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Lack of Interference with Immunogenicity of a Chimeric Alphavirus Replicon Particle-Based Influenza Vaccine by Preexisting Antivector Immunity

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Antivector immunity has been recognized as a potential caveat of using virus-based vaccines. In the present study, an alphavirus-based replicon particle vaccine platform, which has demonstrated robust immunogenicity in animal models, was tested for effects of antivector immunity on immunogenicity against hemagglutinin of influenza virus as a target antigen and efficacy for protection against lethal challenge with the virus. Chimeric alphavirus-based replicon particles, comprising Venezuelan equine encephalitis virus nonstructural and Sindbis virus structural components, induced efficient protective antibody responses, which were not adversely influenced after multiple immunizations with the same vector expressing various antigens.

Over the last 2 decades, replication-defective viral vectors have been drawing attention in the vaccine research field as an alternative delivery system for antigens that address shortcomings of the more classical vaccine strategies of inactivated, subunit, or attenuated vaccines. Alphaviruses are among the viral vectors being developed and undergoing clinical testing due in part to their ability to stimulate humoral, cellular, and mucosal immune responses. Alphaviruses belong to the *Togaviridae* family and contain a positive-sense, single-stranded RNA genome of approximately 12 kb encoding four nonstructural proteins in the 5′ two-thirds of the genome, followed by a strong subgenomic promoter that directs expression of the viral structural proteins in the 3′ one-third of the genome. Upon infection of a cell, the alphavirus nonstructural proteins are immediately translated to form a polymerase complex, which initiates replication of the viral genome and high-level transcription from the subgenomic promoter and translation of the downstream structural protein gene products, which leads to assembly of progeny viral particles.

Vaccine delivery vectors based on alphaviruses have been developed from Semliki Forest virus (SFV) (27), Sindbis (SIN) virus (7, 53), Venezuelan equine encephalitis (VEE) virus (41), and also vector chimeras incorporating desirable properties from both SIN and VEE (38). These alphavirus vectors have a modified RNA genome where the subgenomic coding region for the structural proteins has been replaced with one or more antigen encoding sequences. This modification allows for cytoplasmic replication of the RNA vector but renders defective *de novo* viral particle formation because of the lack of the structural proteins. Such alphavirus vectors are referred to as "replicons." The replicons can be used in the form of DNA, such as plasmid DNA vaccines (13), or alternatively with the "defective" replicon RNA packaged into virus-like particles with the alphavirus capsid and envelope structural proteins. Such particles (replicon particles) can be produced by providing structural proteins to replicon RNA in cultured production cells (7, 27, 39). The replicon particles have been shown to be highly efficient for eliciting antigen-specific immune responses in a variety of animal models (3, 17, 18, 22, 31, 35, 36).

For viral vector vaccine platforms in general, preexisting antivector immune responses of the host may become a complicating issue that should be considered when using the vector-based platform as a general vaccine strategy. Indeed, it has been shown that vaccines using vaccinia virus vector failed to induce strong immune responses in the presence of antivector immunity (28, 46). In the case of adenovirus vectors, interference by preexisting antivector neutralizing antibodies has been vigorously discussed (4, 8, 15, 24, 30) although a few controversial findings have been reported (2). For VEE-based replicon particles, it has been shown that anti-VEE antibodies induced by the particles did not interfere with the induction of protective immunity induced by replicon particles based on the same vector, expressing a different gene of interest (41), although the neutralization titers against the vector were not shown in the report. More recently, the alphavirus-based vaccine strategy has been tested in clinical settings (6, 33). One of these studies reported that immunization with VEE-based replicon particles could successfully break tolerance to self-antigen (a tumor-specific antigen) despite induction of vector-specific neutralizing antibodies.

In this study, we have evaluated VEE/SIN chimera-based replicon particles expressing influenza virus hemagglutinin (HA) as an alternative vaccine strategy to the traditional influenza subunit vaccine preparations. Despite the existence of neutralizing antivector immunity induced by administration of replicon particles encoding an unrelated antigen with higher doses than under the condition used by others (41), we showed that the HA-expressing replicon particles were still able to generate strong humoral anti-

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body responses against the HA antigen and to protect mice from lethal challenge of influenza virus.

MATERIALS AND METHODS

Influenza virus and subunit vaccine preparations. A seed stock of the RESVIR17 (H3N2) strain, a reassortant vaccine strain generated from A/Panama/2007/99 (H3N2) and A/Puerto Rico/8/34 (H1N1), and a bulk lot of monovalent anti-H3N2 subunit influenza vaccine preparation derived from this strain through good manufacturing practice guidelines were provided by the production department of Novartis Vaccines & Diagnostics s.r.l. The antigen concentration was measured as the content of HA in the preparation. A seed stock of A/WS/33 (H1N1) strain was kindly provided by A. R. Douglas, National Institute for Medical Research, United Kingdom. A/WS/33 was propagated from the seed stock in embryonated chicken eggs and titrated by the standard procedure (10). The allantoic fluid was collected, aliquoted, and stored at −80°C.

Generation of VEE/SIN replicon particles. To obtain the sequences encoding HA from the influenza strains, viral seeds were processed for RNA extraction using the RNeasy kit (Qiagen, Düsseldorf, Germany). The extracted RNA was subsequently reverse transcribed, followed by PCR using primers of sequences at the 5′ and 3′ end of the HA coding region. For RESVIR17, the parental A/Panama/2007/99 (H3N2) was used to design PCR primers. Amplified DNA fragments were cloned into pBluescript vector (Stratagene, La Jolla, CA). To avoid selecting sequences containing mutations incorporated during PCR, a consensus sequence of each HA segment was determined from multiple clones. The sequence identical to the consensus sequence from the RESVIR17 (H3N2) strain was further transferred to the VCR-Chim2.1 vector (16, 38). The replicon particles derived from the HA sequence of RESVIR17 (H3N2) were referred to as VEE/SIN-H3. Similarly, the HA and nucleoprotein (NP) sequences from the A/WS/33 (H1N1) were transferred to the VCR-chim2.1 vector, and the replicon particles produced from these constructs were referred to as VEE/SIN-H1 and VEE/SIN-NP, respectively. cDNA encoding severe acute respiratory syndrome (SARS)-spike protein (GenBank accession number Y310120) was also subcloned into the VCR-chim2.1 vector to prepare replicon particles expressing SARS-spike protein (VEE/SIN-SSP). The HN gene of the PIV3 JS strain (48) or the gp140ΔV2 derived from the HIV-1 SF162 strain was generated synthetically and also inserted into the VCR-chim2.1 alphavirus replicon vector to produce VEE/SIN-PIV3 and VEE/SIN-gp140 replicon particles, respectively (38, 39). Generation, purification, and quality control of replicon particles were performed as described elsewhere (17, 18, 38).

Immunization and challenge experiments. Female BALB/c (BALB/cAnNCrl), mice were purchased at the age of 6 weeks from Charles River Laboratories, Calco, Italy. Replicon particles were diluted to appropriate concentrations in phosphate-buffered saline (PBS). Groups of 10 mice were immunized through intramuscular (i.m.) routes with 50 μl of replicon particles (VEE/SIN-H1 and VEE/SIN-H3, 10^2 to 10^6 infectious units [IU]/mouse) at 3-week intervals. For challenge experiments 2 weeks after the last immunization with VEE/SIN-H1, 10 μl of virus solution containing 150 mouse 50% lethal doses (mLD_{50}) of live influenza virus A/WS/33 was inoculated in each nostril (300 mLD_{50}/mouse total) without anesthesia. Survival of the challenged mice was monitored for 14 days after infection. For immunogenicity experiments, serum was prepared from blood from immunized mice 2 weeks after the immunization(s) for serological analyses.

VEE/SIN-SSP particles were administered at 10^7 IU/mouse three or four times at 3-week intervals as hyperimmunization to induce antivector immunity followed by immunization with 10^4 IU/mouse of VEE/SIN-H3 replicon particles twice with an interval of 3 weeks.

For the VEE/SIN-PIV3 priming and VEE/SIN-gp140 boosting experiment, groups of 10 BALB/c mice each were immunized by i.m. administration three times with either inactivated PIV-3 virus at 10 μg per dose or VEE/SIN-PIV3 or VEE/SIN-gp140 (20) at 10^6 IU per dose. Both groups were then given two booster immunizations with VEE/SIN-gp140. The data are presented as anti-gp140 serum IgG1 and IgG2a endpoint antibody titers, with the mean of each group shown as horizontal bars (see Fig. 4).

For rabbit immunizations, two sets of 10 New Zealand White rabbits were used with two priming doses administered i.m. at weeks 0 and 4 followed by two booster doses i.m. at weeks 12 and 24. One group of rabbits received a subtype-B gp140SF162ΔV2 DNA prime i.m. and an o-gp140SF162ΔV2 protein boost i.m. The other group was immunized with VEE/SIN-gp140 for both priming and boosting i.m. Following the prime-boost regimens, the effect of preexisting antivector neutralization was evaluated by administration of three additional doses, 10^8 IU per dose, of VEE/SIN-H3 particles at weeks 31, 35, and 43.

Animals were monitored on a daily basis and euthanized when they exhibited defined humane endpoints (over 20% of body weight loss) that were preestablished for the study in agreement with Novartis Animal Welfare Policies.

ELISA. Circulating influenza H3N2 HA-, SARS-spike-, and HIV-1 gp140-specific antibodies were titrated by endpoint enzyme-linked immunosorbent assay (ELISA), by coating plates (Nunc, Roskilde, Denmark) with monovalent subunit vaccine preparation derived from RESVIR17 (at 2.5 μg/ml), the recombinant spike protein of SARS coronavirus (SARS-CoV) (at 1 μg/ml) (47), or gp140 (at 3 μg/ml). Serial 1:3 dilutions of sera were added to the coated and blocked ELISA plates. Bound anti-HA and anti-SARS-spike antibodies were detected with alkaline phosphatase-conjugated goat anti-mouse IgG (H+L), (Southern Biotechnology Associates, Birmingham, AL), followed by the *p*-nitrophenylphosphate (pNPP) substrate (Sigma/Aldrich, St. Louis, MO). Bound anti-gp140 IgG1 and IgG2a antibodies were detected with peroxidase-conjugated goat anti-mouse IgG1 or IgG2a (Southern Biotechnology Associates, AL), followed by the *o*-phenylenediamine dihydrochloride (OPD) substrate (Sigma/Aldrich, St. Louis, MO). Antibody titers were expressed as reciprocal dilutions giving an optical density at 405 nm (OD_{405}) of more than the mean OD_{405} of blank wells plus three standard deviations (SD). The blanks consistently displayed OD_{405} of <0.1 and <10% variability.

Measurement of anti-VEE/SIN neutralizing antibodies. To evaluate the level of antivector antibodies, a high-throughput assay was developed that utilized BHK-21 cells stably transfected with a β-galactosidase reporter that is activated by VEE/SIN replicon particle-encoded nonstructural proteins.

In this assay, test sera were heat inactivated for 1 h at 56°C and then duplicate samples were serially diluted 10-fold ($1{:}10^1$ to $1{:}10^6$) in Dulbecco's modified Eagle medium (DMEM) supplemented with 1% fetal calf serum (FCS) and penicillin and streptomycin. The sera were then incubated with 4×10^4 IU (multiplicity of infection [MOI], 2) VEE/SIN replicon particles expressing green fluorescent protein (GFP) for 1 h at room temperature. These samples were then used to infect β-galactosidase reporter cells that had been seeded at 2×10^4 cells per well in 96-well plates. The infected samples were incubated at 37°C at 5% CO_2 for 1 h followed by the addition of DMEM containing 10% FCS and overnight incubation at 37°C and 7% CO_2. Assay plate controls included background wells with cells and no added VEE/SIN-GFP/sera and wells with cells infected with 4×10^4 IU VEE/SIN-GFP.

Approximately 16 hours postinfection, the cells were prepared for evaluation using Galacto-Light Plus (Applied Biosystems, Foster City, CA), a chemiluminescent reporter gene assay for β-galactosidase. In this assay, the cell supernatant was removed and the cells rinsed with PBS, lysed using the provided buffer, and then frozen at −80°C. The β-galactosidase reporter assay was completed following the manufacturer's recommendations. Briefly, 0.02 ml of each lysate was added to 0.07 ml of Galacto-Light Plus dilution buffer plus Galacton-Plus and incubated for 30 min at room temperature, at which time 0.1 ml of accelerator was added. Luminosities were determined with a Trilux luminometer and reported as relative light units (RLU). The resulting RLU of each test sample was converted to percent inhibition using the formula % inhibition = [1 − (experimental RLU − background RLU)/(total RLU – back-

ground RLU)] × 100, where total RLU is the reading from the culture well infected with 4×10^4 IU VEE/SIN-GFP. The serum dilution resulting in 90% inhibition was calculated by interpolation.

HIV neutralization assays. Neutralizing antibody titers against SF162 were assessed using an M7-luciferase based neutralization assay (32). The neutralization titer is the highest serum dilution at which the relative luminescence units (RLUs) were reduced 50% compared to measurements in virus control wells (no serum sample) after subtraction of background RLU.

Western blotting. Purified VEE/SIN-H3 particles and control cells were lysed with RIPA buffer (Thermo Scientific, Rockford, IL) according to the manufacturer's instruction. The extracted proteins were separated by electrophoresis using a Criterion XT 4 to 12% Bis-Tris precast polyacrylamide gel (Bio-Rad, Hercules, CA). The protein extract from 1.5×10^5 IU of the replicon particles and 1 μg each of total cell lysates were loaded on the gel. Proteins separated in the gel were transferred to a nitrocellulose membrane using iBlot gel transfer stacks and an iBlot apparatus (Invitrogen, Carlsbad, CA). The membrane was incubated with 1:1,000 diluted mouse anti-RESVIR17 antisera, followed by incubation with 20,000-fold diluted horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (PerkinElmer, Waltham, MA). The blot was further treated with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Rockford, IL). The membrane was exposed to Amersham Hyperfilm ECL (GE Healthcare Limited, United Kingdom) to visualize the HA-specific bands.

Statistics. For statistical analysis, antibody titers were $\log_{10}$ transformed and tested for normal distribution by using the Kolmogorov-Smirnov test for continuous variables. Geometric mean antibody titers (GMTs) and the 95% confidence interval (95% CI) were derived from the anti-$\log_{10}$ of the mean, and of the mean ± SD, of the $\log_{10}$ titer transformations.

RESULTS

Protection against intranasal challenge with lethal live influenza virus following immunizations with VEE/SIN-HA. We first tested whether the chimeric replicon particles indeed protect animals from lethal challenge with live H1N1 influenza virus (A/WS/33) in mice. We prepared chimeric replicon particles with the HA sequence from A/WS/33 virus (VEE/SIN-H1). When we vaccinated BALB/c mice twice with 10^5 and 10^6 IU of VEE/SIN-H1, all mice were protected from influenza virus challenge (data not shown). In a subsequent study, we vaccinated mice either once or twice with 10^2, 10^3, or 10^4 IU of VEE/SIN-H1. Two weeks after the final vaccination, the mice were challenged intranasally (i.n.) with a dose of 300 mLD_{50} of the A/WS/33 virus. The groups of mice that received one and two doses of 10^4 IU were completely protected from the lethal influenza virus challenge. Within the group of mice that was vaccinated twice with 10^3 IU of VEE/SIN-H1, 1 of 10 animals died at 1 day after challenge. Of note, it is unlikely that the one mouse that died on day 1 of the challenge died due to the challenge, since in this model, it takes 4 to 5 days for the influenza virus to cause death. Moreover, following one vaccination with 10^3 IU and two vaccinations with 10^2 IU of VEE/SIN-H1, the mice were only partially protected from the lethal challenge. Vaccination with 10^2 IU only once did not protect the mice (Fig. 1). Based on these data, mice were vaccinated twice with 10^4 IU of VEE/SIN-H1 replicon particles to determine the effect of preexisting antivector immunity.

Effect of hyperimmunization with replicon vector on protection following vaccinations and lethal challenge. In order to induce antivector immunity prior to immunization with VEE/SIN-H1, we subcloned the sequence encoding the spike protein of severe acute respiratory syndrome coronavirus (SARS-CoV), as an antigen unrelated to HA, into the chimeric vector and prepared replicon particles expressing SARS-spike protein (VEE/SIN-SSP). The hyperimmunization induces immune responses to various components of replicon particles, including VEE virus nonstructural proteins, while only anti-envelope (SIN) antibodies are expected to neutralize cellular entry of the replicon particles. Upon replicon particle immunization, the SIN envelope glycoproteins were supplied as molecules associated with the particles and not synthesized *de novo* in infected tissues *in vivo*. Although the adjuvant effect could be expected from the RNA replication event upon particle infection, the amount of the administered envelope glycoproteins of the replicon particles as antigens was much lower than that found with standard protein immunization. For this reason, we vaccinated mice i.m. with these replicon particles repeatedly (three times, at weeks 0, 3, and 6) with a high dose, i.e., 10^7 IU/mouse, which was the highest feasible producible dose at our laboratory. After the hyperimmunization, the mice were vaccinated once or twice at 3-week intervals with 10^4 IU/mouse of VEE/SIN-H1 particles. We observed that mice vaccinated with the VEE/SIN-SSP particles were as protected as animals in the control groups that had been vaccinated with PBS (Fig. 2). Moreover, the possibility that antivector immunity might cross-protect against influenza virus infection may be excluded, as mice vaccinated with VEE/SIN-NP replicon particles were susceptible to influenza virus lethal challenge (data not shown).

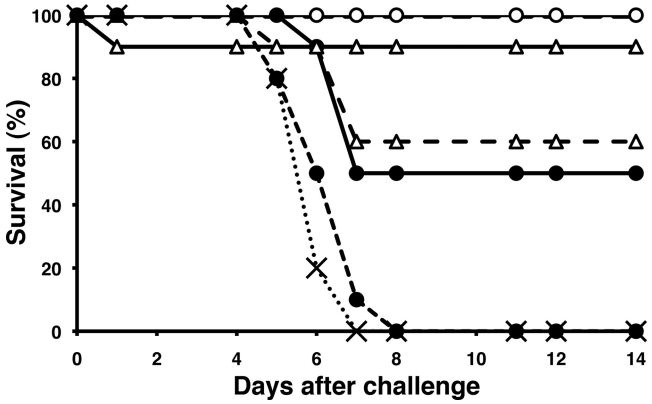

FIG 1 Protection from lethal challenges of A/WS/33 influenza virus immunized with VEE/SIN-H1. Groups of 10 mice were immunized once (dashed lines) or twice (solid lines) with 10^4 IU (open circle) 10^3 IU (open triangles), or 10^2 IU (closed circles). A group was also treated twice with PBS (dotted line with crossed symbols). Two weeks after the final immunization, mice were challenged with 300 mLD_{50} of A/WS/33 influenza virus intranasally. After the challenge, the number of surviving animals from each group was monitored.

No interference of antivector immunity for inducing humoral immune responses by replicon particle vaccination. Due to a lack of reagents to study immunogenicity against the HA protein from the influenza A/WS/33(H1N1) strain, we repeated the above-described experiment with the influenza RESVIR17 (H3N2) strain. We vaccinated mice i.m. with VEE/SIN-SSP replicon particles with the dose of 10^7 IU/mouse four times (weeks 0, 3, 6, and 9). We detected high titers of anti-SARS-spike antibodies in the immunized animals, indicating that the vaccination had been successful (Fig. 3A). The mice were subsequently vaccinated i.m. with 10^4 IU of VEE/SIN-H3, particles prepared with the HA sequence from RESVIR17 (H3N2). HA-specific antibody titers of the sera from these animals were used to evaluate the suppressive

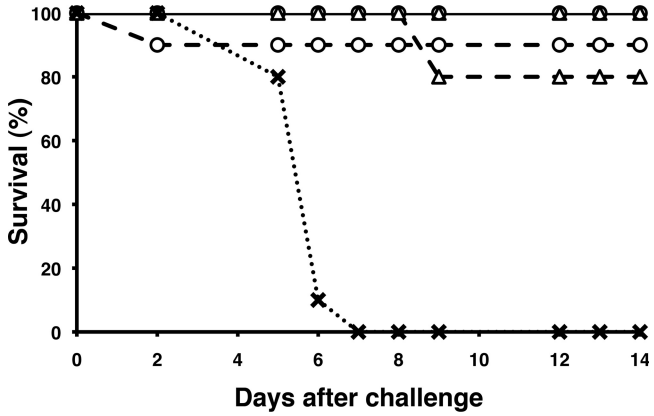

FIG 2 Protection from lethal challenges of A/WS/33 influenza virus hyperimmunized with VEE/SIN-SSP followed by immunization with VEE/SIN-H1. Groups of 10 mice were immunized three times with 10^7 IU of VEE/SIN-SSP replicon particles (open circles) or PBS (open triangles). The treated mice were further immunized once (dashed lines) or twice (solid lines) with 10^4 IU of VEE/SIB-H1 replicon particles. A group was also treated 5 times with PBS (dotted line with crossed symbols). Two weeks after the final immunization, mice were challenged with 300 mLD_{50} of A/WS/33 influenza virus intranasally. After the challenge, the number of surviving animals from each group was monitored.

effect of the prior hyperimmunization. Interestingly, the animals vaccinated with the VEE/SIN-SSP yielded immune responses specific for the H3 protein that were as high as those of the animals from the control group, which were vaccinated with PBS (Fig. 3B). Since RESVIR17 (H3N2) does not infect mice, we measured hemagglutination inhibition (HI) titers to evaluate production of functional antibodies instead of performing challenge experiments. Although before immunization with VEE/SIN-H3 particles, some mice (i.e., one hyperimmunized and all control mice showing HI titers of 40) showed marginal HI activity in serum, hyperimmunized mice developed significant serum HI activity similar to those detected in sera of PBS control mice after VEE/SIN-H3 particle immunization (Table 1). The vector neutralization titers varied from 138 to 20,421. However, there was no correlation between the alphavirus neutralization titer and the HA-specific ELISA titer.

In order to exclude the possibility of HA contaminated in the particle preparation, which would be potentially able to induce anti-HA immune responses, we performed a Western blot experiment and confirmed there is no detectable HA protein in the replicon particles even in larger IU than were administered to each mouse (Fig. 3C). One possible explanation for the unaffected humoral responses in the hyperimmunized mice was that the immunization with VEE/SIN-SSP failed to mount neutralizing antibodies *in vivo* to the SIN virus envelope protein, which the chimeric replicon particles utilize for infection. To address this possibility, we analyzed the sera for neutralizing titers against infection with chimeric replicon particles *in vitro*. Surprisingly, all animals possessed a sufficient number of neutralizing antibodies to prevent infection of replicon particles *in vitro* (Table 1). These data indicate that preexisting antivector immunity does not interfere with protective immune responses after immunization using the same alphavirus replicon vector.

We next sought additional support for the lack of a preexisting-

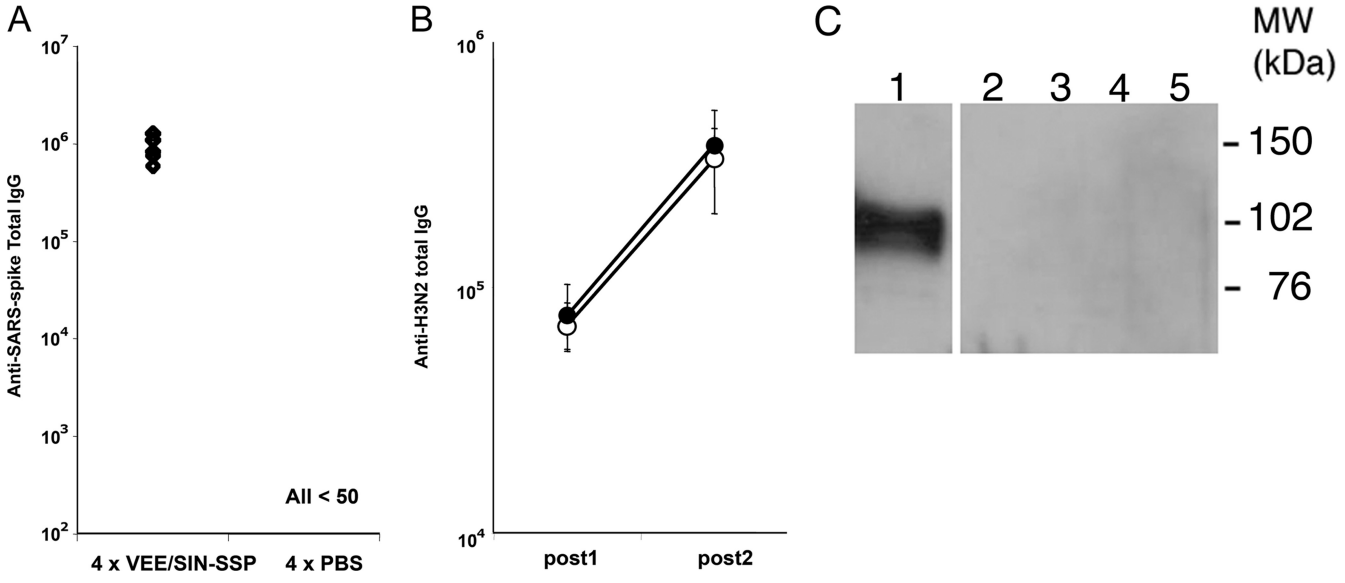

FIG 3 (A) Total IgG titers specific for the spike protein of SARS-CoV. The titer was individually plotted from five hyperimmunized (10^7 IU/mouse, 4 times, i.m.) mice. Sera were assayed after 2 weeks of the hyperimmunization. In the control group (PBS), mice were treated with PBS. (B) Comparison of total immunoglobulin titers specific for the subunit monovalent vaccine preparation derived from RESVIR17 (H3N2) between hyperimmunized and control mice. The titers were indicated as geometric means of results for 5 mice hyperimmunized with VEE/SIN-SSP (open circles) or treated with PBS (closed circles) before immunization with 10^4 IU of VEE/SIN-H3. The 95% CIs are also indicated with vertical bars. Sera were assayed after 2 weeks of the first (post1) and the second (post2) immunization with VEE/SIN-H3. (C) An undetectable level of HA protein contamination in the alphavirus replicon particle preparation. Extracted proteins from the alphavirus replicon particles preparation used in this study (VEE/SIN-H3) and control proteins expressed in BHK cells were separated in a 4 to 12% polyacrylamide gel. The HA-specific band (noncleaved HA0, ca. 85 kDa) was visualized with mouse anti-RESVIR17 (H3N2) serum. Lane 1, BHK lysate infected with VEE/SIN-H3 (MOI, 4); lane 2, BHK lysate infected with VEE/SIN-NP (MOI, 12); lane 3, BHK lysate infected with VEE/SIN-NP (MOI, 4); lane 4, noninfected BHK lysate; lane 5, 1.5×10^5 IU VEE/SIN-H3.

TABLE 1 Anti-replicon neutralization (neut) titers and HI titers of individual sera in hyperimmunized and control mice[a]

Pre-VEE/SIN-SSP anti-replicon neut titers		Post-VEE/SIN-SSP Pre-VEE/SIN-H3				Post-VEE/SIN-H3 HI titers	
		Anti-replicon neut titers		HI titers			
Hyperimmunized	Control	Hyperimmunized	Control	Hyperimmunized	Control	Hyperimmunized	Control
<10	<10	7,656	<10	<10	40	320	160
<10	<10	20,421	<10	<10	40	480	320
<10	<10	171	<10	40	40	480	320
<10	<10	138	<10	<10	40	320	320
<10	<10	2,437	<10	<10	40	320	320

[a] Serum neutralization titers were determined at 90% inhibition of infection of BHK cells. The hemagglutination inhibition (HI) assay (10), which is known to give values correlating with protection, was used to evaluate protection in serum immunized with antigens derived from virus that cannot infect the animal. The hyperimmunized group was treated four times with 10^7 IU of VEE/SIN-SSP replicon particles. In the control group, mice were treated with PBS before immunization with VEE/SIN-H3.

antivector effect on the immune responses following vaccinations with the same vector bearing a different antigen. For this purpose, we determined whether the three priming i.m. vaccinations with VEE/SIN-PIV3 would result in decreased serum antibody responses against two subsequent i.m. vaccinations with VEE/SIN-gp140, i.e., the same VEE/SIN vector expressing HIV-1 gp140 instead of PIV3. We found that there was no statistically significant difference in the anti-gp140 serum IgG1 or IgG2a responses in the group that was vaccinated three times i.m. with inactivated hPIV3 compared to those for the group vaccinated with VEE/SIN-PIV3 (Fig. 4A and B).

To further assess the effect of preexisting antivector responses in different species of animals, we vaccinated i.m. a group of rabbits with four priming doses of VEE/SIN-gp140 followed by three i.m. boosting doses of VEE/SIN-H3. In addition, a second group of rabbits were also vaccinated i.m. with three doses of VEE/SIN-H3. This second group of rabbits was previously vaccinated with two doses of gp140SF162ΔV2 DNA followed by two doses of o-gp140SF162ΔV2 protein and served as an alphavirus-naïve control group. Both the vector-naïve and the vector-preexposed rabbits elicited strong anti-HA antibody responses (Fig. 5). Following the four priming vaccinations, all rabbits from the first group induced high titers of antibodies against SF162 envelope as well as high levels of antivector neutralizing responses (data not shown). These data show that vaccinations of mice or rabbits with VEE/SIN replicon particles encoding one antigen did not cause a decrease in serum antibody titers against a new antigen following subsequent vaccinations with VEE/SIN replicon particles encoding the new antigen.

DISCUSSION

There are a number of publications evaluating alphavirus-based replicon particle-based vaccines for immunogenicity as well as protective efficacy against a variety of infectious agents, including human immunodeficiency virus (11, 12, 19, 20, 50, 52, 54, 55), cytomegalovirus (42, 43), respiratory syncytial virus (9, 14, 31), measles virus (35, 36), parainfluenza virus (17, 18), and influenza virus (1, 22, 41). Furthermore, the VEE/SIN replicon particles have demonstrated higher cellular immune responses over SIN virus-based replicon particles using the gag protein of human immunodeficiency virus (38). The chimeric replicon particles used in the present study were also able to show improved humoral responses compared to SIN virus replicon particles (see Table S1 in the supplemental material) and successfully demonstrated protective immune responses in different preclinical studies (5, 17, 18, 35). The VEE/SIN replicon particles have two major advantageous characteristics. First, the chimeric replicon particle expresses the replicase from VEE virus, which is known to be less sensitive to type 1 interferon (IFN) (38), thus enabling it to replicate more efficiently than the SIN virus-based replicon particle. This mechanism most likely leads to larger amounts of protein expression of the gene of interest. Consequently, the chimeric rep-

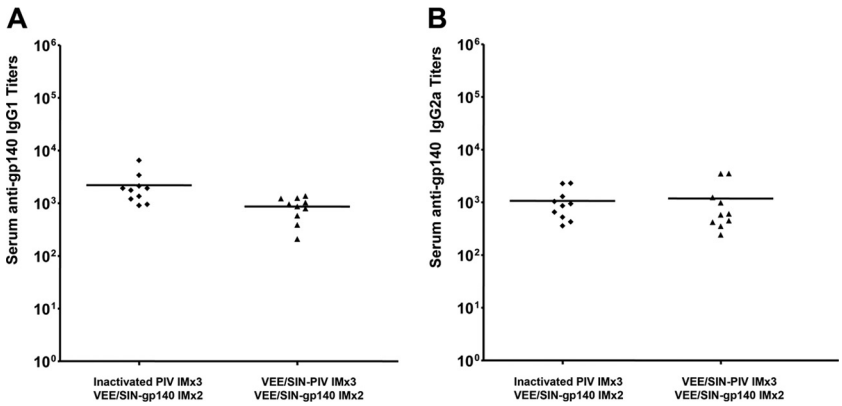

FIG 4 No decrease of immunogenicity against on antigen encoded in the VEE/SIN vector after multiple immunizations with the same vector encoding another antigen. Groups of 10 BALB/c mice each were immunized three times with either inactivated PIV-3 virus or VEE/SIN-PIV3. Both groups were then given two booster immunizations with VEE/SIN-gp140. The data are presented as anti-gp140 serum IgG1 (A) and IgG2a (B) endpoint antibody titers, with the mean of each group as horizontal bars. In both measurements, there were no significant differences in IgG titers ($P > 0.05$).

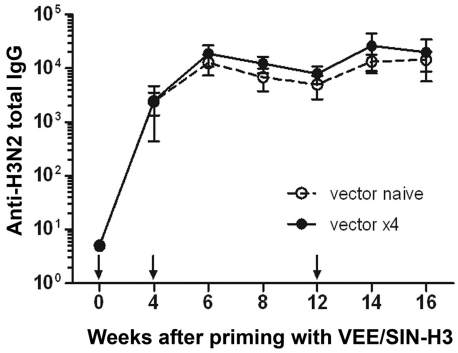

FIG 5 Lack of effect of the antivector immunity in rabbits. The vector-naïve group of New Zealand White rabbits ($n = 10$) were given two priming doses of a subtype-B gp140SF162ΔV2 DNA at weeks 0 and 4, followed by immunization of two booster doses with o-gp140SF162ΔV2 protein at weeks 12 and 24. The vector-immunized group ($n = 10$) were immunized with 10^8 IU of VEE/SIN-gp140 at weeks 0, 4, 12, and 24. Following the prime-boost regimens, the effect of preexisting antivector neutralization was evaluated by administration of three additional doses, 10^8 IU per dose, of VEE/SIN-H3 particles at weeks 31, 35, and 43 (weeks 0, 4, and 12, counting from the first VEE/SIN-H3 administration, indicated with arrows). All immunizing reagents were administered through i.m. routes. HA antibody titers were monitored following immunization with VEE/SIN-H3 in the presence (closed circles) or absence (open circles) of antivector immune responses.

licon particles offer stronger antigen presentation to the host immune system. Second, the envelope proteins of the chimeric replicon particles are derived from those of SIN virus with a point mutation to more efficiently infect dendritic cells (DCs) (16). Therefore, the use of the chimeric replicon particles also adds an advantage to the immunogenicity, since DCs are pivotal professional antigen-presenting cells. When the replicon RNA starts to replicate in the host cell, it forms double-stranded RNA intermediates, which are the ligand of toll-like receptor (TLR) 3. The signaling through the TLR leads to production of interleukin 12, which is an upstream cytokine leading to IFN secretion in turn leading to Th1 and subsequently to cytotoxic T cell responses (23, 26, 34). The scenario of immune activation by replicon particles is thus more natural for the immune system, by mimicking viral infection, but without causing actual disease.

Although the high potency of alphavirus replicon particles is advantageous to induce better protective immunity, such robust immune responses might induce antivector immunity that would reduce vaccine efficacy when administered repeatedly. The present study addressed this issue. To set up challenge and immunization conditions we selected a high lethal challenge dose (300 mLD_{50}/mouse) of influenza virus and determined an immunization dose to ensure the detection of decreased efficacy due to antivector immunity. We observed that vaccinations with relatively small doses of replicon particles are enough to protect against the lethal challenge with a relatively excessive dose of A/WS/33 influenza virus (Fig. 1). The protective replicon particle doses depend on the challenge dose (data not shown); therefore, it is possible to further reduce immunization doses if mice are challenged with lower virus doses. The virus was administered i.n. without anesthesia, in order to target and infect the upper respiratory tract. The replicon particles were also protective when mice were challenged with anesthesia to infect the lower respiratory tract (data not shown).

The robust immunogenicity induced with alphavirus replicon particles shown in the present study as well as in the past report (38) offers a potential benefit for immunodeficient individuals such as newborns, the elderly, and individuals with immunosuppressive conditions. The efficacy of VEE-based replicon particles in the immature immune system was demonstrated in chicken embryos and young birds (45). The data lead to the hypothesis that alphavirus replicon particles may also be a good alternative for induction of effective immune responses in immunocompromised individuals. Hence, testing alphavirus replicon particles in different age groups in mammals is warranted.

Interference of preexisting antivector immunity with vaccine efficacy is one of the most important obstacles against the application of viral vectors as vaccine delivery systems. For example, a significant proportion of the human population already has antibodies against a number of adenovirus serotypes (37). The broad seroprevalence of the human population may limit application of the vector. In the case of alphaviruses, the viruses are not commonly infecting the human population and most individuals are negative for anti-alphavirus immunity. Furthermore, the present study using HA of influenza virus as a vaccine antigen showed that repetitive immunization of the chimeric replicon particles induced antivector immunity, whereas efficacy of the vaccine was not affected (Fig. 2). Although contaminated HA protein was not detected in the replicon particle preparation used for the immunization, it is still possible to argue that a trace amount of protein incorporated in the particles might be sufficient to induce immune responses. However, in the absence of adjuvant, the subunit vaccine preparation cannot yield specific anti-HA IgG responses as high as those of replicon particle-based immunization (see Table S1 in the supplemental material). Moreover, VEE/SIN-H3 that had been accidentally stored on dry ice for 2 days and had lost their infectivity were immunized to mice but failed to induce specific humoral immune responses to HA (data not shown). These data and observation further suggest that the presence of neutralizing antibodies against the VEE/SIN vector (SIN envelope) did not affect the induction of antibodies against HA encoded in the replicon particles.

We also confirmed the lack of the effect of antivector immunity using a different antigen combination (Fig. 4) and different animal species (Fig. 5). Rhesus macaques could also be boosted to increase immune responses by multiple administrations of replicon particles, although the effects of antivector immunity were not directly addressed (54). Thus, the alphavirus replicon particles may be applicable to humans for multiple administrations without decreasing efficacies. This characteristic is of particular advantage for some pathogens, such as influenza viruses, which very often change their antigenicity (antigenic drift); anti-influenza vaccines must be prepared according to the prediction of an outbreak every year. Moreover, the alphavirus replicon particle platform is suitable for preparing vaccines against influenza pandemics; it handles only a part of the genetic material from aggressive influenza strains, similar to the revolutionary reverse genetics (RG) approach (21). While the RG is used with live viruses (although they are supposed to be attenuated) to isolate subunit proteins, there is no step of producing infectious influenza virus in the replicon platform. This approach, therefore, grants further safety for preparing the vaccines. Virus-like particles (VLPs) constitute another potential vaccine platform (29, 40, 44). The technology is similar in safety to the replicon platform, as no influenza

virus is produced, although production of VLPs requires more genes from influenza virus (HA, NA, and M1).

Our results are supported by a study demonstrating that newborn animals that possessed maternal anti-VEE virus neutralizing antibodies successfully mounted anti-dengue immune responses by immunizing VEE-based replicon particles expressing a dengue virus antigen (51). More recent studies demonstrated that multiple administrations of alphavirus replicon particles induced both vector neutralization responses and functional antibodies against the products from the genes of interest encoded in the vector (6, 33). However, in both studies antivector immunity was elicited with immune responses to the gene products of interest simultaneously. The present study directly addressed the effect of preexisting antivector immunity.

It is possible to argue that the hyperimmunization did not induce antivector immunity. *In vitro* neutralization might be attributed rather to antibodies against molecules that function as receptors for alphavirus entry. In fact, the replicon particles are prepared using BHK-V cells, a cell line derived from BHK-21 cells, which are used for the SIN neutralization assay in the present study. We cannot directly exclude the possibility of carryover of receptor molecules from production cell lines during the particle preparation. However, we did not detect anti-bovine serum antigen (BSA) antibodies from particle-immunized mouse sera (data not shown). If the particle preparations eliminated BSA, the major component of the culture media, and no anti-BSA antibodies were induced in immunized mice, it was less likely that antibodies against other molecules like cellular receptors were induced.

How can replicon particles stimulate the host immune system in the presence of anti-alphavirus neutralizing antibodies? One possible explanation is that the site of immunization (the muscle tissue) is not very accessible for neutralizing antibodies. Another possibility is that molecules involved in host-particle interaction *in vivo* may not be the same *in vitro*. It is known that some tissues express Fc receptors, which could be instrumental for antibody-mediated alphavirus particle entry to the target cells and could bypass the viral receptor-mediated infection *in vivo*. A recent report showed that the alphavirus replicon can migrate quickly to draining lymph nodes after administration (49). The authors speculated that DCs in muscle tissue may play a role in such migration, although it was not discussed whether DCs can circumvent particle neutralization from antivector antibodies. To understand the exact mechanism, studies of the microenvironment at the site of particle administration, including localization of expression of virally encoded proteins, and kinetics of inflammatory cell recruitment would also be required.

Although safety issues of alphaviruses as a vaccine vector are not addressed in this paper, it is also critical for clinical application of the platform. It has been stated that no pathogenic effect was observed upon replicon particle immunization (25). In the present study, we noticed early deaths after challenge in the group immunized with 10^3 IU (Fig. 1) and the group of hyperimmunized animals (10^7 IU with VEE/SIN-SSP followed by 10^4 IU with VEE/SIN-H1) (Fig. 2). Are these a sign of adverse effects caused by particle immunization? We think that this is unlikely the case because there is no dose dependence and even animals from PBS control groups died 1 day after challenge in independent experiments (data not shown). We consider that the early death event may be associated with the handling of animals when they are challenged. Otherwise we observed neither abnormal behaviors nor pathological signs in animals vaccinated with replicon particles. A recent clinical safety study of VEE replicon particles showed that only one serious adverse event, which was not associated with the study agent, had been observed in a total of 32 healthy volunteers who received as many as 1×10^8 IU of replicon particles three times (6).

In conclusion, our data demonstrated that the replicon particles can be used repeatedly because preexisting anti-alphavirus immunity did not affect the efficacy of the replicon particles as vaccines. Therefore, the chimeric replicon particles are a promising vaccine delivery technology platform.

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