A Viral Vaccine Vector That Expresses Foreign Genes in Lymph Nodes and Protects against Mucosal Challenge

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A candidate live-virus vaccine strain of Venezuelan equine encephalitis virus (VEE) was configured as a replication-competent vector for in vivo expression of heterologous immunogens. Three features of VEE recommend it for use as a vaccine vector. (i) Most human and animal populations are not already immune to VEE, so preexisting immunity to the vector would not limit expression of the heterologous antigen. (ii) VEE replicates first in local lymphoid tissue, a site favoring the induction of an effective immune response. (iii) Parenteral immunization of rodents and humans with live, attenuated VEE vaccines protects against mucosal challenge, suggesting that VEE vaccine vectors might be used successfully to protect against mucosal pathogens. Upon subcutaneous (s.c.) inoculation into the footpad of mice, a VEE vector containing the complete influenza virus hemagglutinin (HA) gene expressed HA in the draining lymph node and induced anti-HA immunoglobulin G (IgG) and IgA serum antibodies, the levels of which could be increased by s.c. booster inoculation. When immunized mice were challenged intranasally with a virulent strain of influenza virus, replication of challenge virus in their lungs was restricted, and they were completely protected from signs of disease. Significant reduction of influenza virus replication in the nasal epithelia of HA vector-immunized mice suggested an effective immunity at the mucosal surface. VEE vaccine vectors represent an alternative vaccination strategy when killed or subunit vaccines are ineffective or when the use of a live attenuated vaccine might be unsafe.

Vaccination has proved to be the most effective means of controlling viral disease, especially when live attenuated vaccines, such as those for smallpox, poliomyelitis, and measles, have been employed. In these instances, virus mutants replicate within the vaccinated host without causing disease, while inducing protective and long-lasting immunity to the naturally occurring pathogenic virus. This approach has been extended by using vaccine virus genomes as vectors to express the genes of other pathogens in vivo (2, 12, 20, 25). In this report, we describe the construction of an expression system based on a candidate Venezuelan equine encephalitis virus (VEE) vaccine strain and its ability to express a heterologous gene both in vitro and in vivo. The influenza virus hemagglutinin (HA) gene was used in this study because it afforded a stringent test of mucosal protection in the mouse. Mice vaccinated with the VEE HA vector not only were protected against clinical disease following intranasal influenza virus challenge, but also exhibited resistance to influenza virus replication in the lung and at mucosal surfaces of the upper respiratory tract.

VEE is a member of the *Alphavirus* genus, a group which includes Sindbis virus and Semliki Forest virus as prototypes (35). Several properties of alphaviruses have made them useful tools for high-level expression of foreign genes both in cell culture and in animals (21, 23, 24, 28, 29, 37, 39). These viruses can infect a wide range of vertebrate and invertebrate cells to initiate a prolific, cytoplasmic replication cycle. Their single-stranded positive-sense RNA genomes (between 11,000 and 12,000 nucleotides) are infectious, as are genome replicas derived from full-length cDNA clones (35). Self-replicating RNA genomes serve as mRNA for an enzyme complex with both RNA replicase and transcriptase activities, and transcription of

a subgenomic mRNA for the structural proteins is driven by a very strong, highly conserved promoter sequence. Taking advantage of this high-level promoter, alphavirus expression vectors either contain a foreign gene in place of the viral structural protein genes (37) or contain a duplicate subgenomic mRNA promoter that drives expression of a foreign gene (13).

Three biological features of VEE suggest that it could be an unusually effective in vivo expression vector for vaccination with heterologous gene products. First, parenteral immunization of rodents and humans with live, attenuated VEE vaccines results in protection not only against parenteral challenge but also against intranasal (i.n.) and aerosol challenges (5, 7, 16, 17). This predicts that a delivery system based on a VEE vaccine could induce protection from invasion at mucosal surfaces, which are important routes of entry for many pathogens, including human immunodeficiency virus. Second, in contrast to most other alphaviruses, VEE replicates first in the lymph nodes draining the site of inoculation (10, 11, 15), where high-level synthesis of a heterologous antigen might result in efficient immunization. Finally, and in contrast to viral vectors based on vaccinia virus or vaccine strains of adenovirus and poliovirus, most human and animal populations are not already immune to VEE. Therefore, in vivo expression of a heterologous immunogen from a VEE-based vaccine vector would not be limited by prior immunity to the vector itself.

MATERIALS AND METHODS

Clones and viruses. Plasmid pV3014, a derivative of the full-length cDNA clone of the virulent Trinidad donkey strain of VEE with two attenuating mutations (E2 [Lys-209] and E1 [Thr-272]; described in references 8 and 11), was altered to allow expression of heterologous genes. A duplicate 26S RNA promoter followed by a unique *Clal* site was inserted immediately downstream of the E1 gene by oligonucleotide-directed mutagenesis of a subclone of the structural protein genes in M13 (18). The promoter sequence and its ability to function in an abnormal context were predicted from previous work with Sindbis virus (13, 19). The VEE structural gene region with the auxiliary 26S subgenomic

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RNA promoter was subcloned into pUC118. The multiple cloning region of the *Clal*2 adaptor plasmid (14) (the kind gift of S. Hughes, National Cancer Institute), flanked by *Clal* sites, was inserted into the unique *Clal* site to form a shuttle vector. The cloned cDNA of the complete HA gene from influenza virus strain PR/8/34, type H1N1, kindly supplied by P. Palese, Mt. Sinai School of Medicine, New York, was inserted into the multiple cloning region of the shuttle vector. The HA gene was then transferred by using *Clal* into the full-length VEE vector clone. Infectious RNA genome equivalents were transcribed *in vitro* from linearized plasmids with T7 RNA polymerase and used in electroporation of baby hamster kidney (BHK) cells to produce stocks of progeny virus (9, 22).

An egg-grown stock of influenza virus strain PR/8/34, generously supplied by H. van Campen, University of Wyoming, Laramie, was used for *i.n.* challenge of mice. A similar virus preparation grown from ATCC seed stock VR-95 in embryonated eggs was used for neutralization assays and BHK cell infections.

Immunization and challenge of mice. Adult (4- or 6-week old), female CD-1 mice (Charles River) were put under light anesthesia by inhalation of Metofane (Pitman-Moore) and immunized by subcutaneous (s.c.) inoculation of 10^4 PFU of vector without insert or HA vector in a 10- μ l volume of diluent (phosphate-buffered saline [PBS] containing 1% donor calf serum) into each rear footpad. Mock-immunized controls received diluent alone. Booster inoculations were done 3 weeks postimmunization and were identical to the initial immunization except that the total dose of HA vector or vector alone was 2×10^5 PFU. Twenty-one days following the primary immunization or 18 days following the boost, a challenge dose of 10^5 50% egg infectious doses of egg-grown influenza virus strain PR/8/34 in a total volume of 20 μ l of PBS was introduced into the nares under light Metofane anesthesia. Under the conditions used here, this dose represented one 50% lethal dose but induced obvious clinical signs in 100% of inoculated naive animals.

Antibody assays. Serum samples were obtained by limited bleeding from tail veins. Enzyme-linked immunosorbent assays (ELISAs) included gradient-purified PR/8/34 influenza virus as the antigen and horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG) or HRP-conjugated goat anti-mouse IgA as the second antibody. Positive controls were the H1-specific IgG2a monoclonal antibody 37-85, produced by W. Gerhard, Wistar Institute (34), and obtained through S. Clarke, University of North Carolina (UNC), Chapel Hill, and an H1-specific IgA monoclonal antibody kindly supplied by P. Small, University of Florida, Gainesville. In these ELISAs, an optical density at 450 nm (OD_{450}) of more than 0.2 above the background (wells without serum) was scored as positive. Preimmunization mouse sera and sera from control mice inoculated with PBS or vector alone showed variable reactivity at a 1:50 dilution in the influenza virus ELISA, possibly caused by nonspecific binding of sialylated serum immunoglobulins to the influenza virus HA. Therefore, positive reactivity at dilutions of less than 1:100 were considered background. Plaque reduction neutralization assays were carried out essentially as described by Olmsted et al. (27). Virus was incubated for 30 min at 37°C with dilutions of serum in the presence of 5% guinea pig serum (Cedarlane) as a source of complement, followed by a second 30-min incubation at 37°C with a 1:200 dilution of goat antiserum to mouse immunoglobulin (Cappel). Standard plaque assay of serum-treated virus was done on Madin-Darby canine kidney (MDCK) cells overlaid with agarose without serum but containing trypsin (1 μ g/ml). Titers represent the highest dilution that reduced plaque number by 60%.

In situ hybridization analysis. Tissues were prepared for *in situ* hybridization as described before (6, 11). Popliteal lymph nodes were taken 24 h after s.c. inoculation with VEE vector, while heads were processed 48 h after *i.n.* challenge with influenza virus, following perfusion with 4% paraformaldehyde in PBS. Similarly sized, [35 S]UTP-labeled, negative-sense riboprobes were prepared from transcription vectors containing either the identical HA gene used in the construction of the VEE HA vector, a subclone of the VEE structural genes, or a subclone of the Epstein-Barr virus genome, obtained from N. Raab-Traub, UNC, Chapel Hill. The lengths of the respiratory epithelial surface in coronal sections incubated with the HA-specific riboprobe, both total length and that associated with positive *in situ* signal, were measured with a Nikon FXA microscope and a Macintosh Quadra 840 AV computer with the public-domain National Institutes of Health (NIH) Image program (developed at the NIH and available from the Internet by anonymous file transfer protocol [zipper.nimh.nih.gov] or on floppy disk from National Technical Information Service, Springfield, Va. [PB95-500195GEI]).

RESULTS

Construction of an attenuated VEE expression vector containing the influenza virus HA gene. A full-length cDNA clone of the VEE RNA genome was the basis for construction of a panel of live, attenuated VEE vaccine candidates (8). A representative vaccine candidate, V3014, contains two attenuating mutations (E2 [Lys-209] and E1 [Thr-272]), is avirulent following s.c. inoculation of adult mice, and grows to high titers in the draining lymph node without extensive pathology (8, 11). V3014 was converted to a replication-competent VEE vaccine

vector by placing a second copy of the 26S subgenomic RNA promoter and a unique *Clal* site at the exact 3' end of the structural gene region. To test this vector for its ability to express a heterologous gene, the cloned influenza virus HA gene from strain PR/8/34 was introduced at the unique *Clal* site downstream of the second subgenomic promoter. The influenza virus HA protein is well characterized, as is the disease course following *i.n.* inoculation of mice with strain PR/8/34 (38). Thus, this system provides (i) a mouse model for direct assessment of the ability of the VEE vaccine vector to induce an immune response not only to VEE proteins but also to a heterologous protein and (ii) a challenge model in which to test the capacity of that response to protect against influenza virus challenge across a mucosal surface.

VEE vector virus without an insert, HA vector virus, and AH vector virus (containing the HA gene in the noncoding orientation) were produced by transfection of BHK cells with *in vitro*-synthesized RNA transcripts of the appropriate cDNA clones. Immunoprecipitation and immunocytochemistry were used to show that authentic HA protein was synthesized in BHK cells infected by the HA vector. HA vector-infected cells but not cells infected with AH vector or vector alone contained a protein that was precipitated by anti-influenza virus rabbit polyclonal antibody (obtained from R. Webster and B. Meyer, St. Jude Medical Center, Memphis, Tenn.) and that migrated on sodium dodecyl sulfate (SDS)-containing polyacrylamide gels similarly to HA protein precipitated from BHK cells infected with influenza virus (data not shown). Although high levels of HA were produced in HA vector-infected cells, VEE virions produced by these cells contained no detectable HA protein (data not shown). Immunocytochemical staining with an HA-specific monoclonal antibody (34) demonstrated that the amount and intracellular location of HA were similar for HA vector-infected BHK cells and influenza virus-infected cells (data not shown). Cells infected with VEE vector alone did not bind the HA-specific monoclonal antibody. About one-fourth of the HA vector-infected cells (estimated from the proportion of cells reacting with anti-VEE antibody) did not react with the anti-HA monoclonal antibody. This may reflect a lower detection limit for the polyclonal anti-VEE antibody than for the HA monoclonal antibody or indicate that the HA gene was not expressed in all HA vector-infected cells. The possibility that the large HA gene (1.7 kb) was deleted from the VEE vector during vector replication in some cells is suggested by results with some large inserts in the analogous Sindbis virus vector system (13).

Expression of HA in draining lymph nodes of HA vector-inoculated mice. The major site of replication of the parent attenuated virus, V3014, in CD-1 mice inoculated in the rear footpad is the draining popliteal lymph node (11). V3014 does not cause significant tissue destruction in the lymph node, although it replicates to titers equivalent to those of its virulent progenitor within 24 h postinfection. To determine whether the VEE double promoter vector replicated and expressed a heterologous gene at this site, popliteal lymph nodes taken from mice 24 h after a primary inoculation in the rear footpads with 2×10^4 PFU of either vector alone or the HA vector were analyzed for the presence of virus-specific RNA by *in situ* hybridization. The lymph node from the HA vector-inoculated mouse bound both the VEE- and HA-specific negative-sense riboprobes, whereas the tissue from animals inoculated with vector alone bound only the VEE riboprobe (Fig. 1). No signal was detected in any of the negative controls, which included similar sections from these lymph nodes incubated with an irrelevant, similarly sized riboprobe containing Epstein-Barr virus sequences and lymph node sections from mock-inocu-

FIG. 1. Detection of coding-sense HA RNA sequences in the popliteal lymph nodes of mice inoculated with the HA vector. Popliteal lymph nodes from mice inoculated with vector alone (A and C) or HA vector (B and D) were harvested at 24 h postinoculation and processed for in situ hybridization. Similar sections were probed with a ^{35}S -labeled HA-specific riboprobe (A and B) or a ^{35}S -labeled VEE-specific riboprobe (C and D). Magnification, $\times 183$. Bars, 100 μm .

lated mice incubated with all of the riboprobes. In situ hybridization analysis of spleen and decalcified legs taken at 24 h postinoculation with the HA vector also showed specific hybridization with both the HA and VEE riboprobes, while parallel preparations from mice inoculated with vector alone reacted only with the VEE riboprobe (not shown). Therefore, as predicted from the phenotype of the attenuated VEE parent, the VEE HA vector replicated at the site of inoculation, in the draining lymph node, and to a lesser degree in the spleen and expressed the inserted HA gene at all of these sites.

Anti-HA antibody levels in serum of VEE vector-immunized mice. A correlation between the presence of anti-influenza virus antibody in serum and protection of mice against influenza virus-induced pneumonitis and death has been shown previously (for an example, see reference 31). The delivery of HA to the lymphoid tissues of the HA vector-immunized mice was effective in eliciting a measurable humoral immune response to HA. Groups of 12 6-week-old mice were given a primary immunization by s.c. inoculation of 2×10^4 PFU of vector alone or HA vector or an equal volume of diluent into the rear footpads and 3 weeks later were given s.c. booster inoculations. The boosters contained, for both vector- and HA vector-immunized mice, 10 times the original dose of virus, or 2×10^5 PFU. Sera collected pre-immunization, 3 weeks following the primary immunization, and 12 days after the booster immunization were tested by ELISA for anti-HA serum IgG and IgA. After a single inoculation, 75% of the HA vector-inoculated mice had positive anti-influenza virus serum IgG titers, and 25% had positive anti-influenza virus serum

IgA titers (Table 1). The booster inoculation increased these titers and gave a 100% positive response for IgG and a 75% positive response for IgA. Inoculation with vector alone or HA vector induced a strong primary anti-VEE humoral antibody response (Table 1). Although the vigorous anti-VEE immune response probably limited the replication of the booster inoculum, sufficient HA protein was expressed from the second inoculum of HA vector to increase the anti-influenza virus titers substantially (Table 1).

Serum samples taken 12 days after the booster immunization were also tested for neutralizing activity against the PR/

TABLE 1. Serum antibody titers in HA vector-immunized mice^a

Serum sample	Geometric mean titer			
	Anti-HA		Anti-VEE ^b	
	IgG	IgA	IgG	IgA
Post-primary inoculation	270	126	16,000	6,600
Postboost	562	467	25,400	8,980

^a Geometric mean titers for samples giving an OD_{450} of ≥ 0.2 at dilutions of 1:100 or greater. Preimmunization titers were consistently < 100 . Of 24 animals in the control groups, 22 had anti-HA serum IgG and IgA titers of < 100 , one had an IgG titer of 100 before but not after the boost, and one had an IgA titer of 100 following the boost.

^b Mean anti-VEE titers for mice immunized with the HA vector (shown here) or vector alone (not shown) were not significantly different from each other (Student's *t* test; *P* values ranged from 0.11 to 0.94).

8/34 strain of influenza virus in a plaque reduction assay. Sera from mice in the two control groups (those inoculated with diluent or with vector alone) did not neutralize influenza virus at a dilution of 1:20, the lowest tested. However, all sera from the HA vector-immunized mice showed neutralizing activity. Ten of the 12 sera from this group gave a geometric mean 60% plaque reduction titer of 1,175, while samples from 2 of the 12 mice showed 30 and 56% plaque reduction at the lowest dilution. As a benchmark comparison, convalescent-phase sera (post-challenge sera from four surviving mock-immunized mice) gave a geometric mean 60% plaque reduction titer of 460.

Protection of HA vector-immunized mice against intranasal challenge. (i) **Protection from clinical disease.** Three weeks after receiving a single s.c. dose of diluent, 2×10^4 PFU of vector alone, or 2×10^4 PFU of HA vector, mice were challenged i.n. with influenza virus strain PR/8/34, a strain that induces a lethal pneumonitis in mice infected by this route (38). Results recorded over a 2-week observation period showed that inoculation with the HA vector conferred significant protection against mortality ($P < 0.01$) and morbidity ($P < 10^{-4}$). Mice in the two control groups suffered 54% mortality overall, and all of the survivors showed moderate to severe signs of disease for an average of 5.1 ± 1.9 days. In contrast, 7 of 12 immunized animals showed no signs of infection, and 3 other animals showed very mild signs for an average of 2.3 ± 1.2 days. One HA vector-immunized mouse died, and one showed severe signs for 1 day prior to recovery. These two individuals had undetectable anti-influenza virus serum IgG prior to challenge.

In a separate experiment, three groups of 12 mice received both a primary and booster immunization of either vector alone or HA vector or were mock immunized with diluent (antibody titers in Table 1). At 18 days following the boost, these mice were challenged i.n. with influenza virus strain PR/8/34. Four mice in each group were sacrificed at 48 h postchallenge for in situ hybridization analysis of nasal tissue (see below). For the remaining mice, results recorded over a 22-day observation period showed that immunization with the HA vector conferred complete protection against mortality and morbidity (Fig. 2). Mice in the two control groups suffered 44% mortality overall, and all of the survivors showed clinical signs of disease for an average of 4.3 ± 1.7 days. In contrast, none of the HA vector-immunized animals died or showed any clinical signs of disease.

Protection of HA vector-immunized mice against illness was also indicated by individual daily weight measurements. Mice receiving two inoculations of diluent or vector alone showed an average maximum weight loss (percent of initial weight) of $23.4 \pm 5.6\%$ and $26.0 \pm 11.2\%$, respectively, while mice immunized twice with HA vector lost only $8.4 \pm 5.0\%$. By 9 days postchallenge, HA vector-immunized mice on average had recovered their initial weight, while surviving mice that had received diluent or vector alone still weighed an average of 19.5 ± 6.2 and $21.7 \pm 6.2\%$ less than their initial weights, respectively.

As would be predicted from previous results with V3014 and other attenuated VEE strains (7, 8, 16, 17), mice inoculated with a single s.c. dose of 2×10^4 PFU of vector alone or HA vector were completely protected against VEE-induced disease following either intraperitoneal or i.n. lethal challenge with 10^4 PFU of virulent VEE (data not shown).

(ii) **Restriction of challenge virus replication in vivo.** Parenteral immunization with the HA vector also reduced the level of influenza virus replication in the lung (Fig. 3). Mice received a single s.c. inoculation of vector alone, HA vector, or diluent and were challenged i.n. with virulent influenza virus 3

FIG. 2. Protection resulting from primary and booster immunizations with VEE HA vector against i.n. challenge with virulent influenza virus. Mice received primary and booster inoculations of diluent (A), vector alone (B), or HA vector (C), were challenged i.n. with influenza virus PR/8/34 at 18 days following the boost, and were observed for 22 days. Open bars, clinically normal mice; solid bars, dead mice. Mildly affected mice showed slight ruffling (open hatching). Moderately ill mice showed ruffling, hunching, and slowed movement (dense hatching). Severely ill mice showed lack of movement, obvious wasting, and labored breathing (crosshatching).

FIG. 3. Restriction of influenza virus replication in lungs of HA vector-immunized mice. Mice were inoculated s.c. with diluent, vector alone, or HA vector and 26 days later were challenged i.n. with influenza virus PR/8/34. Mice were killed on day 4 postchallenge, and lungs were homogenized in PBS with 0.1% (wt/vol) bovine serum albumin to give a 20% (wt/vol) suspension, clarified by centrifugation, and stored at -70°C . A standard plaque assay on MDCK cells was performed with duplicate aliquots. The limit of detection was approximately 25 PFU per average lung. Geometric mean titers (solid circles) were calculated for control mice with measurable virus titers.

weeks later. At 4 days postchallenge, no influenza virus was detected in the lungs of 12 HA vector-immunized mice. Eight of 12 diluent controls and 5 of 12 vector-alone controls were positive for influenza virus replication in the lungs, with geometric mean titers of 3.0×10^6 and 1.9×10^6 PFU/g, respectively.

Replication of the challenge virus in the nasal epithelium was measured directly by in situ hybridization with an HA-specific riboprobe. Mice were immunized with primary and booster doses of vector alone or HA vector, or mock immunized, and challenged i.n. with virulent influenza virus as described above. Forty-eight hours after challenge, the animals were killed, and the heads were prepared and sectioned for in situ hybridization (6). Four mice were selected from each group. With the serum neutralizing antibody level used as a general indicator of the overall HA-specific response, HA vector-immunized mice with different intensities of response were chosen. One in situ sample was from the HA vector-immunized mouse with the lowest level of serum neutralizing antibody and minimal reactivity in ELISA, while the other three mice had 60% plaque reduction neutralizing titers of 500, 1,000, and 5,000. Intense confluent stretches of positive in situ signal marked the epithelial surface of the upper respiratory tract in sections from mice inoculated with vector alone or diluent (Fig. 4A), indicating vigorous replication and cell-to-cell spread of the challenge virus. In contrast, sections from HA vector-immunized mice showed fewer and smaller foci of positive in situ signal (Fig. 4B), indicating restriction of replication and limitation of spread of the challenge virus.

A morphometric analysis of the coronal sections used for in situ hybridization was performed to compare the percentage of the upper respiratory epithelial surface that bound the HA riboprobe among animals in the three groups. The mean percentage for the combined control groups was $16.21 \pm 5.43\%$. This value and the percentages for individual HA vector-immunized mice were compared with respect to their corresponding serum neutralizing antibody titers (Fig. 4C). The upper respiratory tract epithelium from the HA vector-immunized mouse with the lowest neutralizing antibody titer (<20) was among the least resistant to replication of the challenge

FIG. 4. Detection of HA mRNA sequences in the nasal epithelia of mice challenged i.n. with virulent influenza virus. Mice were immunized and challenged as described in the legend to Fig. 2. At 48 h postchallenge, heads were processed for in situ hybridization. (A and B) Coronal sections from mice inoculated with diluent (A) or HA vector (B) were incubated with a ^{35}S -labeled, negative-sense HA-specific riboprobe. The percent respiratory surface involved for the HA vector-immunized mouse (B) was 4.7%, similar to the average of 3.65% for mice with a strong anti-HA immune response. No signal was detected in similar sections incubated with a VEE virus-specific riboprobe. Magnification, $\times 123$. Bar, 100 μm . (C) Percent respiratory surface involved (the length of respiratory surface in each section showing positive in situ signal divided by the total length $\times 100$) was calculated for individual HA vector-immunized mice (solid bars), averaged for controls (open bar), and plotted relative to titers of anti-influenza virus neutralizing antibody.

influenza virus, while sections from the three mice with strong anti-HA immune responses showed a significant reduction in the extent of challenge virus replication (mean percentage, $3.65 \pm 3.12\%$). Therefore, successful immunization with the HA vector, indicated by high levels of anti-HA serum neutralizing antibody, conferred significant protection against replication of virulent influenza virus in the target cells of the upper respiratory mucosa. In a separate experiment with mice immunized only once with vector alone or HA vector, the combined percentage of upper respiratory epithelial involvement was reduced by greater than 50% in the HA vector-immunized mice (data not shown). These results show that the protective effect of a parenteral immunization with the HA vector extended to a vulnerable mucosal surface. The immunological mediators of that protection remain to be determined but are presumed to include HA-specific antibody at the mucosal surface.

DISCUSSION

The properties of VEE vaccine strains in humans and in rodent models predicted two characteristics of a VEE vaccine vector expressing a heterologous gene. The first prediction was that a heterologous gene would be expressed in the lymph node draining the site of inoculation, based on the finding that the parental VEE strain and its attenuated mutant, V3014, replicate extensively at this site. In work presented here, we showed that the HA vector, derived from V3014, also replicated and expressed the HA gene in lymph nodes, a site expected to favor the efficient induction of an immune response. In fact, the average level of anti-influenza virus serum neutralizing antibody induced by the VEE HA vector was in excess of that found among control animals that survived influenza virus-induced disease.

The second prediction was that protection against challenge at a mucosal surface would be evident even when the VEE vaccine was administered parenterally. Live attenuated VEE vaccines can induce protection against i.n. or aerosol challenge regardless of the route of administration. Protection against disease caused by virus invasion across a mucosal surface may occur either at the level of the mucosal surface itself, by preventing the initial infection of epithelial target cells, or at a later step, by preventing spread of the virus and the resulting clinical signs. In work not presented here, s.c. immunization of mice with V3014 induced a vigorous humoral antibody response, substantial anti-VEE IgA on the vaginal surface, detectable anti-VEE IgA in nasal secretions, and complete protection of the nasal neuroepithelium from infection following i.n. challenge with virulent VEE virus (5). This represents a case in which specific antibodies are detected both in the serum and at the mucosa and strong protective immunity is observed at the level of the mucosal surface. Likewise, we have now demonstrated that immunization with the HA vector derived from V3014 provided significant protection at the level of the nasal mucosa upon i.n. influenza virus challenge. When effective protection is observed at the level of the mucosal surface, it may involve one or more distinct elements, including secretory IgA antibody, transudated humoral IgA and/or IgG antibodies, or a specific cellular immune response (reviewed in reference 26). Although the exact contribution of each element of the immune response to this protection remains to be determined, we have detected low levels of HA-specific IgA in vaginal wash samples from mice immunized by s.c. inoculation with the HA vector (unpublished results).

As might be expected for a virus with a highly structured icosahedral capsid (35), there is a limit to the amount of het-

erologous genetic material that can be stably accommodated in the alphavirus genome. Our results suggest that a proportion of HA vector viruses lose the property of HA expression during successive rounds of replication in cultured cells. However, other inserted genes, not described here, are more stably expressed from this vector (4). It appears that multiple factors, including but not limited to the length of the heterologous sequence, determine stability of expression. The HA protein may be unusually detrimental to VEE replication, which increases the selective pressure for its deletion. Therefore, results with the HA gene may represent a minimal estimate of the immunizing efficacy of VEE vaccine vectors.

The influenza virus challenge model in mice was used here as a feasibility test for protective immunization with replication-competent VEE vaccine vectors. There is good potential for human use of an attenuated VEE vaccine configured as a vaccine vector, based on long experience with an investigational live attenuated VEE vaccine (TC-83), the only such alphavirus vaccine tested extensively in humans (3). More recent recombinant live attenuated VEE vaccine candidates are superior to TC-83 in both safety and efficacy in rodents (8), horses (1), and primates (30). Unintentional spread of an engineered VEE strain by the mosquito vector, an important safety issue, is made extremely unlikely in that V3014, the attenuated parent of the vector described here, does not produce a serum viremia in rodents (11), and even the virulent Trinidad donkey strain of VEE produces a viremia in humans that is too low to support transmission by mosquitoes (33). Other attenuating mutations under consideration appear to reduce specifically the efficiency of transmission by the mosquito vector (36).

Engineering live virus vaccines for immunization against heterologous antigens is an active area of experimentation. To this end, expression of foreign genes has been accomplished with members of several virus families, e.g., poxviruses, adenoviruses, picornaviruses, herpesviruses, and orthomyxoviruses, as well as alphaviruses (reviewed in reference 32). In fact, it is very unlikely that one vector system will be optimal in all respects, such as the length of heterologous sequence that can be maintained stably in the vector, level of efficacy, induction of mucosal protection, potential problems associated with pre-existing immunity to the vector, and issues of safety, such as the potential for latent infection. Moreover, the generation of a strong immune response to the vector itself will allow only a limited number of applications of a specific vaccine vector in a given population. Therefore, efforts to produce an ideal vector for all applications are likely to be unproductive. Rather, a strong case can be made for parallel development of multiple virus vaccine vectors, including VEE, which will make available the unique advantages of each for specific applications.

In summary, we have described the generation of a replication-competent VEE expression vector capable of driving the efficient synthesis of a heterologous protein in cell culture and in vivo. This vector replicated in lymphoid tissue draining the s.c. inoculation site, where it expressed an inserted influenza virus HA gene. The resulting immune response was characterized by high levels of anti-HA serum antibody. Following i.n. challenge with virulent influenza virus, we observed complete protection against clinical signs of disease, restriction of challenge virus replication in the lungs, and significant protection of the nasal mucosal surfaces. These findings suggest that a new generation of rationally engineered, molecularly cloned live VEE vaccines may serve as effective and safe vaccine vectors, protecting against systemic and mucosal pathogens for which other vaccine strategies are not effective or feasible.

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