuated isolates shared the property of accelerated penetration of BHK cells [14, 36]. When VEEV was passaged under a specific selection for rapid penetration, 70% of isolates from as early as the fourth passage were rapidly penetrating and attenuated in mice [29]. In contrast, 83 blind passages were required for attenuation of the VEE vaccine strain, TC-83. Sequencing of the glycoprotein genes of a number of these isolates revealed a panel of candidate attenuating mutations [16].

In parallel, a full-length cDNA clone of the VEEV single-stranded RNA genome was constructed [18]. Genetic manipulations could be performed at the level of the cDNA clone, and those changes could be recovered in the genomes of infectious virus generated from the clone. This process depends on the fact that the genomic RNA of VEEV is message sense, and purified genomic RNA extracted from the virus can initiate viral infection when introduced artificially into susceptible cells. In the cDNA clone, the VEEV genomic sequences were placed downstream of a phage T7 promoter. Linearization of the clone at a unique restriction site downstream of the VEEV sequences and *in vitro* transcription with

T7 polymerase generated VEEV genomic replicas that could be electroporated into cells, resulting in the production of infectious virus. Using this genetic system, individual candidate attenuating mutations could be introduced into the clone at the DNA level and then tested as viruses for their effect on virulence and pathogenesis in the mouse model [22]. In addition, the balance between attenuation and overattenuation could be sustained by judicious choice of individual mutations, which were attenuated to different degrees, and by combining two or three attenuating mutations in the same virus to minimize the chances of reversion [29].

Selection of attenuating point mutations based on studies of viral pathogenesis

Our studies of VEEV pathogenesis have utilized molecularly cloned viruses to minimize the effects of cell culture passage on pathogenetic phenotypes and to take advantage of specific mutations introduced into the clone. This approach has defined a pathogenetic pathway in the mouse, by which VEEV infection progresses from the site of inoculation in the left rear footpad to eventual death by encephalitis [15, 22]. The virus infects Langerhans cells at the site of inoculation, and these cells move rapidly to the draining lymph node where replication can be detected within 4 hrs post-inoculation [35]. Near peak levels of viremia are established by 12 hrs, and all the lymphoid tissues are infected by 18 hrs. Replication in the brain is detectable between 24 and 36 hrs. Invasion of the central nervous system (CNS) occurs by infection of peripheral nerves and subsequent progression into the CNS. Virus appears first in olfactory tracts of the brain, suggesting that the earliest entry into the CNS is via infection of olfactory nerves in the nasal neuroepithelium [10]. If the nasal neuroepithelium is artificially ablated, then the earliest appearance of virus in the CNS is in areas enervated by the trigeminal nerve.

The kinetics with which the virus progresses through the mouse suggested a tentative pathogenetic pathway leading ultimately to encephalitis and death. By analogy to a biochemical pathway, further elucidation of the pathogenetic pathway has been accomplished through the study of avirulent mutants. By definition, these mutants must be defective or blocked in one or more of the steps required to complete the progression to lethal encephalitis. Identification of the block points for multiple mutants make possible the elucidation of the pathogenetic pathway for the wild-type virus. An illustration of this is the avirulent mutant V3010, in which the glutamic acid at E2 amino acid 76 is replaced with lysine. The defect in this virus was manifested as a delay in reaching the draining lymph node [1, 22]. Virus could be isolated sporadically from serum, from other lymphoid organs and from the brain. However, half of the isolates from the draining lymph node and all the isolates downstream of the draining lymph node in the pathogenetic pathway were revertant, in that they contained sequence changes associated with reversion or displayed a virulence phenotype distinct from the inoculated V3010 virus.

There were important differences among the different revertants depending on the tissue source of the isolation [1]. Revertants from serum and the contralateral

lymph node were second-site revertants which retained the original V3010 mutation at E2 76 but had an additional substitution of glutamic acid for lysine at E2 116. To demonstrate that the E2 116 mutation altered the V3010 phenotype, the putative second-site mutation was introduced into the V3010 clone, and the cloned virus containing both mutations (V3533) was compared to the biological isolates. The cloned and biological viruses shared the same pathogenesis phenotype. In V3533, the original block in reaching the draining lymph node was abrogated, spread and replication in the periphery was analogous to that of wild-type virus, the CNS was invaded, all the animals displayed clinical disease with neurological signs, but all recovered from the infection without obvious sequelae. Therefore, selective pressure for reversion *in vivo* is actually at the point of the genetic blockade imposed by the attenuating mutation. There is no selective pressure *per se* for reversion to wild-type virulence.

Vaccine lessons from studies of viral pathogenesis

What lessons can be learned from these experiments with respect to design of live attenuated vaccines? First, one should select point mutations which block early in the pathogenetic pathway, as the selection pressure to bypass the mutant block would not necessarily lead to a fully virulent revertant. However, if the block point imposed by a given mutation is close to the end of the pathway, i.e. near the point of death, then selection for reversion to bypass the block will be tantamount to selection for reversion to wild-type virulence. Second, genetic stability will be improved if multiple point mutations are used. Third, multiple defined mutations which block at different steps in the pathway may provide a more stable attenuated phenotype than multiple mutations blocking the same step. Finally, vaccine mutations which block too early in the pathway may result in too little replication for induction of protective immune responses.

Lethal mutations in vaccine design

Alphaviruses provide an example where a lethal mutation is an important element in a live attenuated vaccine strategy. These viruses initially produce one of their structural glycoproteins (E2) as a precursor molecule, PE2, which consists of E2 with an amino terminal extension of 55–65 amino acids (E3; Fig. 1). At the carboxy terminus of E3 is a canonical furin protease cleavage site, and in infected cells PE2 is cleaved by furin in the late Golgi resulting in the quantitative incorporation of E2 into budded virions. One of the attenuating mutations identified from a rapid penetration selection with another alphavirus, South African Arbovirus 86 (S.A.Ar-86) involved the addition of an N-linked carbohydrate chain at the first amino acid residue of E2, and this sterically prevented maturation of the E2 glycoprotein through furin-mediated cleavage of PE2 [41]. The resulting virus particles contained PE2 in place of E2, they infected cultured cells more efficiently, but they were highly attenuated in mice. This mutation was reproduced in a full-length cDNA clone of the prototype alphavirus, Sindbis. Electroporation of

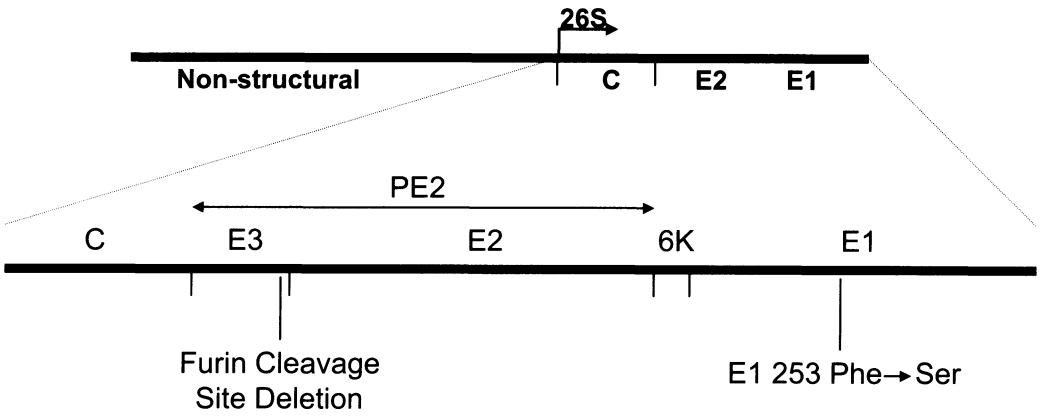

Fig. 1. VEE 3526: resuscitated cleavage site deletion vaccine. Resuscitated for cell culture growth attenuated in mice and non-human primates

the resulting *in vitro* RNA transcripts into cells programmed normal viral RNA synthesis, production of viral structural proteins and the release of viral particles containing PE2 instead of E2 [6, 24–26, 37]. Unlike the S.A.Ar-86 mutants, prototype Sindbis virions containing PE2 were not viable due to their failure to penetrate BHK cells. However, it was possible to select revertants of two types which were viable in cell culture: 1) Mutation in the N-X-S/T N-linked glycosylation signal abrogated glycosylation at position 1 of E2 and allowed PE2 cleavage. And 2) Second-site mutants retained the N-terminal glycosylation and the consequent inclusion of PE2 in the mutant virions but had a second mutation in either E2 or E3 that resuscitated the virus for the cell culture penetration phenotype. These resuscitated viruses were uniformly attenuated in animals.

In the VEEV clone, the four amino acid furin cleavage site was deleted by site directed mutagenesis, resulting in a virus genome which, when electroporated into permissive cells, could program normal RNA replication and the assembly of PE2-containing virions [12]. However, these virions were not infectious due to their inability to effect penetration of other new cells. As with Sindbis virus, VEEV second-site revertants in either E2 or E1 were isolated in cell culture. In the case of the V3526 double mutant, the lethal furin cleavage site deletion was retained, and a second, independently attenuating mutation in E1 restored cell culture infectivity (Fig. 1). V3526 was highly attenuated in mouse and primate models and it induced protection against both parenteral and aerosol challenge with virulent VEEV [38]. Compared to the existing vaccine TC-83, V3526 induced higher levels of antibody in a larger percentage of animals, was less reactogenic, and conferred stronger protection against challenge. The genetic stability of V3526 stems from the very low probability that the deletion will be repaired and from the fact that reversion of the second-site mutation to the wild-type amino acid will result in a dead virus rather than a virulent one. A cGMP lot of V3526 has been prepared and is currently undergoing requisite toxicity testing in anticipation of a phase I human trial.

The lethal deletion/resuscitating mutation strategy may be generally applicable in designing effective live attenuated vaccines with very low propensities for reversion to virulence. Recently, Kofler et al. [30, 31] created lethal deletions in the capsid protein of tick-borne encephalitis virus and found resuscitating mutations which render the double mutant viable in cell culture yet highly attenuated in animal models of disease.

Use of VEEV as a vaccine vector

Using viruses to express exogenous genes has its roots in bacteriophage transduction experiments, in which phage are used to move bacterial genes from one strain to another. In terms of vaccination, a number of different animal and human viruses have been utilized to express an immunizing gene from a given pathogen to effect protective immunization against that pathogen. Zoonotic viruses are especially well suited for this purpose. These viruses have the capacity for replication in humans, but unlike viruses that widely infect humans (e.g. adenovirus) or viruses to which large human populations are vaccinated (e.g. polio and vaccinia), only a small number of persons are already immune to most zoonotic viruses. This suggests that vectors derived from zoonotic viruses will express well in humans but that interference due to pre-existing immunity will be minimal.

The VEEV genome, when introduced into a cell, can be viewed as inducing a highly efficient machine for the production of large amounts of its own structural proteins through the transcription of a subgenomic mRNA encoding these proteins. If a gene of interest is substituted for the structural protein genes,

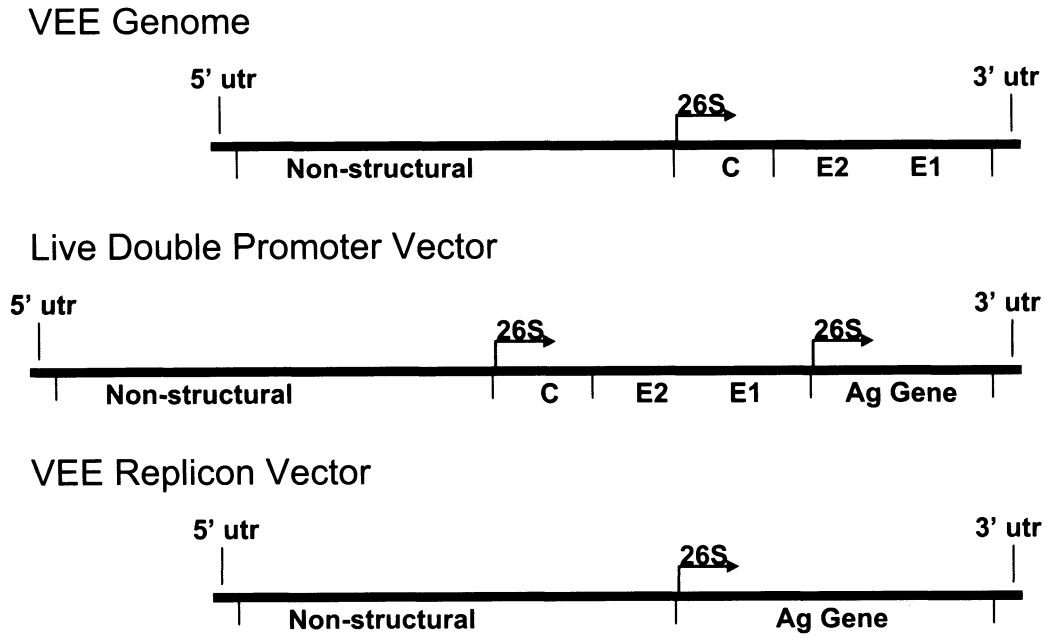

Fig. 2. VEE vaccine vectors

or if a gene of interest is inserted downstream of a second copy of the subgenomic promoter, then the VEEV machine expresses high levels of that gene product.

The vaccine vectors based on VEEV are of two types. In the first, an additional copy of the 26S subgenomic promoter was inserted between the end of the E1 gene and the start of the 3′ untranslated region (Fig. 2) [8, 9, 11]. This results in a viable virus capable of expressing an immunizing gene inserted after the second 26S promoter.

VEEV replicon vaccine vectors

The second type is a Areplicon vector in which the immunizing gene is substituted for the structural protein genes (Fig. 2) [40]. Upon electroporation of replicon RNA into cells, the VEEV replication machinery produces high levels of the subgenomic mRNA and hence high levels of the vectored gene product. However, as the structural protein genes are not present in the replicon RNA, no new virus particles are released. One can package the replicon in virions by supplying the structural proteins *in trans* from helper RNAs. The replicase proteins encoded on the replicon RNA provide the machinery for replicating the helper RNAs (Fig. 3), while the structural proteins encoded by the helpers encapsidate and envelope the replicon RNA. Only the replicon RNA is packaged into VEEV replicon particles (VRP), because the replicon RNA contains the *cis*-acting packaging signal deleted from

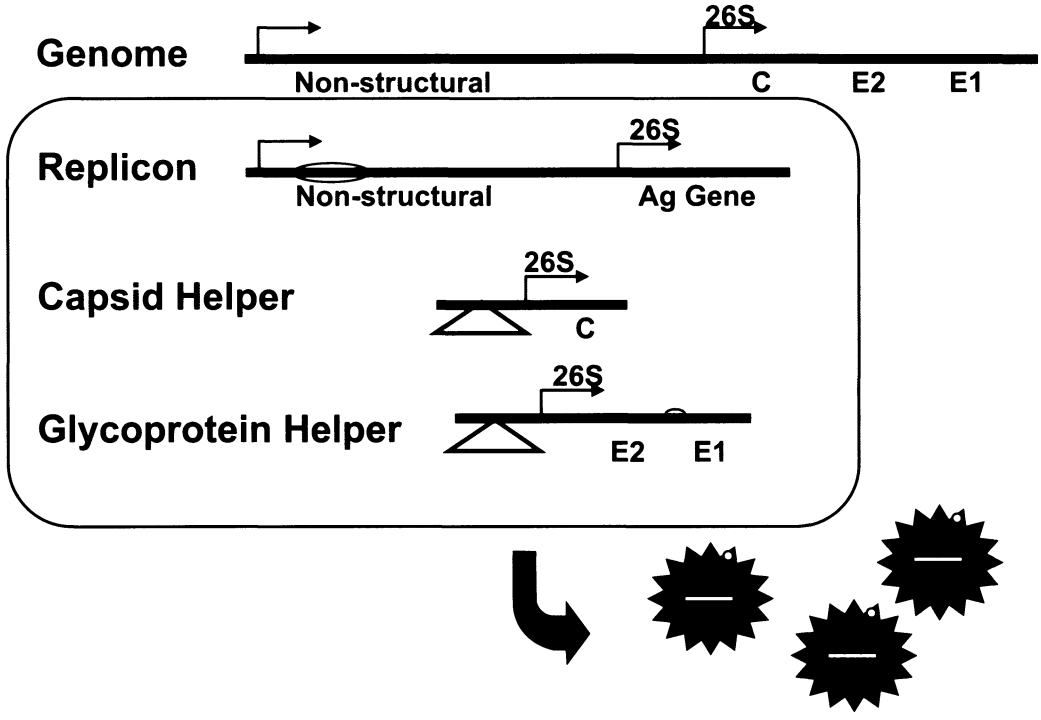

Fig. 3. Replicon expression and packaging

the helper RNAs. When the resulting VRP infect another cell, either in culture or *in vivo*, the replicon RNA is introduced, high levels of the immunizing gene product are synthesized, but no new particles are formed due the absence of the structural genes. Therefore, these single-cycle vectors are expressed only in the first cell infected.

Features of VEEV replicon vaccines

Safety in animal models: Redundant safety features are embedded into the replicon system. First, over one-third of the genome has been deleted. Second, helper functions are supplied in the form of two separate RNAs to greatly reduce the chances of re-generating an infectious virus through RNA-RNA recombination. Third, attenuating mutations can be introduced into the glycoprotein helper RNA so that even if a viable recombinant were formed, the worst case scenario would be a live attenuated VEEV vaccine strain. Finally, sensitive tests have been devised for the detection of propagation competent viruses in VRP preparations. After parenteral inoculation of various VRP in a number of laboratories, no clinical signs have been observed in experimental animals (rabbits, guinea pigs, macaques), in natural hosts of VEEV (adult mice, horses), in animals lacking a fully developed immune system (neonatal mice, one-day-old chickens, 19 day chicken embryos), and animals genetically incapable of cellular and humoral immune responses ($RAG^{-/-}$ mice).

High level gene expression: In a VEEV infected cell, the virus produces large amounts of its structural proteins to facilitate assembly of new virus particles. In a cell infected by VRP, this same machinery produces high levels of the immunizing gene product. For cytoplasmic proteins, such as the Lassa N protein [40] or the Gag protein of human (HIV) or simian (SIV) immunodeficiency viruses [13], up to 20% of the total cell protein is from the replicon expressed gene.

Induction of humoral and cellular immune responses: Both the humoral and cellular arms of the immune response are induced upon immunization with VRP [13]. This has been demonstrated in a variety of animal models including rodents and non-human primates.

Induction of mucosal immunity after parenteral inoculation: Mucosal immunity, in the form of mucosally produced IgA, is an important contributor to protection against a large number of pathogens. Usually, application of an immunogen to a mucosal site is required for induction of a mucosal response. However, VRP possess the ability to induce mucosal responses after parenteral inoculation [23]. Although the mechanism underlying this phenotype remains unclear, cells secreting IgA specific for the vectored gene have been detected in mucosal tissues of animals immunized subcutaneously with VRP (E. Richmond et al., University of North Carolina, unpublished). This suggests that a true mucosal response was elicited by VRP rather than simply the transudation of IgA antibodies from the plasma to a mucosal surface.

Induction of anti-vector (anti-VEEV) immune responses: Anti-VEEV antibodies can be detected in both rodent and primate models following immunization

with VRP. However, in vaccination regimens involving multiple inoculations, boosted responses were observed with second and third immunizations. The effect of pre-existing anti-VEEV responses was tested in two experiments. In the first, groups of mice were immunized with diluent or V3014, a molecularly cloned virus containing an attenuating mutation in each of the glycoprotein genes. The 80% plaque reduction neutralization titers ($PRNT_{80}$) averaged 10,000 in the vaccinated group. Both control and vaccinated animals were subsequently immunized with priming and boosting inoculations of VRP expressing the influenza HA gene. The resulting ELISA titers against influenza were approximately 10,000 in both control and V3014 immunized groups.

In a second experiment, one group of mice received two consecutive inoculations of VRP expressing SIVgp140 followed by two consecutive inoculations of VRP expressing a portion of the HIV Gag gene. A control group received four inoculations of diluent. Then, both groups of animals received two consecutive inoculations of VRP expressing influenza HA. The resulting anti-influenza ELISA titers were 3200 and 1800 for the control and pre-immunized groups, respectively. At least in mice therefore, neither sequential vaccination with VRP nor the presence of pre-existing neutralizing antibody to VEEV affected the induction of immune responses by the VEEV replicon vectors.

Targeting of VRP to dendritic cells: Replication of VEEV virus in the mouse is first detected in the draining lymph node, and this feature of VEEV biology strongly suggested that vaccine vectors based on VEEV would be especially effective. Using VRP expressing the green fluorescent protein (GFP) and taking advantage of the fact that VRP express only in the first cell they infect, we demonstrated the initial infection of Langerhans cells at the site of inoculation, the rapid migration of these cells to the draining lymph node, and the continued expression of GFP in dendritic cells (DC) for 4–5 days thereafter [35]. The efficiency of DC infection *in vivo* is strongly influenced by the VEEV glycoprotein envelope of the VRP tested, with different glycoprotein mutants varying with respect to this parameter (A. West et al., University of North Carolina, unpublished). DC targeting also was influenced by the site and route of inoculation. However, with each of these variables, the ability to target DC was positively correlated with the induction of both cellular and humoral responses. Given that DC are among the most potent antigen presenting cells in the body, we feel that the innate ability of VEEV to target these cells is largely responsible for their ability to successfully immunize animals. Analogous studies in non-human primates are in progress.

Vaccine efficacy: VEEV replicon vectors have been used in successful immunization or challenge experiments for a wide range of pathogens and animal model systems. Especially in acute infection models, the VEEV vectors demonstrate the capacity for strong and protective immunization. Examples include influenza viruses in rodents [40] and chickens (H5N1, 42), bacteria and bacterial toxins in rodents [21, 32–34, 43], Ebola virus in rodents [39], Marburg virus in rodents and primates [27, 28], equine arteritis virus in horses [2, 3], Norwalk virus in rodents [23], feline immunodeficiency virus in cats [7], a

human papilloma virus tumor model in mice [20, 44], as well as HIV and SIV in rodents [19], in primates [13], and as a contemplated human vaccine for HIV [17, 45].

Conclusions

Study of the molecular genetics of VEEV and its pathogenesis in animal models has provided a number of insights applicable to the design of live attenuated vaccines for VEEV and other emerging zoonotic viruses. Pathogenesis of these viruses may be considered in terms of a pathogenesis pathway providing a working framework for the genetic dissection of pathogenesis using wild-type virus and individual, defined, avirulent mutants, each of which interdicts the pathway at a specific stage(s). Although all avirulent mutants could be considered candidate live attenuated vaccines, pathogenesis studies suggest that: 1) Each mutation will block progression through the pathway at a specific step. 2) There is no selective pressure for reversion to wild-type virulence *per se*, only for reversion in the sense of bypassing the particular genetic blockade imposed by that specific mutation. 3) Preferred attenuating vaccine mutations are more likely found, therefore, among those that block early stages of the pathway. For mutants blocked at a late stage, reversion to bypass the genetic blockade may be tantamount to reversion to wild-type virulence. 4) Mutations which block too early in the pathway may be overattenuated. And 5) Multiple attenuating mutations, each blocking a different step in the pathway, will provide genetic stability to a live attenuated vaccine.

Deletion mutations provide very stable phenotypes, but in the context of a viral genome are often lethal for viral growth even in cell culture. However, the plasticity of viral genomes, especially RNA virus genomes, can give rise to second-site point mutations that partially compensate for a primary deletion. In VEEV mutants deleted for the furin cleavage site required for maturation of the E2 glycoprotein, selection of such second-site resuscitating mutations in cell culture resulted in viruses that were able to grow *in vitro* but were highly attenuated in rodent and primate models. This principle, creating a lethal deletion and then selecting for resuscitating point mutations, seems to apply also to flaviviruses, in which lethal deletions in the capsid gene can be compensated by the selection of point mutations elsewhere to generate a virus which is avirulent in animals [31]. These candidate vaccine strains display remarkable genetic stability, because the deletion is unlikely to be repaired and same-site reversion of the resuscitating point mutation will result in a dead virus rather than a virulent one.

Finally, the VEEV system illustrates the advantages of using a zoonotic virus as a vaccine vector system. Live, attenuated VEEV strains carrying a second 26S promoter and VEEV replicon particles, expressing a variety of immunizing genes from both bacterial and viral pathogens, have proven to be safe and effective vaccines in animal models.

Vaccine strategies do not yet exist for a number of significant human pathogens. The emergence of entirely new human pathogens such as HIV and severe acute

respiratory syndrome (SARS) has been both dramatic and frightening, taking an enormous toll on human health, social interactions and economic activity. Because many of these consequences are most evident in poorer developing countries, western governments and business interests have not fully appreciated the urgency of these infectious disease emergencies, nor have they fully accepted the concept of a global imperative for the discovery and application of new vaccine strategies to combat them. We hope that the VEEV model can and will be helpful in the scientific development of additional safe, effective and affordable vaccine strategies in support of global public health needs.

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References

1. Aronson JF, Davis NL, Grieder FB, Charles PC, Knott TA, Brown K, Johnston RE (2000) A single-site mutant and revertants arising *in vivo* define early steps in the pathogenesis of Venezuelan equine encephalitis virus. Virology 270: 111–123
2. Balasuriya UBR, Heidner HW, Davis NL, Wagner HM, Hullinger PJ, Hedges JF, Williams JC, Johnston RE, Wilson WD, Liu IK, MacLachlan NJ (2002) Alphavirus replicon particles expressing the two major envelope proteins of equine arteritis virus induce high level protection against challenge with virulent virus in vaccinated horses. Vaccine 20: 1609–1617
3. Balasuriya UBR, Heidner HW, Hedges JF, Williams JC, Davis NL, Johnston RE, MacLachlan NJ (2000) Expression of the two major envelope proteins of equine arteritis virus as a heterodimer is necessary for induction of neutralizing antibodies in mice immunized with recombinant Venezuelan equine encephalitis virus replicon particles. J Virol 74: 10623–10630
4. Baric RS, Moore DB, Johnston RE (1980) *In vitro* selection of an attenuated variant of Sindbis virus. ICN-UCLA Symp Mol Cell Biol 18: 685–694
5. Baric RS, Trent DW, Johnston RE (1981) A Sindbis virus variant with a cell determined latent period. Virology 110: 237–242
6. Boehme KW, Williams JC, Johnston RE, Heidner HW (2000) Linkage of an alphavirus host-range restriction to the carbohydrate processing phenotypes of the host cells. J Gen Virol 81: 161–170
7. Burkhard MJ, Valenski L, Leavell S, Dean GA, Tompkins WA (2002) Evaluation of FIV protein-expressing VEEV-replicon vaccine vectors in cats. Vaccine 21: 258–268
8. Caley IJ, Betts MR, Davis NL, Swanstrom R, Frelinger JA, Johnston RE (1999) Venezuelan equine encephalitis virus vectors expressing HIV-1 proteins: vector design strategies for improved vaccine efficacy. Vaccine 17: 3124–3135
9. Caley IJ, Betts MR, Irlbeck DM, Davis NL, Swanstrom R, Frelinger JA, Johnston RE (1997) Humoral, mucosal and cellular immunity in response to a human

immunodeficiency virus type 1 immunogen expressed by a Venezuelan equine encephalitis virus vaccine vector. J Virol 71: 3031–3038
10. Charles PC, Walters E, Margolis F, Johnston RE (1995) Mechanism of neuroinvasion of Venezuelan equine encephalitis virus in the mouse. Virology 208: 662–671
11. Davis NL, Brown K, Johnston RE (1996) A viral vaccine vector that expresses foreign genes in lymph nodes and protects against mucosal challenge. J Virol 70: 3781–3787
12. Davis NL, Brown KW, Greenwald GF, Zajac AJ, Zacny VL, Smith JF, Johnston RE (1995) Attenuated mutants of Venezuelan equine encephalitis virus containing lethal mutations in the PE2 cleavage signal combined with a second-site suppressor mutation in E1. Virology 212: 102–110
13. Davis NL, Caley IJ, Brown KW, Betts MR, Irlbeck DM, McGrath KM, Connell MJ, Montefiori DC, Frelinger JA, Swanstrom R, Johnson PR, Johnston RE (2000) Vaccination of macaques against pathogenic simian immunodeficiency virus with Venezuelan equine encephalitis virus replicon particles. J Virol 74: 371–378
14. Davis NL, Fuller FJ, Dougherty WG, Olmsted RA, Johnston RE (1986) A single nucleotide change in the E2 glycoprotein gene of Sindbis virus affects penetration rate in cell culture and virulence in neonatal mice. Proc Natl Acad Sci USA 83: 6771–6775
15. Davis NL, Grieder FB, Smith JF, Greenwald GF, Valenski ML, Sellon DC, Charles PC, Johnston RE (1994) A molecular genetic approach to the study of Venezuelan equine encephalitis virus pathogenesis. Arch Virol Suppl 9: 99–109
16. Davis NL, Powell N, Greenwald GF, Willis LV, Johnson BJB, Smith JF, Johnston RE (1991) Attenuating mutations in the E2 glycoprotein gene of Venezuelan equine encephalitis virus: Construction of single and multiple mutants in a full-length cDNA clone. Virology 183: 20–31
17. Davis NL, West A, Reap E, MacDonald G, Collier M, Dryga S, Maughan M, Connell M, Walker C, McGrath K, Cecil C, Ping L-H, Frelinger J, Olmsted R, Keith P, Swanstrom R, Williamson C, Johnson P, Montefiori D, Johnston RE (2002) Alphavirus replicon particles as candidate HIV vaccines. IUBMB Life 53: 209–211
18. Davis NL, Willis LV, Smith JF, Johnston RE (1989) *In vitro* synthesis of infectious Venezuelan equine encephalitis virus RNA from a cDNA clone: Analysis of a viable deletion mutant. Virology 171: 189–204
19. Dong M, Zhang PF, Grieder F, Lee J, Krishnamurthy G, VanCott T, Broder C, Polonis VR, Yu XF, Shao Y, Faix D, Valente P, Quinnan GV Jr (2003) Induction of primary virus-cross-reactive human immunodeficiency virus type 1-neutralizing antibodies in small animals by using an alphavirus-derived *in vivo* expression system. J Virol 77: 3119–3130
20. Eiben GL, Velders MP, Schreiber H, Cassetti MC, Pullen JK, Smith LR, Kast WM (2002) Establishment of an HLA-A*0201 human papillomavirus type 16 tumor model to determine the efficacy of vaccination strategies in HLA-A*0201 transgenic mice. Cancer Res 62: 5792–5799
21. Gipson CL, Davis NL, Johnston RE, deSilva AM (2003) Evaluation of Venezuelan equine encephalitis (VEEV) replicon-based outer surface protein A (OspA) vaccines in a tick challenge mouse model of Lyme disease. Vaccine 21: 3875–3884
22. Grieder FB, Davis NL, Aronson JF, Charles PC, Sellon DC, Suzuki K, Johnston RE (1995) Specific restrictions in the progression of Venezuelan equine encephalitis virus induced disease resulting from single amino acid changes in the glycoproteins. Virology 206: 994–1006
23. Harrington PR, Yount B, Johnston RE, Davis NL, Moe C, Baric RS (2002) Systemic, mucosal and heterotypic immune induction in mice inoculated with Venezuelan

equine encephalitis replicons expressing Norwalk virus-like particles. J Virol 76: 730–742
24. Heidner HW, Johnston RE (1994) The amino-terminal residue of Sindbis virus glycoprotein E2 influences virus maturation, specific infectivity for BHK cells, and virulence in mice. J Virol 68: 8064–8070
25. Heidner HW, Knott TA, Johnston RE (1996) Differential processing of Sindbis virus glycoprotein PE2 in cultured vertebrate and arthropod cells. J Virol 70: 2069–2073
26. Heidner HW, McKnight KL, Davis NL, Johnston RE (1994) Lethality of PE2 incorporation into Sindbis virus can be suppressed by second-site mutations in E3 and E2. J Virol 68: 2683–2692
27. Hevey M, Negley D, Pushko P, Smith J, Schmaljohn A (1998) Marburg virus vaccines based upon alphavirus replicons protect guinea pigs and nonhuman primates. Virology 251: 28–37
28. Hevey M, Negley D, VanderZanden L, Tammariello RF, Geisbert J, Schmaljohn C, Smith JF, Jahrling PB, Schmaljohn AL (2001) Marburg virus vaccines: comparing classical and new approaches. Vaccine 20: 586–593
29. Johnston RE, Smith JF (1988) Selection for accelerated penetration in cell culture co-selects for attenuated mutants of Venezuelan equine encephalitis virus. Virology 162: 437–443
30. Kofler RM, Heinz FX, Mandl CW (2002) Capsid protein C of tick-borne encephalitis virus tolerates large internal deletions and is a favorable target for attenuation of virulence. J Virol 76: 3534–3543
31. Kofler RM, Leitner A, O'Riordain G, Heinz FX, Mandl CW (2003) Spontaneous mutations restore the viability of tick-borne encephalitis virus mutants with large deletions in protein C. J Virol 77: 443–451
32. Lee JS, Dyas BK, Nystrom SS, Lind CM, Smith JF, Ulrich RG (2002) Immune protection against staphylococcal enterotoxin-induced toxic shock by vaccination with a Venezuelan equine encephalitis virus replicon. J Infect Dis 185: 1192–1196
33. Lee JS, Hadjipanayis AG, Welkos SL (2003) Venezuelan equine encephalitis virus-vectored vaccines protect mice against anthrax spore challenge. Infect Immun 71: 1491–1496
34. Lee JS, Pushko P, Parker MD, Dertzbaugh MT, Smith LA, Smith JF (2001) Candidate vaccine against botulinum neurotoxin serotype A derived from a Venezuelan equine encephalitis virus vector system. Infect Immun 69: 5709–5715
35. MacDonald GH, Johnston RE (2000) The role of dendritic cell targeting in Venezuelan equine encephalitis virus pathogenesis. J Virol 74: 914–922
36. Olmsted RA, Baric RS, Sawyer BA, Johnston RE (1984) Sindbis virus mutants selected for rapid growth in cell culture display attenuated virulence in animals. Science 225: 424–427
37. Paredes AM, Heidner H, Thuman-Commike P, Prasad BVV, Johnston RE, Chiu W (1998) Structural localization of the E3 glycoprotein in attenuated Sindbis virus mutants. J Virol 72: 1534–1541
38. Pratt WD, Davis NL, Johnston RE, Smith JF (2003) Genetically engineered live attenuated vaccines for Venezuelan equine encephalitis: testing in animal models. Vaccine 21: 3854–3862
39. Pushko P, Bray M, Ludwig GV, Parker M, Schmaljohn A, Sanchez A, Jahrling PB, Smith JF (2000) Recombinant RNA replicons derived from attenuated Venezuelan equine encephalitis virus protect guinea pigs and mice from Ebola hemorrhagic fever virus. Vaccine 19: 142–153

40. Pushko P, Parker M, Ludwig GV, Davis NL, Johnston RE, Smith JF (1997) Replicon-helper systems from attenuated Venezuelan equine encephalitis virus: expression of heterologous genes *in vitro* and immunization against heterologous pathogens *in vivo*. Virology 239: 389–401
41. Russell DL, Dalrymple JM, Johnston RE (1989) Sindbis virus mutations which coordinately affect glycoprotein processing, penetration and virulence in mice. J Virol 63: 1619–1629
42. Schultz-Cherry S, Dybing JK, Davis NL, Williamson C, Suarez D, Johnston R, Perdue ML (2000) Influenza virus (A/HK/156/97) hemagglutinin expressed by an alphavirus replicon system protects chickens against lethal infection with Hong Kong-origin H5N1 viruses. Virology 278: 55–59
43. Thomas CE, Van Dam CN, Zhu W, Davis NL, Johnston RE, Sparling PF (2004) Vaccination of mice with gonococcal antigens expressed from Venezuelan equine encephalitis viral replicon particles. Inf Immun (submitted)
44. Velders MP, McElhiney S, Cassetti MC, Eiben GL, Higgins T, Kovacs GR, Elmishad AG, Kast WM, Smith LR (2001) Eradication of established tumors by vaccination with Venezuelan equine encephalitis virus replicon particles delivering human papillomavirus 16 E7 RNA. Cancer Res 61: 7861–7867
45. Williamson C, Johnston RE, Williamson AL (2002) HIV-1 vaccine candidates for South Africa. South African J HIV Med (March): 10–14

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