VIROLOGY 239, 389–401 (1997)
ARTICLE NO. VY978878

Replicon-Helper Systems from Attenuated Venezuelan Equine Encephalitis Virus: Expression of Heterologous Genes *in Vitro* and Immunization against Heterologous Pathogens *in Vivo*

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Received July 26, 1996; returned to author for revision June 5, 1997; accepted October 1, 1997

A replicon vaccine vector system was developed from an attenuated strain of Venezuelan equine encephalitis virus (VEE). The replicon RNA consists of the *cis*-acting 5′ and 3′ ends of the VEE genome, the complete nonstructural protein gene region, and the subgenomic 26S promoter. The genes encoding the VEE structural proteins were replaced with the influenza virus hemagglutinin (HA) or the Lassa virus nucleocapsid (N) gene, and upon transfection into eukaryotic cells by electroporation, these replicon RNAs directed the efficient, high-level synthesis of the HA or N proteins. For packaging of replicon RNAs into VEE replicon particles (VRP), the VEE capsid and glycoproteins were supplied *in trans* by expression from helper RNA(s) coelectroporated with the replicon. A number of different helper constructs, expressing the VEE structural proteins from a single or two separate helper RNAs, were derived from attenuated VEE strains. Regeneration of infectious virus was not detected when replicons were packaged using a bipartite helper system encoding the VEE capsid protein and glycoproteins on two separate RNAs. Subcutaneous immunization of BALB/c mice with VRP expressing the influenza HA or Lassa virus N gene (HA-VRP or N-VRP, respectively) induced antibody responses to the expressed protein. After two inoculations of HA-VRP, complete protection against intranasal challenge with influenza was observed. Furthermore, sequential immunization of mice with two inoculations of N-VRP prior to two inoculations of HA-VRP induced an immune response to both HA and N equivalent to immunization with either VRP construct alone. Protection against influenza challenge was unaffected by previous N-VRP immunization. Therefore, the VEE replicon system was characterized by high-level expression of heterologous genes in cultured cells, little or no regeneration of plaque-forming virus particles, the capability for sequential immunization to multiple pathogens in the same host, and induction of protective immunity against a mucosal pathogen.

INTRODUCTION

The alphaviruses, a genus in the family *Togaviridae,* possess a single-stranded, positive-sense RNA genome enclosed in a relatively simple virion structure (reviewed in Strauss and Strauss, 1994). The icosahedral nucleocapsid consists of 240 copies of a single capsid protein and is surrounded by a lipoprotein envelope containing two viral glycoproteins, E1 and E2. The capsid, E1, and E2 genes are expressed from a subgenomic 26S mRNA produced in infected cells. The 26S mRNA promoter drives transcription of the subgenomic RNA such that it is present at up to 10 times the amount of genomic (or 42S) RNA on a molar basis. Capsid monomers and the genomic RNA assemble into nucleocapsids in the cytoplasm. Glycoproteins E1 and PE2, the precursor to E2, are inserted into the endoplasmic reticulum and processed through the Golgi where a cellular protease, furin, cleaves PE2 into E2 and E3, the amino-terminal portion of PE2.

Vector systems for the expression of heterologous genes have been developed from full-length cDNA clones of three members of the alphavirus genus, Sindbis virus, Semliki Forest virus (SFV), and Venezuelan equine encephalitis virus (VEE) (Xiong *et al.,* 1989; Huang *et al.,* 1989; Liljeström and Garoff, 1991; Bredenbeek *et al.,* 1993; Zhou *et al.,* 1994; Davis *et al.,* 1996; Dryga *et al.,* 1996; reviewed in Frolov *et al.,* 1996). The systems are of two general types: "double promoter" vectors and "replicon" vectors. In the double promoter vectors, a second copy of the 26S promoter is inserted into the genome either immediately upstream of the authentic promoter or between the E1 gene and the beginning of the 3′ untranslated region. A heterologous gene is then inserted into the genome just downstream of the second 26S promoter such that a second subgenomic mRNA containing the heterologous gene is transcribed. These vectors replicate in infected cells and assemble into infectious particles which can spread to other cells and again express the heterologous gene. When such vectors are based on vaccine strains of alphaviruses, they can be utilized *in vivo* for immunization against both the alphavirus vector and the pathogen from which the heterologous gene was derived (Hahn *et al.,* 1992; Davis *et al.,* 1996; Caley *et al.,* 1997).

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0042-6822/97 $25.00

In alphavirus replicon vectors, the viral structural protein genes are deleted and replaced by a heterologous gene under the control of the 26S promoter. The structural genes are provided *in trans* from a helper construct(s) consisting of a partial clone of the viral genome which is missing all or part of the genes for the nonstructural proteins, including the putative *cis*-acting RNA packaging signals (Weiss *et al.,* 1989, 1994). Both the replicon and the helper RNAs retain the *cis*-acting terminal sequences required for genome replication and the promoter for transcription of the subgenomic mRNA. The replicon RNA supplies the nonstructural proteins *in trans* for the expression of the helper RNA. However, only the replicon RNA retains the packaging signal(s), and it is packaged into replicon particles by the viral structural proteins provided *in trans* by the helper. Infection of cells by these replicon particles results in amplification of replicon RNA and expression of the heterologous gene, but there is no further spread to other cells.

Alphavirus replicon vectors have been utilized for vaccination against microbial pathogens (Zhou *et al.,* 1995; Mossman *et al.,* 1996) and offer several potential advantages as vaccine delivery systems. Replicons typically express heterologous genes to high levels, suggesting that a relatively low dose of replicon particles would produce a large dose of immunogen *in vivo.* They do not contain a complete complement of viral genes, so that after replication and expression of the heterologous gene in the cells initially infected, no additional infectious particles would be produced to spread to other tissues. This property contributes significantly to the inherent safety of alphavirus replicon vectors. In addition, the self-limiting nature of the replicon particle infection and the lack of structural protein expression should minimize the induction of an immune response to the vector, allowing the sequential use of these vectors for immunization of the same individual with immunogens of different pathogens. In practice however, infectious virus is frequently regenerated through copackaging of helper and replicon RNAs (Geigenmüller-Gnirke *et al.,* 1991) or recombination between these molecules (Weiss and Schlesinger, 1991; Raju *et al.,* 1995). The presence of copackaged and/or recombinant virus in replicon preparations may increase the chance of inducing immunity to the vector itself and limit its usefulness for subsequent vaccination against other pathogens. In addition, there remain substantial concerns regarding the consequences of disease due to infection by recombinant replication-competent virus in a vaccine preparation (Willems *et al.,* 1979).

VEE is the only alphavirus for which a live, attenuated vaccine strain (TC-83) has been developed for veterinary and human use (Jahrling and Stephenson, 1984; Kinney *et al.,* 1993). New live, attenuated VEE vaccines, which offer significant improvements over TC-83, have been developed from a full-length cDNA clone (Davis *et al.,* 1989), by the introduction of multiple attenuating mutations into the structural protein genes. Thus, vectors derived from such vaccine strains are inherently safer than those derived from wild-type virus. Several of these strains, including V3014, replicate in lymphoid tissue without causing disease (Grieder *et al.,* 1995; Davis *et al.,* 1996). In the context of a vector, the VEE glycoproteins will preferentially target heterologous gene expression to lymphoid tissues (Davis *et al.,* 1996; Caley *et al.,* 1997), which may result in increased immunogenicity of the heterologous gene product.

In this study, two strategies were examined for improving the safety and efficacy of a VEE-based replicon vector. One strategy was to include previously defined attenuating mutations in the replicon and/or its helper. In this way, any viable recombinant virus is prevented from initiating a virulent infection. A second approach used a bipartite helper to supply structural proteins for packaging of the replicon into particles, thus requiring at least two recombination events for the generation of viable virus. This strategy greatly reduced the probability that a viable recombinant VEE virus would be generated during packaging. Using a VEE replicon particle (VRP) vaccine prepared in this manner, we have demonstrated the induction of high-level, protective immunity to a heterologous mucosal pathogen in naive animals and have achieved an equally high level, protective response in animals previously immunized with VRP-expressing genes from another pathogen.

MATERIALS AND METHODS

Cell lines and plasmids

Primary chicken (CEF) and duck (DEF) embryo fibroblasts, baby hamster kidney (BHK), and Vero cell lines were maintained in minimal essential medium with Earle's salts, 10% fetal bovine serum, 200 U/ml penicillin, 200 μg/ml streptomycin, and 10 μg/ml gentamicin sulfate. Full-length VEE clones pV3014, pV3519, and pV3526 were previously derived by mutagenesis of pV3000, a cDNA clone of the Trinidad donkey strain of VEE (Davis *et al.,* 1989, 1991, 1995; Grieder *et al.,* 1995). The VEE cDNA is downstream from a T7 RNA polymerase promoter such that linearization of the clone downstream of the VEE sequences, and subsequent *in vitro* transcription with T7 polymerase, yields infectious VEE genomic replicas (Davis *et al.,* 1989, 1991, 1995) (Fig. 1A). Plasmid pLS5560-7A, containing the N gene of the Josiah strain of the Lassa virus, was obtained from D. Auperin (CDC, Atlanta). The cloned HA gene of influenza A/PR/8/34 (H1N1) was obtained from P. Palese (Mount Sinai School of Medicine, New York).

The VEE replicon was prepared from a plasmid carrying a complete cDNA copy of the VEE genome modified to contain a second 26S promoter followed by a multiple cloning site from Cla12 adaptor plasmid (Hughes *et al.,*

1987). The double promoter clone was digested with *Apa*I, which cleaves within the 26S promoters bracketing the structural protein genes. Religation reconstituted a single 26S promoter followed by a multiple cloning site, which was used for insertion of the influenza HA or the Lassa N genes (Fig. 1B). To facilitate the cloning of these and other genes, a shuttle vector also was constructed (Davis *et al.,* 1996).

The helper constructs were derived from the pV3014, pV3519, and pV3526 clones by partial deletion of the genes encoding the VEE nonstructural proteins (Fig. 1C). When necessary, incompatible 5′ and 3′ overhanging ends were made blunt by treatment with T4 DNA polymerase prior to religation of the plasmid. As cDNA clones of three attenuated strains of VEE were used, the helpers differed not only in the length of the deletion, but also in the mutations within the genes encoding the virus glycoproteins (Table 1). Because certain relatively short in-frame deletions in the nonstructural genes are compatible with viability (Davis *et al.,* 1989), frame-shift mutations were inserted in V3014Δ2307–3055 and V3014Δ1951–3359 at the site of the deletions in order to terminate translation of the residual downstream reading frame.

The bipartite helper system consisted of individual Capsid (C)- and glycoprotein (GP)-helper RNAs which were constructed from V3014Δ520–7505. In the C-helper, nt 8495–11229 were deleted by digestion of V3014Δ520–7505 with *Hpa*I and religation of the 3.8-kb DNA fragment. In the GP-helper, nt 7565–8386 were deleted by digestion of V3014Δ520–7505 with *Tth*111I and *Spe*I followed by ligation of the 5.7-kb DNA fragment with the synthetic double-stranded oligonucleotide 5′-TAGTCTAGTCCGCCAAGATGTCA-3′. This oligonucleotide contained *Tth*111I and *Spe*I overhanging ends at the 5′ and 3′ ends, respectively, and reconstituted the 26S promoter downstream from the *Tth*111I site, the initiation codon normally used for the capsid protein, and the first codon of E3.

Transcription and transfection

Plasmid templates were linearized by digestion with *Not*I at a unique site downstream from the VEE cDNA sequence, and capped run-off transcripts were prepared *in vitro* with the RiboMAX T7 RNA polymerase kit (Promega). BHK cells were transfected by electroporation (Liljeström and Garoff, 1991) and incubated in 75-cm^2 flasks at 37°C in 5% CO_2. For the preparation of VRP, transcripts of both the replicon and the helper plasmids were coelectroporated into BHK cells, and the culture supernatants were harvested at 30 hrs after transfection.

Analysis of expression products and titration of VRP

Subconfluent cell monolayers were infected with VRP (m.o.i. = 5) or transfected with RNA by electroporation. Cells were harvested at the times indicated in the text, and expressed polypeptides were separated by polyacrylamide gel electrophoresis (Laemmli, 1970) and examined by Western blotting (Towbin *et al.,* 1979). To determine transfection efficiencies or the titers of various VRP preparations, BHK cells were transfected with RNA or infected with serial dilutions of VRP and incubated in eight-chamber slides (Nunc) for 20 h at 37°C to allow expression of HA, N, or VEE proteins. Antigen-positive cells were then enumerated for Lassa N protein by direct immunofluorescence using a FITC-conjugated monkey anti-Lassa serum or for influenza HA protein by indirect immunofluorescence as previously described (Pifat *et al.,* 1988) using the HA-specific monoclonal antibody 37-85 (Staudt and Gerhard, 1983) obtained from S. Clarke, University of North Carolina.

Immunization of mice with VRP

VRP were prepared by clarification of culture supernatants from cotransfected cells at 4000*g* for 10 min. Alternatively, VRP were purified further and concentrated by centrifugation at 39,000 rpm in an SW41 rotor through a cushion of 20% sucrose in PBS. The VRP preparations were diluted in Hank's balanced salt solution (HBSS), and groups of 6- to 8-week old BALB/c mice were inoculated with either a single dose of 0.2 ml subcutaneously (sc; HA-VRP) or 0.1 ml sc and 0.1 ml intraperitoneally (N-VRP). For booster immunizations, animals received identical inoculations at the times indicated. Control mice were inoculated with HBSS. The animals were observed daily and bled at 7- to 14-day intervals.

Enzyme-linked immunosorbent assay (ELISA)

Quantitation of VRP-induced serum antibodies was performed by ELISA essentially as described previously (Ludwig *et al.,* 1996), except that purified and inactivated Lassa virus or live VEE or influenza virus (A/PR8/34 (H1N1) was diluted in buffer containing 0.01 M Na_2CO_3 and 0.035 M $NaHCO_3$, pH 9.6, and 400 ng/well was adsorbed to plates for 12–15 h. Residual binding sites were blocked with PBS containing 5% fish gelatin and 0.1% Tween 20. Negative control wells received serum from mock immunized mice. Serum titers were expressed as the highest serum dilution resulting in optical densities greater than the mean of the optical densities of the negative controls plus three standard deviations.

Challenge with influenza virus

Animals were anesthetized with Metofane (Pitman-Moore) and challenged by administration of 1×10^5 to 2×10^5 50% egg infectious doses (EID_{50}) of influenza A/PR/8/34 intranasally (i.n.) in 20 μl (10 μl in each naris). Mice were observed and weighed daily for 14 days after challenge.

RESULTS

The objectives of these experiments were (1) to construct VEE replicon RNAs capable of programming their own replication and expressing a heterologous gene to high level; (2) to develop packaging systems for the production of VRP, which would greatly reduce the rate of recombination between helper and replicon RNAs; (3) to demonstrate the utility of VRP for induction of protective immune responses to a heterologous pathogen; and (4) to determine whether VRP may be used sequentially in the same individual for immunization against multiple pathogens.

Replicon and single helper constructs

Figure 1 shows the structures of the replicon and helper constructs. The replicon RNAs retained all of the *cis*-acting signals present in the VEE RNA genome, while the genes for the structural proteins were deleted. The influenza HA gene or the N gene from Lassa virus was inserted into the replicon downstream of the 26S promoter.

The helper constructs were derived from full-length VEE cDNA clones which harbored different constellations of attenuating mutations. This precaution was taken so that in the event that recombination between helper and replicon RNAs resulted in plaque-forming virus, an attenuated VEE vaccine strain rather than virulent virus would be generated. Three full-length cDNA clones were assessed as possible sources of the helper RNAs (Fig. 1). V3014 and V3519 contain substitution mutations within the E2 and E1 glycoproteins (Davis *et al.,* 1991; Grieder *et al.,* 1995). V3526, in addition to an attenuating amino acid change within E1, has a deletion of the consensus furin cleavage site between the E3 and E2 glycoproteins so that V3526 virions incorporate the unprocessed pE2 glycoprotein precursor in place of the E2 glycoprotein (Davis *et al.,* 1995). The helper constructs were prepared by various partial deletions of the nonstructural protein genes of the pV3014, pV3519, and pV3526 clones (Fig. 1C).

Expression of heterologous proteins from replicon RNA

The expression in BHK cells of influenza virus HA and Lassa virus N proteins from HA-replicon RNA and N-replicon RNA, respectively, and of the VEE structural proteins from the V3014Δ520–7505 RNA helper is shown in Fig. 2. In this study, BHK cells were transfected by electroporation, incubated for 30 h at 37°C, and analyzed by Western blotting. In BHK cells transfected with N-replicon RNA alone (data not shown) or cotransfected with N-replicon RNA and the V3014Δ520–7505 helper (Fig. 2, lane 3), a predominant polypeptide species of the expected molecular mass (63 kDa) was detected with monkey anti-Lassa antisera. In parallel studies with HA-replicon RNA, the HA migrated as two species with apparent molecular masses of 75–80 kDa. These probably represented glycosylation variants, as both bands reacted with the HA-specific monoclonal antibody, 37–85 (Fig. 2, lane 1). The antibodies were unreactive with control cells which were electroporated without RNA (Fig. 2, lanes 2 and 4). The anti-VEE antiserum was reactive with a protein in the control cell extract (Fig. 2, lane 9), but as would be expected, neither a recombinant replicon (Fig. 2, lane 7) nor a helper RNA alone (Fig. 2, lane 8) directed the synthesis of VEE structural proteins when transfected separately into BHK cells. However, VEE structural proteins were produced in large amounts in cells cotransfected with helper and replicon RNAs (Fig. 2, lane 6), and these proteins comigrated with authentic V3014 proteins (Fig. 2, lane 5).

Packaging of VEE replicon RNAs in cotransfected cells

Packaging signals for alphavirus RNAs have been described in both nonstructural (Weiss *et al.,* 1989, 1994) and structural genes (Rumenapf *et al.,* 1995), but remain undefined for VEE. However, efficient packaging of N-replicon and HA-replicon RNAs with several of the helper RNAs (Table 1) demonstrates that packaging signals are present in the VEE replicon RNAs, presumably in the nonstructural genes, as shown for Sindbis virus (Weiss *et al.,* 1989) and SFV (Liljeström and Garoff, 1991). To determine whether the size or position of the nonstructural gene deletions in the helper RNAs influenced the efficiency of packaging, the N-replicon RNA was packaged by coelectroporation with a series of helpers prepared by deleting varying portions of the nonstructural protein genes (Table 1). Titration of released VRP was readily accomplished by enumeration of antigen-positive cells by immunofluorescence. Titers approaching 10^8 IU/ml were obtained when either the Lassa N or the influenza HA replicon was coelectroporated with a V3014-derived helper from which nt 520–7505 (90%) of the nonstructural genes were deleted. The titers were reduced when helpers had shorter deletions or were derived from V3519 or V3526 strains of VEE. The reasons for the lower packaging efficiency with these helpers are unknown. However, previous studies which examined complementation among alphavirus temperature-sensitive mutants also found that complementation was highly dependent on the specific virus and specific strains used (Burge and Pfefferkorn, 1966; Tan *et al.,* 1969; Keränen and Kääriänen, 1974; Atkins *et al.,* 1974; Strauss *et al.,* 1976; Hashimoto and Simizu, 1978; Maeda *et al.,* 1979; reviewed in Strauss and Strauss, 1980).

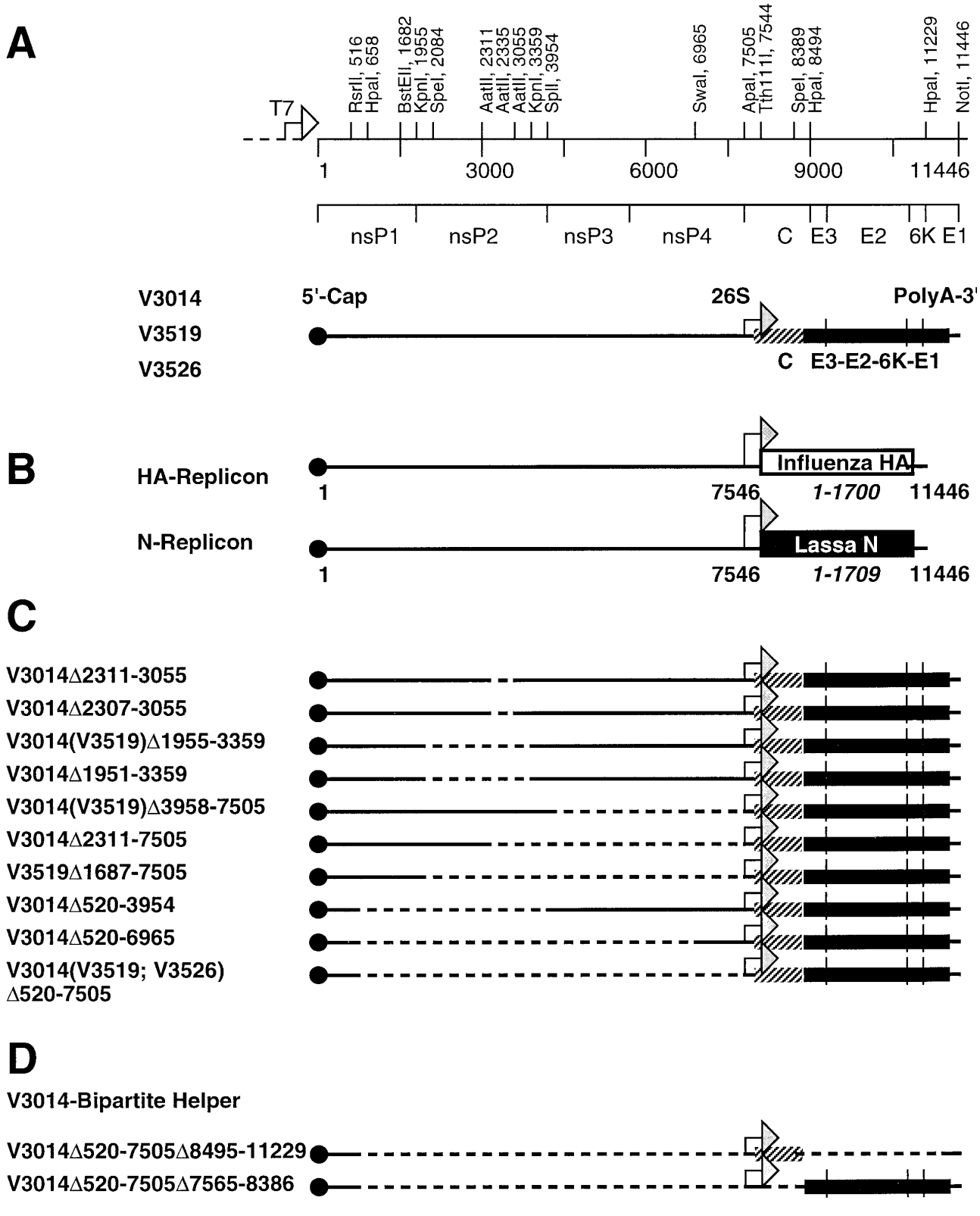

FIG. 1. Structure of VEE replicon and helper RNAs. (A) VEE full-length cDNA clones and infectious RNA. Restriction sites and numbering are according to Kinney *et al.*, (1993). Open arrow shows the position of the T7 RNA polymerase promoter and direction of *in vitro* transcription. Also indicated are the 5′-cap, 26S promoter (shaded arrow), genes encoding nonstructural (solid line) and structural proteins (capsid, hashed bar; glycoproteins, solid bar), and the 3′-poly(A). (B) VEE replicons. The HA-replicon and N-replicon RNAs were similar to the full-length RNA except that the open reading frame encoding the VEE structural proteins in the full-length RNA was replaced with the HA or N genes, respectively. Nucleotides of VEE, as well as of the HA and N genes (italics) are shown. (C) Helper constructs. The helper RNAs are isogenic to the VEE full-length RNA, with the exception of the deletions (dashed lines) in the nonstructural region. The parental attenuated VEE strains and the deletions are listed on the left. The deletions were generated using the restriction sites shown in A. (D) Bipartite helper RNAs. The bipartite helper system consisted of two helper RNAs derived from the V3014Δ520–7505 monopartite helper. One RNA expressed the VEE capsid gene, and the second RNA expressed the glycoprotein genes.

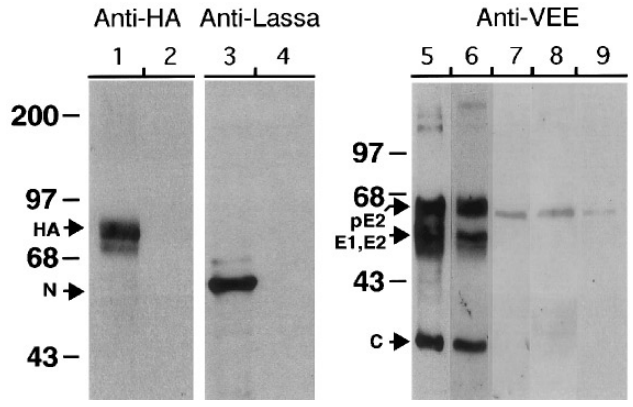

FIG. 2. Synthesis of influenza HA, Lassa N, and VEE structural proteins in transfected BHK cells. BHK cells were coelectroporated with RNAs of the HA-replicon and V3014Δ520–7505 helper (lane 1) or N-replicon and V3014Δ520–7505 (lanes 3 and 6) or electroporated with individual RNAs of V3014 (lane 5), N-replicon (lane 7), or V3014Δ520–7505 (lane 8). Lanes 2, 4, and 9 represent BHK cells electroporated without RNA. Cell extracts were collected at 30 h postelectroporation as described under Materials and Methods. Each lane was loaded with the equivalent of 5×10^4 cells. Proteins were visualized by immunoblotting with an anti-HA monoclonal antibody (37–85; lanes 1 and 2) or monkey anti-Lassa serum (lanes 3 and 4). VEE structural proteins were visualized by immunoblotting with rabbit anti-VEE serum. Positions of VEE PE2, E1, E2, and capsid proteins are shown. The minor band present in all lanes likely represents a cellular protein cross-reacting with the anti-VEE serum.

Expression of heterologous proteins in different cell lines infected with VRP

Lassa N-replicon RNAs were packaged using the V3014Δ520–7505 helper, and the resulting N-VRP were used to infect BHK, Vero, CEF, and DEF cells at a m.o.i. of 5 (Fig. 3). By 48 h postinfection, large amounts of N were evident. Densitometric analysis of several such gels from BHK cell extracts indicated that approximately 20% of the total cellular protein was Lassa N. This study shows that VRP have a broad host range as specified by the VEE glycoproteins and can induce high-level protein expression in a variety of cell types.

Recombination between replicon and monopartite helper RNAs

Existing alphavirus replicon systems produce significant levels of live, plaque-forming virus in addition to packaged replicons due to recombination between the replicon and the helper RNAs (Weiss and Schlesinger, 1991; Bredenbeek *et al.,* 1993). Significant amounts of infectious virus also were evident in cells transfected with the VEE replicon and the various monopartite helper RNAs. As shown in Table 1, infectious virus titers of $<10^2$ to 2×10^5 PFU/ml (limit of detection $= 10^2$) were produced, and the amount of plaque-forming virus appeared to be dependent on the specific helper used (Table 1). Although the packaging of replicons was much less efficient with V3519-derived helpers, no infectious virus was detected by direct plaque assay of the replicon preparations (Table 1). However, when BHK cells were inoculated with media from cells coelectroporated with the replicon and the V3519Δ520–7505 helper, cytopathology was evident by 48 h, and virus was then readily detectable by plaque assay (data not shown).

TABLE 1

Replicon Packaging in BHK Cells Cotransfected with Lassa N-Replicon and Helper RNAs[a]

VEE strain	Mutations[b]	Helper[c]	IU per ml of medium[d]	PFU per ml of medium[e]
V3519	E2 E76K; E2 E209K	V3519 Δ1955–3359	<10	<100
	E1 A272T	V3519 Δ1687–7505	nd[f]	<100
		V3519 Δ3958–7505	<10	<100
		V3519 Δ520–7505	6×10^5	<100
V3526	E3 Δ56RKRR59	V3526 Δ520–7505	3×10^4	3×10^2
	E1 F253S			
V3014	E2 E209K; E2 I239N	V3014 Δ2307–3055	nd	1×10^4
	E1 A272T	V3014 Δ2311–3055	nd	1×10^4
		V3014 Δ1951–3359	nd	2×10^5
		V3014 Δ1955–3359	6×10^6	1×10^5
		V3014 Δ3958–7505	1×10^4	2×10^5
		V3014 Δ2311–7505	4×10^4	3×10^4
		V3014 Δ520–3954	2×10^7	4×10^3
		V3014 Δ520–6965	5×10^5	2×10^3
		V3014 Δ520–7505	8×10^7	2×10^3
		V3014-Bipartite helper	1×10^8	<100

[a] Cotransfection of BHK cells with the Lassa virus N-replicon and the helper indicated; cotransfections with the HA-replicon yielded similar titers.
[b] Amino acid substitutions compared to the V3000 sequence.
[c] Designated by the parental VEE strain used for construction and the region of the VEE NS region deleted.
[d] As determined by infection of BHK cells with VRP and enumeration of infected cells by immunofluorescence.
[e] As determined by direct plaque assay in Vero cells.
[f] nd, not determined.

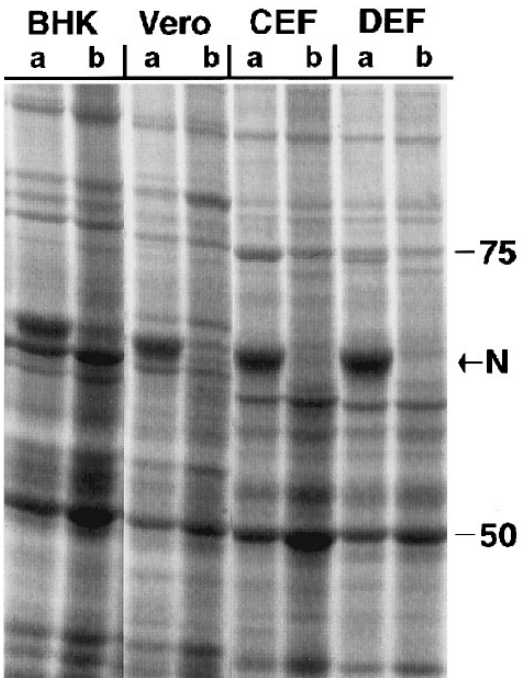

FIG. 3. Expression of Lassa N in cultured cells infected with N-VRP. N-VRP were prepared in BHK cells using the V3014Δ520–7505 helper. Subconfluent monolayers were infected at an m.o.i. of 5, and cells were harvested 48 h postinfection and processed for electrophoresis and staining with Coomassie Blue dye as described under Materials and Methods. Lysates of infected and uninfected cells are indicated with (a) and (b), respectively. Position of the Lassa N protein is indicated.

Although decreasing the size of the available homologous sequences between replicon and helper RNA appeared to reduce the levels of plaque-forming virus, infectious virus was found with each of the helper RNAs tested, even when 90% of the nonstructural gene region was deleted as in the V3014Δ520–7505 helper. Partial sequence analysis of three plaque isolates from different packaging experiments with the V3014Δ520–7505 helper demonstrated that in each case, the virus was a recombinant rather than the result of copackaging of helper and replicon RNAs (Table 2). For each isolate, nonhomologous recombination had occurred at a different point within the heterologous gene insert, resulting in the substitution of the 3′ portion of the HA gene insert and the 3′ untranslated region of the replicon RNA with a second 26S promoter, the VEE structural protein genes and the 3′ untranslated region of the helper RNA.

TABLE 2

Recombination Points in Plaque-Forming Viruses

5′ nucleotide from HA replicon[a]	3′ nucleotide from helper V3014Δ520–7505[b]
nt + 788	nt 391
nt + 189	nt 425
nt −25[c]	nt 441

[a] Nucleotide immediately 5′ of the recombination point; numbering upstream (−) or downstream (+) from the first nucleotide of the HA gene coding sequence.

[b] Nucleotide immediately 3′ of the recombination point; numbering from the first nucleotide of the helper RNA.

[c] The promoter site is in the polylinker downstream of the 26S promoter and upstream of the HA gene coding sequence.

TABLE 3

Intracerebral Inoculation of Infant Mice with HA-VRP Packaged with Single and Bipartite Helpers

Inoculum[a]	Dose (IU)[b]	PFU[c]	Survivors/total[d]
Diluent	0	0	10/10
HA-VRP (BH)	5×10^5	0	10/10
HA-VRP (BH)	5×10^7	0	10/10
HA-VRP (SH)	5×10^5	500	1/11
HA-VRP (SH)	5×10^7	50000	0/10

[a] HA-VRP packaged with single (SH) and bipartite (BH) helper systems.

[b] Infectious units were determined as in Table 1.

[c] Plaque-forming units inoculated; PFU were determined by direct plaque assay in Vero cells.

[d] Survivors/total number of animals inoculated. Samples of replicon preparations (25 μl/animal) were inoculated ic into litters of 3-day-old outbred ICR mice. Although VEE virus strain 3014 is avirulent in adult mice inoculated sc at doses up to 2×10^5 (Davis *et al.,* 1991), its LD_{50} following ic inoculation of infant mice was 15 PFU (data not shown; Reed and Muench, 1938).

Generation of VRP using bipartite helper RNAs

To decrease the probability of recombination, a bipartite packaging system was engineered to increase the number of recombination events required to regenerate an infectious virus. The bipartite helper encoded the VEE capsid and the V3014 mutant glycoproteins on two separate helper RNAs (Fig. 1D), analogous to a helper system independently developed for packaging of Sindbis replicons (Frolov *et al.,* 1997). Coelectroporation of the three RNAs (either HA or N replicon RNA and the two RNAs composing the bipartite helper) resulted in replicon packaging at levels equivalent to the best yields obtained when a monopartite helper was used (Table 1). In addition, infectious virus was not detected in these experiments by plaque assay (limit of detection was 20 PFU/ml). Blind passage of supernatants from transfected cells and infectious center assays also detected no viable virus (limit of detection was 1 PFU/ml and 5 PFU/ml, respectively). Intracerebral (ic) inoculation of suckling mice (Table 3) also failed to provide any evidence of the regeneration of virulent infectious virus (limit of detection was 64 mouse lethal doses/ml). These results demonstrated that the preparation of high-titer VRP, free of detectable plaque-forming virus and offering a significantly enhanced level of safety, was feasible with a bipartite helper system.

TABLE 4

Induction of Serum Antibody by HA-VRP or N-VRP (Monopartite Helper) and Protection of BALB/c Mice against Influenza[a]

Immunogen		Dose (IU)[b]	Anti-HA antibody[c]	Anti-N antibody[c]	Anti-VEE antibody[c]	Morbidity after challenge[d] (sick total)
HBSS	Primary (0)	0	$<10^2$ (0/8)	$<10^2$ (0/8)	$<10^2$ (0/8)	
	Booster (32)	0	$<10^2$ (0/8)	$<10^2$ (0/8)	$<10^2$ (0/8)	8/8
HA-VRP	Primary (0)	3×10^7	1×10^3 (9/9)		$<10^2$ (0/9)	
	Booster (32)	3×10^7	4×10^4 (9/9)		1×10^3 (4/9)	0/8
HA-VRP	Primary (0)	3×10^5	2×10^2 (9/9)		$<10^2$ (0/9)	
	Booster (32)	3×10^5	4×10^3 (9/9)		2×10^2 (8/9)	0/9
HA-VRP	Primary (0)	3×10^3	$<10^2$ (0/9)		$<10^2$ (0/9)	
	Booster (32)	3×10^3	$<10^2$ (2/9)		$<10^2$ (0/9)	8/8
N-VRP	Primary (0)	4×10^7		1×10^3 (5/5)	3×10^3 (5/5)	
	Booster (28)	4×10^7		5×10^4 (5/5)	8×10^3 (5/5)	nd[e]
N-VRP	Primary (0)	4×10^5		$<10^2$ (0/5)	$<10^2$ (0/5)	
	Booster (28)	4×10^5		2×10^3 (4/4)	2×10^2 (3/4)	nd

[a] VRP were prepared in BHK cells with the V3014Δ520–7505 monopartite helper.

[b] Primary and booster immunizations were administered sc (HBSS; HA-VRP) or sc and ip (N-VRP) on the days indicated in parentheses.

[c] Geometric mean titers were determined by ELISA using purified influenza, Lassa, or VEE virion antigens. In parentheses, ratio of antibody-positive/total animals is shown. Responses after primary and secondary inoculation were determined at days 28 and 70 postimmunization, respectively.

[d] A challenge dose of 10^5 EID_{50} of influenza A/PR/8/34 was administered intranasally (day 116 postinoculation). The ratio of sick/total animals is shown. Illness was determined by loss of at least 10% of body weight when measured at day 7 postchallenge.

[e] nd, not determined.

Antibody responses in mice to HA, N, and VEE induced by HA-VRP and N-VRP

The immunogenicity of VRP-vectored influenza HA or Lassa N was determined by measuring antibody responses to the HA or N proteins following immunization of BALB/c mice (Table 4). In the first set of experiments, the HA-VRP and N-VRP used were prepared using the monopartite V3014Δ520–7505 helper. Despite a low level of contaminating infectious virus in these preparations (Table 1), no clinical signs of infection were observed in any of the inoculated animals regardless of the dose, demonstrating the margin of safety afforded by the attenuating mutations present in the V3014-derived helper.

Single sc inoculations of 3×10^5 or 3×10^7 IU of HA-VRP induced dose-dependent, serum antibody responses to HA in all animals, and booster immunizations resulted in significant increases in antibody titer (Table 4). In contrast, two inoculations of 3×10^3 IU of HA-VRP induced little, if any, serum antibody in response to HA-VRP. Thus, in this experiment, the minimum dose required to induce seroconversion in 100% of the mice with a single inoculation was approximately 3×10^5 IU of HA-VRP. Similarly, serum antibodies to Lassa N were elicited by a single administration of 4×10^7 IU of N-VRP or two inoculations of 4×10^5 IU. Serum antibodies to VEE were present in a majority of the animals receiving immunizing doses of HA- or N-VRP as a primary immunization or following a booster (Table 4). The induction of antibodies to VEE may have resulted from infection by regenerated virus present in these preparations (Table 1) or may have reflected an immune response to the structural components of the HA- or N-VRP themselves.

Sequential immunization with HA-VRP and N-VRP prepared with the bipartite helper system

In contrast to VRP prepared with the monopartite helper, regeneration of plaque-forming virus was not detected in VRP prepared with the bipartite helper system (Table 1). Groups of mice, which received two inoculations of 3×10^4 or 3×10^6 IU of N-VRP followed by two inoculations of 2×10^5 IU of HA-VRP, all responded with serum antibodies to both antigens (Fig. 4). A control group of mice, which received two inoculations of HBSS followed by two inoculations of 2×10^5 of HA-VRP, responded with serum anti-HA antibody at a titer equivalent to that in the sequentially inoculated animals. Control mice inoculated only with HBSS were negative for antibodies to HA, N, or VEE. Therefore, with VRP prepared with a bipartite helper, immunization with VRP expressing Lassa N protein did not interfere with the development of a response to subsequent primary immunization with VRP expressing the influenza HA.

ELISA antibody titer to VEE after four inoculations of VRP prepared with the bipartite helper was below the limit of detection (1:100) in 23 of 24 mice which received VRP in this experiment, and the response in the remaining animal was at a low level (1:200). Therefore, these data suggest that the antibody responses to VEE detected in the previous experiment, in which animals were immunized with VRP produced with the monopartite helper, were due to infection by attenuated, plaque-form-

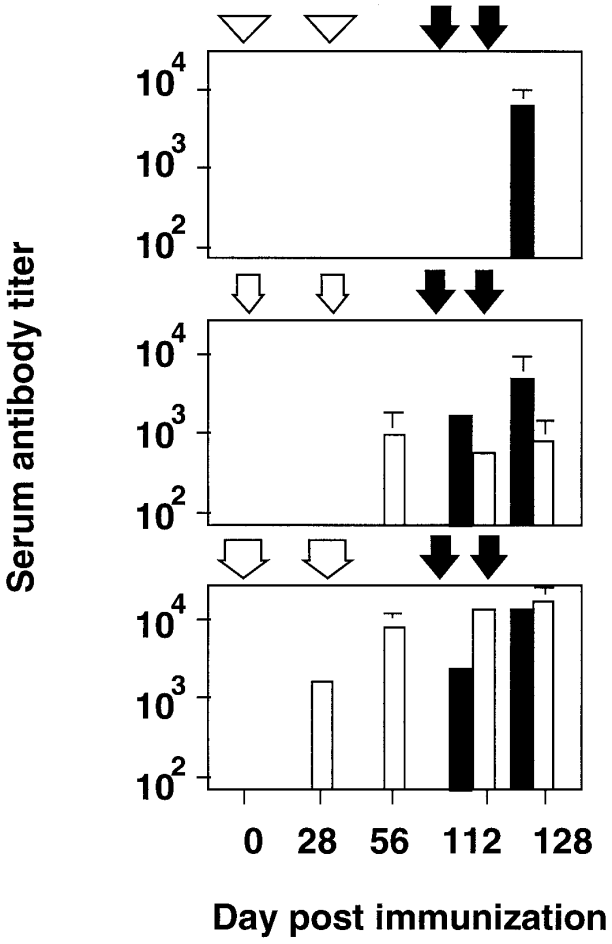

FIG. 4. Sequential immunization of individual mice with N-VRP and HA-VRP. Groups of BALB/c mice were inoculated sc on days 0 and 32 with either 3×10^4 IU (middle, small open arrows) or 3×10^6 IU (bottom, large open arrows) of N-VRP (10 mice/group). Control animals received HBSS at 0 and 32 days (top, open arrowheads; 5 mice/group). On days 84 and 112, all animals received 2×10^5 IU of HA-VRP (solid arrows). Animals were bled on the days indicated on the abscissa. Bars show geometric mean serum antibody titers as measured by ELISA; error bars represent one standard deviation. Open bars, titer to Lassa N. Solid bars, titer to influenza HA.

ing virus rather than the result of repeated exposure to the structural components of the VRP themselves.

Protection against a mucosal challenge with influenza virus

Live, attenuated VEE vaccines elicit protection against both parenteral and aerosol challenge with virulent VEE (Jahrling and Stephenson, 1984). In a mouse model using sc immunization with V3014 and i.n. challenge with wild-type VEE, protection was demonstrated at nasal mucosal surfaces (Charles *et al.,* 1997). To determine if sc administration of VRP was capable of eliciting a protective immune response to a mucosal pathogen, mice were immunized with HA-VRP and subsequently challenged by i.n. inoculation with influenza A/PR/8/34. Control mice inoculated with HBSS or 3×10^3 IU of HA-VRP showed no protection and significant weight loss after influenza challenge (Table 4). In contrast, animals immunized with two doses of 3×10^5 or 3×10^7 IU of HA-VRP showed no symptoms of disease after challenge, demonstrating that complete protection against mucosal challenge could be elicited by parenteral inoculation with HA-VRP.

Complete protection against influenza virus challenge also was achieved in mice which received sequential immunizations with N-VRP and HA-VRP prepared with the bipartite helper (Fig. 4). In this experiment, surviving control animals lost 20% of their body weight, and two of four control mice died. No sickness, weight loss, or death was seen in any of the mice which received immunizing doses of the HA-VRP, regardless of whether they had received prior immunizations with VRP expressing the Lassa N protein.

DISCUSSION

Alphavirus expression systems hold great promise as vaccine delivery vehicles, and the VEE-based replicon-helper system described in this report is especially well suited for this purpose. The VRP efficiently infect a variety of cultured cells in which they program high-level synthesis of the vectored gene product, induce protective immunity in experimental animals, and do so in a way which allows the sequential use of different VRP for immunization of the same individuals against multiple pathogens. Moreover, the combination of previously defined attenuating mutations and the bipartite helper system make VRP an inherently safe means of immunization.

Expression in cultured cells

Like the SFV and Sindbis replicon systems, the VEE vectors program high-level synthesis of heterologous proteins in a wide range of cell types from different species, including cells certified for production of human vaccine products. An important determinant of high-level expression of heterologous genes in Sindbis and SFV replicon systems is the presence of a translational enhancer sequence embedded in the 5′ end of the capsid gene (Frolov and Schlesinger, 1994; Sjöberg and Garoff, 1996). The characteristic stem–loop structure of the SFV and Sindbis translational enhancers is not present in the VEE sequence. The VEE vectors described here retained 14 nucleotides of the authentic VEE 26S RNA followed by a multiple cloning site into which the heterologous gene was inserted. The Lassa N and influenza HA genes utilized their native AUG initiator codons. Nevertheless, the VEE replicon vectors expressed the Lassa N gene to a level approximating 20% of the total cell protein. Therefore, either efficient translation of the VEE subgenomic 26S mRNA does not require an enhancer element or an

element with enhancer function is present in the primary sequence of the 5′ 14 nucleotides of the VEE 26S mRNA.

Sequential immunization with VEE replicon particles

High levels of serum antibody to influenza HA and Lassa N were induced by their respective VRP, and complete protection against i.n. challenge with influenza was obtained. One possible reason for the strength of the response induced by VRP is that the glycoproteins of VEE and VEE-based vectors target the virus to lymphoid tissue (Gleiser *et al.,* 1962; Gorelkin, 1973; Jackson *et al.,* 1991; Grieder *et al.,* 1995; Davis *et al.,* 1996; Caley *et al.,* 1997), an anatomical site highly favorable to induction of immune responses. Moreover, immunization with HA-VRP was demonstrated in animals which had been previously inoculated with N-VRP, and similar levels of humoral antibody to influenza were induced whether or not an individual animal had been immunized with N-VRP previously. Preexisting or induced immunity to other virus vectors, such as vaccinia, adenovirus, adeno-associated virus, and herpesviruses, may limit their usefulness for successive gene therapy or vaccine applications in a given individual. Conversely, the most probable explanation for the success of sequential VRP immunization was the low level of antibody to the VEE structural proteins in animals inoculated up to four times in succession with VRP prepared with the bipartite helpers.

Several aspects of the VEE system may have contributed to the low level of anti-VEE antibody induction. First, VRP stocks packaged with the bipartite helpers contained very few if any plaque-forming virus particles, and the VEE-specific antigenic mass represented by the VRP themselves may have been insufficient for induction of significant anti-VEE antibodies. Second, the titers of the VRP preparations were high relative to the dose used for immunization. Thus, any contaminating cell debris, which would have contained significant levels of VEE structural proteins, would have been removed by purification and diluted by several orders of magnitude prior to inoculation. Third, one would anticipate that the response to both the expressed heterologous protein and the vector itself would be affected significantly by the route of inoculation as well as the species immunized. While the influence of these parameters, and of prior immunity to VEE, is under active investigation, the present results clearly demonstrate conditions where the VRP system can be used for successful immunization against multiple pathogens.

Copackaging and recombination of replicon and helper RNAs

The presence of plaque-forming virus in alphavirus replicon preparations is undesirable with respect to immunization against the vector as well as safety. The bipartite packaging system was designed to reduce the probability that such virus would be generated either by recombination or by copackaging of replicon and helper RNAs in the same virion. In Sindbis systems, packaging of RNAs into particles appears to be somewhat promiscuous. Sindbis replicon preparations have particles which apparently contain helper RNAs (Frolov *et al.,* 1997) and plaque-forming particles in which replicon and helper RNAs are copackaged (Geigenmüller-Gnirke *et al.,* 1991). In the former case, co-infection of cells with helper-only RNA particles along with replicon particles leads to the high-level expression of the structural proteins. In the latter case, structural protein expression will occur after infection with copackaged particles, even at low m.o.i. *In vivo,* either situation could result in the induction of immunity to the vector. In contrast to Sindbis systems, no evidence for copackaging of VEE replicon and helper RNAs was observed.

Alphavirus recombination was first demonstrated experimentally with Sindbis virus (Weiss and Schlesinger, 1991), and sequence comparisons suggest that western equine encephalitis virus is a naturally occurring recombinant between Sindbis and eastern equine encephalitis virus (Hahn *et al.,* 1988). Recombination between alphavirus replicon and helper RNAs during packaging also can result in the generation of plaque-forming virus. Although recombination occurs in infected cells at low frequency, any recombinants generated during replicon packaging will have a very large selective growth advantage. This is of significant concern in the context of a replicon particle vaccine, as contaminating recombinant virus could compromise the safety of the replicon preparation, and *in vivo,* growth of recombinants will induce antibody to the virion structural proteins. In fact, Mossman *et al.,* (1996) observed high titers of neutralizing antibody to SFV in animals immunized with protease-activated SFV replicon particles (Berglund *et al.,* 1993), a result which may have reflected infection by activated recombinants or possibly infectious SFV regenerated by second-site mutation (Salminen *et al.,* 1992; Heidner *et al.,* 1994; Davis *et al.,* 1995).

When VEE replicons were packaged by the V3014-derived monopartite helper RNAs, the infectious virus titers were, in general, inversely related to the size of the deletion in the nonstructural protein genes. However, exceptions to this generalization were evident, and significant amounts of plaque-forming virus were obtained even when the helper RNA contained less than 1 kb of nonstructural gene sequence (V3014Δ520–7505). Of three such plaque isolates sequenced, each derived from an independent packaging experiment, all were nonhomologous recombinants. Nonhomologous recombination was observed previously between Sindbis RNAs (Weiss and Schlesinger, 1991) and SFV RNAs (Thomson and Dimmock, 1994). Each VEE recombinant contained a 5′ to 3′ fusion between the replicon and

helper RNAs at different sites within the HA insert (Table 2). Thus, the formation of viable recombinants did not require the precise regeneration of the natural VEE sequence, as each recombinant contained two 26S promoters with the second driving the transcription of the complete structural gene region. Reducing the size of homologous regions may have reduced the recombination rate, but sequence analysis of the recombinants suggests the possibility that deletion of specific sequences in the helper RNA may have affected the rate of nonhomologous recombination independently of deletion size. Regardless of the mechanism, it is clear that deletion of large portions of the nonstructural protein genes from the monopartite helpers did not prevent the regeneration of plaque-forming virus.

Replicon packaging with bipartite helper RNAs

It seems likely from our results and independent experiments of others (Frolov *et al.,* 1997) that although recombination was occurring in the packaging cells, the recombination requirements imposed by the use of bipartite helper RNAs greatly reduced the probability that a plaque-forming, recombinant virus would be generated.

It is possible that a VRP preparation could contain single recombinants in which the replicon RNA had recombined with only one of the structural protein helper RNAs. Such single recombinants would not have registered as plaque-forming virus but would express a subset of the VEE structural proteins upon infection of cells. The incidence of single recombinants in the VRP preparation would be predicted at less than the frequency of plaque-forming viruses found with the monopartite helpers, as these putative single recombinants would not have been amplified by propagation subsequent to the recombination event.

Role of attenuating mutations in the helper glycoprotein genes

Although no plaque-forming virus was detectable in these experiments, we anticipate that viable recombinant viruses could be formed at some very low but still finite frequency, and this likelihood necessitates additional precautions prior to use of alphavirus replicon particles for immunization or gene therapy in humans. To provide this extra margin of safety, we have utilized helper glycoprotein genes which contain two strongly attenuating mutations derived from V3014, a recombinant VEE vaccine strain. V3014 is avirulent upon sc inoculation of mice (Grieder *et al.,* 1995) and horses (Smith *et al.,* unpublished observations), and therefore, any recombinant generated during VRP packaging is very likely to be at least as attenuated as V3014.

In summary, we have demonstrated that VRP program high-level expression of a vectored gene in cultured cells and *in vivo* can induce an efficacious immune response against the pathogen from which that gene was derived. The VRP are inherently safe in that they are limited to a single cycle of infection, and this level of safety is enhanced by the inclusion of attenuating mutations in the helper glycoproteins and the use of a bipartite helper system which greatly reduces the generation of infectious, plaque-forming virus. This property also potentiates the use of these vectors for sequential immunization of a given individual against multiple pathogens. All of these characteristics make the VEE replicon system with the bipartite helper a flexible and attractive vaccine vector, especially in situations where a live, attenuated vaccine might be undesirable or difficult to develop.

ACKNOWLEDGMENTS

We thank C. Lind for technical assistance and P. Jahrling and J. Geisbert for providing Lassa antigen. P.P. was a recipient of a National Research Council-USAMRIID Research Associateship. N.L.D. and R.E.J. were supported by Department of Defense Grant DAMD 17-94-J-4430. The views in this report are those of the authors and should not be considered as Department of the Army positions. In conducting research using animals, we adhered to the "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 86-23).

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