Generation of a More Immunogenic Measles Vaccine by Increasing Its Hemagglutinin Expression

Emily Julik, Jorge Reyes-del Valle
School of Life Sciences, Arizona State University, Tempe, Arizona, USA

ABSTRACT
Imported measles virus (MV) outbreaks are maintained by poor vaccine responders and unvaccinated people. A convenient but more immunogenic vaccination strategy would enhance vaccine performance, contributing to measles eradication efforts. We report here the generation of alternative pediatric vaccines against MV with increased expression of the H protein in the background of the current MV vaccine strain. We generated two recombinants: MVvac2-H2, with increased full-length H expression resulting in a 3-fold increase in H incorporation into virions, and MVvac2-Hsol, vectoring a truncated, soluble form of the H protein that is secreted into the supernatants of infected cells. Replication fitness was conserved despite the duplication of the H cistron for both vectors. The modification to the envelope of MVvac2-H2 conferred upon this virus a measurable level of resistance to in vitro neutralization by MV polyclonal immune sera without altering its thermostability. Most interestingly, both recombinant MVs with enhanced H expression were significantly more immunogenic than their parental strain in outbred mice, while MVvac2-H2 additionally proved more immunogenic after a single, human-range dose in genetically modified MV-susceptible mice.

IMPORTANCE
Measles incidence was reduced drastically following the introduction of attenuated vaccines, but progress toward the eradication of this virus has stalled, and MV still threatens unvaccinated populations. Due to the contributions of primary vaccine failures and too-young-to-be-vaccinated infants to this problem, more immunogenic measles vaccines are highly desirable. We generated two experimental MV vaccines based on a current vaccine’s genome but with enriched production of the H protein, the main MV antigen in provoking immunity. One vaccine incorporated H at higher rates in the viral envelope, and the other secreted a soluble H protein from infected cells. The increased expression of H by these vectors improved neutralizing responses induced in two small-animal models of MV immunogenicity. The enhanced immunogenicity of these vectors, mainly from the MV that incorporates additional H, suggests their value as potential alternative pediatric MV vaccines.

Despite the existence of an effective vaccine, measles virus (MV) infections remain an insidious threat to global health. After decades of reduction in measles-related mortality due to vaccination, this has stalled at between 110,000 and 120,000 deaths/year since 2012. Approximately 20 million people are infected by measles each year, with significant morbidity and up to 1% mortality (1). In 2014, the United States saw the highest number of annual measles cases since native transmission was declared locally eliminated in 2000 (2) and experienced a large multistate outbreak in the first months of 2015 (3).

Protecting young infants against measles is an important goal to attain to improve the current MV vaccine (4). The highest fatality rates due to MV infection occur in infants <1 year of age (5). Ironically, increasing vaccine coverage also increases the proportion of young infants who are susceptible to MV infection. Infants retain antibodies passed from their mothers across the placenta and in breast milk for months after birth, and these antibodies protect against early-life infection but interfere with early vaccination (6). Only ~85% of 9-month-olds (7) and 65% of 6-month-olds mount antibody responses to the vaccine (8). This level of efficiency proves prohibitive for a public health intervention that must balance cost against protection; infants are therefore vaccinated at 9 months of age in areas where there is great risk of measles transmission or at 12 to 15 months of age where there is not a significant risk of infection.

Young infants can, however, mount protective immune responses to current MV vaccines. Clinical trials revealed a dose-dependent, statistically significant increase in seroconversion with increasing measles vaccine titers (9), thus demonstrating that the increased immunogenic stimulus of the higher-titer vaccine was sufficient to overcome barriers to MV immunization in young infants. Although efficient, the use of high-titer MV vaccines was withdrawn following observations of increased mortality rates not related to wild-type MV infection in female recipients (10–14; reviewed in reference 15). Here, we show a strategy to present an enhanced immunogenic stimulus within the safe context of the current vaccine dosage.

For the Paramyxoviridae, the progressive transcriptional attenuation from the 3′-end leader region of the negative-sense genome determines the expression level of viral proteins. Cistrons encoding the structural envelope proteins, which are the principal targets of protective neutralizing immunity, are located in the second- or third-to-last positions of the genomes of paramyxoviruses, resulting in relatively low expression levels (16). Envelope
protein expression can be augmented by increasing the copy number of their coding cistrons and/or by moving these cistrons proximally within the viral genome (17–27). To develop an MV vaccine containing a greater immunogenic stimulus within the standard WHO-approved dosage, we constructed two recombinant MVs that express an additional copy of the hemagglutinin (H) protein in a membrane-bound or soluble form from the second position of a Moraten strain-equivalent genome, resulting in increased expression and incorporation or secretion of H while maintaining replicative fitness and stability. We show here that both viruses induce significantly stronger neutralizing antibody responses in non-MV-susceptible mice. The vector expressing additional envelope-bound H also proved more immunogenic in an MV-susceptible mouse model and shows resistance to marginal levels of neutralizing antibodies in vitro.

**MATERIALS AND METHODS**

**Cells and viruses.** Vero/hSLAM cells (28, 29) were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with 5% fetal bovine serum (FBS; Atlanta Biologicals, Flowery Branch, GA), 1% penicillin-streptomycin (PS; Sigma-Aldrich, St. Louis, MO), and 0.5 mg/ml G418 (Enzo Life Sciences, Farmingdale, NY). Helper 293-3–46 cells (30) were maintained in DMEM with 10% FBS, 1% PS, and 1.2 mg/ml G418.

Recombinant MVs were rescued according to the method of Radecke et al. (30), as modified by Parks et al. (31). After detection of cytopathic effects in mixed cultures of Helper 293-3–46 and Vero/hSLAM cells, individual syncytia were transferred to and propagated in Vero/hSLAM cells. To prepare stocks of the viral clones thus generated, Vero/hSLAM cells were infected at a multiplicity of infection (MOI) of 0.03 and incubated at 37°C. When ~80% cytopathic effect was observed, cells were scraped into Opti-MEM (Life Technologies, Grand Island, NY), and viral particles were released by two freeze-thaw cycles.

Multistep growth kinetics of the viruses were measured by infecting Vero/hSLAM cells at an MOI of 0.03 and incubating them at 37°C. Supernatants and infected cells were collected and lysed by a single freeze-thaw cycle at the prescribed times postinfection, and the 50% tissue culture infective dose (TCID₅₀) was assessed in Vero/hSLAM cells by using the Spearman-Karber endpoint dilution method (32).

**Construction and recovery of recombinant MVs.** The recombinant MVs were constructed in the background of pH(+)MVvac2(HBsAg)N (33). The MV genome coding capacity of this plasmid is identical to those of the Moraten and Schwartz vaccine strains (34), with an additional transcription unit (ATU) inserted downstream of the nucleocapsid (N) cistron to direct the expression of an inserted foreign gene. To obtain the recombinant MVs with higher H expression levels, MluI and AatII digestion was used to swap the HBsAg insert with a modified H cistron obtained by PCR. To generate the insertion for pH(+)MVvac2(H2), the H coding sequence was amplified by PCR from pH(+)MVvac2(HBsAg)N using forward primer 5′-ACTGACGGTCAAGGGTGCAAGATCATCGA3′ and reverse primer 5′-ACTGACGGTTCACTCTAGTGGGATGGTCTGGTCTCGAGTTCTCCTTTGAC3′ to amplify the entire transcribed region of H with 18 added nucleotides encoding a carboxy-terminal tag composed of six histidines (His tag). To generate the insertion for pB(+)-MVvac2-H2, the same reverse primer was used in conjunction with forward primer 5′-ACTGACGGTATTTGTACAGGATGCAACTTCTGTGTCATCGATCGCAGCACGCGTTCTGTGCTGATGGTCTGGTCTGAAATCGGCAC3′ to amplify the portion of the H cistron corresponding to the H stalk and ectodomain with an additional sequence encoding a carboxy-terminal His tag and an amino-terminal signal sequence from interleukin-2 (the MluI and AatII restriction sites are underlined). Sanger sequencing confirmed the correct junctions and coding sequences within the ATUs.

We constructed in the background of pB(+)MVvac2(ATU)N two additional viruses used as controls here, each encoding a fluorescent protein at the same locus as the insertions in pB(+)MVvac2-H2 and pB(+)MVvac2-Hsol. We inserted an enhanced green fluorescent protein (EGFP) coding sequence as well as a red fluorescent protein (RFP) (tandem dimer Tomato, obtained initially by PCR from pCS2-dTO) coding sequence into the ATU downstream of the N cistron. Expression of both proteins in cells infected with the corresponding virus was confirmed by epifluorescence microscopy.

**Western blot analysis.** For protein expression analysis, 1 × 10⁶ Vero/hSLAM cells were seeded into 100-mm-diameter dishes and infected at an MOI of 0.3 with MVvac2-H2 or MVvac2(EGFP)N or were mock infected. Forty hours after infection, cells were washed three times with phosphate-buffered saline (PBS) and then lysed with RSB–NP-40 buffer (1.5 mM MgCl₂, 10 mM Tris–HCl, 10 mM NaCl, and 1% Nonidet P-40; Sigma-Aldrich, St. Louis, MO) plus protease inhibitors (Complete Mini protease inhibitor tablets; Roche Diagnostics, Manheim, Germany). The protein extracts were mixed with Laemmli buffer (Bio–Rad Laboratories, Hercules, CA) containing β-mercaptoethanol and denatured at 96°C for 10 min.

To purify viral particles for protein incorporation analysis, the supernatants of 5 × 10⁶ cells infected with the parental strain MVvac2, MVvac2-H2, or MVvac2(RFP)N were initially clarified by centrifugation at 5,000 × g for 15 min in an SW28 rotor. The clarified supernatants were layered over a 2-ml 20% sucrose cushion, and viral particles were pelleted by ultracentrifugation at 104,000 × g for 2 h in an SW28 rotor. The pellets were resuspended in TNE buffer (10 mM Tris [pH 7.8], 100 mM NaCl, 1 mM EDTA), and the infectivity of each particle preparation was determined by an endpoint dilution assay. Purified particles were mixed with 6X sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) sample buffer (0.35 M Tris [pH 6.8], 30% glycerol, 10.28% SDS, 0.6 M dithiothreitol, 0.012% bromphenol blue) and denatured at 96°C for 10 min.

Protein extracts or purified virion lysates were separated by SDS-PAGE in a 4 to 15% or 10% acrylamide gel, respectively. Protein extracts were loaded in volumes that achieved equalized expression levels of F and N, while for the viral particles, 2.5 × 10⁶ TCID₅₀ were loaded for each sample. Following electrophoretic separation, proteins were transferred to nitrocellulose for immunoblotting using a 1:20,000 dilution of rabbit polyclonal anti-MV N, a 1:7,500 dilution of rabbit polyclonal anti-MV F, and a 1:10,000 dilution of rabbit polyclonal anti-MV H (35), or a 1:10,000 dilution of mouse antiserum (Sigma–Aldrich, St. Louis, MO), followed by the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (GE Healthcare, Little Chalfont, United Kingdom). Additional immunoblotting was performed by using a 1:5,000 dilution of anti-His(C terminus)-HRP (Invitrogen, Carlsbad, CA). Reactions were developed using a chemiluminescence kit (SuperSignal West Pico chemiluminescent substrate; Pierce Biotechnology, Rockford, IL).

**Immobilized cobalt ion affinity chromatography.** To isolate 6XHis-tagged soluble H protein from the supernatants of MVvac2-Hsol-infected cells, 100-mm-diameter dishes were seeded with 1 × 10⁶ Vero/hSLAM cells and infected with MVvac2 or MVvac2-Hsol at an MOI of 0.1. Supernatants and cells were collected at 24-h intervals and lysed by a single freeze-thaw cycle, and supernatants were clarified by centrifugation at 3,000 × g for 5 min in an SW28 rotor. Talon Superflow nickel affinity resin (Clontech Laboratories, Mountain View, CA) equilibrated in interaction buffer (50 mM NaH₂PO₄ [pH 8], 200 mM NaCl, 1% Triton X-100) was added to the clarified supernatants for 2 h of batch interaction. The resin was subsequently washed four times in washing buffer (50 mM NaH₂PO₄ [pH 8], 500 mM NaCl, 5 mM imidazole, 1% Triton X-100), followed by a final wash in 0.5× PBS. The resin was resuspended in 6X SDS-PAGE sample buffer, incubated at 96°C for 10 min, and then separated by SDS-PAGE in a 10% acrylamide gel. The electrophoretically separated proteins were visualized by staining with Coomassie brilliant blue G-250. A replicate gel was transferred to nitrocellulose and subjected to immunoblotting for the 6XHis tag as described above to visualize soluble H protein.

**MATERIALS AND METHODS**
Preparation of viral particles for thermostability assays and animal experiments. To generate high-titer preparations of purified viral particles, virus stocks were layered over 1 ml 20% sucrose cushions through which viral particles were pelleted by ultracentrifugation at 147,000 x g for 2 h in an M-50 rotor. Pellets were resuspended in TNE buffer and titrated by an endpoint dilution assay.

Thermostability assay. To test the thermostability of recombinant MVs, particles of MVvac2 and MVvac2-H2 were diluted in Opti-MEM to generate aliquots of ~1 x 10^5 TCID_{50}/mL. Aliquots of each virus were treated by incubation at 4°C, 24°C, 37°C, 45°C, or 96°C for 1 h. Additionally, one aliquot of each virus was titrated immediately to determine the baseline titers. Titers were determined by measurement of PFU generated in plaque assays. Briefly, serial 10-fold dilutions of the untreated or treated viruses were inoculated in duplicate onto Vero/hSLAM cells in 24-well plates. After allowing 2 h for infection, the plates were overlaid with carboxymethylcellulose (CMC) in DMEM to bring the final concentration of CMC in the plate to 0.75%. Five days later, the titer was determined by counting the plaques per milliliter virus applied in the first well with discrete countable plaques, averaging the titers from the two tests. Infectivity was calculated as the PFU per milliliter in the treated sample as a percentage of the PFU per milliliter in the untreated sample for that virus.

In vitro neutralization of viruses with MV-immune sera. To test the susceptibility of our recombinant MVs to neutralization by anti-MV antibodies, we performed a modified logarithmic neutralization index assay using sera of defined MV neutralization titers from mice that had been immunized with MVvac2. In a 96-well plate, 11000 dilutions of sera (MV neutralization titers of 1:33, 1:80, 1:133, 1:213, 1:427, 1:640, and 1:1066) in Opti-MEM were incubated with 1 x 10^5 TCID_{50} of MVvac2, MVvac2-H2, or MVvac2-Hsol for 1 h at 37°C. Following incubation, serial 10-fold dilutions of the serum-virus mixture were generated and then transferred onto Vero/hSLAM cells and incubated at 37°C for 3 days, at which time infectivity in the samples was assessed as the TCID_{50}.

Mouse inoculations. All experimental procedures were performed according to a protocol approved by the Arizona State University Institutional Animal Care and Use Committee. To determine the immunogenicity of recombinant MVs in a nonsusceptible small-animal host, groups of eight CD1 mice (Jackson Laboratories, ME) were inoculated initially by the intraperitoneal (i.p.) route with 10^3 TCID_{50} of purified viral particles in 100 μl of Opti-MEM. Three weeks after the initial inoculation, the mice received a second i.p. dose of 10^4 TCID_{50} of purified particles in Opti-MEM. Two weeks after the second dose, mice were bled. Sera were separated and heat inactivated at 56°C for 1 h. To determine the immunogenicity of recombinant viruses in an MV-susceptible model, groups of six HuCD46Ge-IFNarKO mice (36) were inoculated by the i.p. route with 10^6 TCID_{50} of the viruses in 100 μl of Opti-MEM. Mice were bled at 28 days postinoculation, and sera were separated and heat inactivated.

MV microneutralization assay. Serial 2-fold dilutions of heat-inactivated sera in Opti-MEM were incubated with 100 TCID_{50} of MVvac2 for 1 h at 37°C. Vero/hSLAM cells were added to the serum-virus mixtures, and the plates were then incubated at 37°C for 3 days, at which time the neutralization titer was assessed as the highest dilution of serum capable of complete neutralization of infectivity, defined as the absence of cytopathic effect. Neutralization titers were reported as the averages of determinations made in triplicate.

MV ELISA. Overall anti-MV IgG reactivity from available individual samples was determined with a mouse-adapted semiquantitative enzyme-linked immunosorbent assay (ELISA) kit (Alpha Diagnostic International, San Antonio, TX) and expressed as arbitrary ELISA units (AEU) per milliliter by comparison with internal standards supplied by the manufacturer.

RESULTS

Generation of measles vaccine vectors encoding additional H protein in the second position of the genome. We hypothesized that H expression could be augmented by inserting an additional copy of the H-encoding sequence at a high-expression locus in the viral genome. To generate recombinant MV vaccines with increased expression of H, we made use of a previously described MV cDNA identical in amino acid sequence to the Moraten vaccine strain with an inserted additional transcriptional unit (ATU) downstream from the N cistron (34). Initially, an additional copy of the full-length Moraten H-encoding sequence was cloned into this ATU, yielding pB(+)MVvac2-H2 (Fig. 1A). To distinguish expression at the ATU from expression of H at its native locus in the viral genome, we tagged the extracellular carboxy terminus of H with six histidines (His tag), increasing the length of the additional H copy to 623 amino acids. By this approach, we expected pB(+)/MVvac2-H2 to generate recombinant MVs incorporating additional H into the viral envelope. We then generated a second vector encoding a modified copy of H from the same ATU locus. Here, we exchanged the amino-terminal cytoplasmic tail and transmembrane region of the additional H (58 amino acids) with 20 amino acids corresponding to the signal sequence of interleukin-2. The modified copy of H was again marked with a carboxy-terminal His tag for a total length of 565 amino acids following cleavage of the signal sequence. We expected this recombinant genome to direct the secretion of soluble forms of H (Hsol) from infected cells and thus named it pB(+)MVvac2-Hsol (Fig. 1A).

To test if the increased cellular expression of H resulted in increased incorporation of H into virions, we performed a similar analysis using purified particles of MVvac2-H2, with MVvac2 and MVvac2(RFP)N as controls. Western blot detection of 2.5 x 10^9
TCID_{50} of purified particles of each virus demonstrated increased levels of H in MVvac2-H2 particles (Fig. 2B). While expression levels of N and F were also slightly higher in MVvac2-H2 particles, densitometric analysis of the blots revealed an ~1.5- to 3-fold increase in the ratio of H to the other viral proteins in MVvac2-H2 relative to MVvac2, supporting the increased incorporation of H into MVvac2-H2 virions. The bona fide nature of the analyzed material is supported by the absence of the immature form of F, as seen in lysates of infected cells. Additionally, immunoblotting using an anti-His tag monoclonal antibody showed a band of ~78 kDa, the expected size of the H protein, in MVvac2-H2 particles. Taken together, these results indicate that the expression of additional His-tagged H protein from an ATU downstream of N in MVvac2-H2 results in increased cellular expression of H and enhanced incorporation of H into MVvac2-H2 particles.

To test whether the enhanced H expression by MVvac2-Hsol induces the secretion of soluble H forms upon infection and to purify this protein using affinity chromatography, we collected supernatants of cells infected at an MOI of 0.1 at 24-h intervals and interacted their clarified supernatants with immobilized cobalt ion resin using a batch approach to isolate His-tagged proteins. We used the supernatant from MVvac2-infected cells as a control. We were able to isolate by affinity chromatography a protein doublet with electrophoretic migration of 73 and 70 kDa specifically in supernatants of MVvac2-Hsol-infected cells from 48 to 96 h postinfection (Fig. 2C, left). Quantitatively, according to our Coomassie stain analysis of the purified material, we estimated a yield of ~250 ng/ml at 96 h postinfection (intracellular virus replication peaked at 48 to 72 h postinfection for this experiment). Furthermore, immunoblotting using anti-His tag antibodies to

FIG 1 Generation and multistep growth kinetics of recombinant MVs expressing additional H protein. (A) Diagram of MVvac2, MVvac2-H2, and MVvac2-Hsol. In the second and third viruses, an additional, engineered H coding sequence was inserted as an ATU downstream of the N cistron. MV genes are indicated in gray, and the added copies of H are in black. The lighter gray bar represents the signal sequence for interleukin-2 (IL-2 ss), replacing the transmembrane region and cytoplasmic tail coding sequence of the added H cistron in MVvac2-Hsol to direct the secretion of a truncated, soluble form of the H protein. Each added copy of H was modified to encode a six-histidine tag (6×His-COOH) at its carboxy terminus, as indicated. nts, nucleotides. (B) Time course of cell-associated (top) and cell-free (bottom) virus production at the indicated time points after Vero/hSLAM cells grown at 37°C were infected at an MOI of 0.03 with either the parental strain MVvac2 (black circles), MVvac2-H2 (gray squares), or MVvac2-Hsol (gray diamonds). Averages and standard deviations of data from three independent experiments are shown. (C) Growth kinetic profile of cell-associated (top) and cell-free (bottom) MVs obtained from primary chicken embryo fibroblasts grown at 32°C and infected at an MOI of 0.03. The symbols are the same as those described above for panel B. (D) Viral plaque appearance in Vero/hSLAM cells infected with the indicated MV. Plaques were visualized at 5 days postinfection when ethanol-fixed cells were stained with naphthol blue-black.
probe the affinity chromatography-purified material documented that the 73-kDa-band protein results from H secretion driven by MVvac2-Hsol infection from 48 to 96 h postinfection (Fig. 2C, left). These results indicated that soluble His-tagged H protein is expressed from the ATU of MVvac2-Hsol and secreted from cells; it is possible that the His tag addition altered its electrophoretic migration, as observed in the Coomassie-stained gel. Alternatively, immature unglycosylated His-tagged H was secreted from infected cells.

Recombinant MV with enhanced H incorporation is as thermostable as the parental MV and possesses a different kinetic of neutralization by measles-immune sera. To test the stability of MVvac2-H2 at temperatures ranging from 4°C to 96°C, aliquots of purified viral particles, each with an infectivity of \( \sim 10^5 \) TCID50, were incubated at increasing temperatures for 1 h, using MVvac2 as a control. Following treatment, the remaining infectivity in each sample was determined by a plaque assay and compared to the pretreatment titer. As shown in Fig. 3, MVvac2-H2 responded to heat treatment in a manner similar to that of MVvac2, maintaining most of its infectivity at 20°C for 1 h, while infectivity was reduced to 21% (MVvac2) or 25% (MVvac2-H2) of the pretreatment titer by heating the viral particles at 37°C for the same period of time. MVvac2-H2 thus possesses thermostability equivalent to that of MVvac2 despite the enhanced H incorporation in its envelope.

MV H is the major target of neutralizing antibodies against MV (37). We therefore aimed to test whether the susceptibility to neutralization of our recombinant MVs expressing additional H differed from that of their parental strain. We hypothesized that in the presence of marginal levels of anti-MV antibodies, additional envelope-bound H in MVvac2-H2 could remain free to bind receptors or to alter the ratio of H antigen to neutralizing antibodies, increasing the amount of antibodies required to prevent the virus-cell interaction that triggers entry. We assessed this by comparing the sensitivities of MVvac2, MVvac2-H2, and MVvac2-Hsol to neutralization in vitro by polyclonal mouse sera with various neutralizing capacities generated by immunization with MVvac2. Aliquots of recombinant MVs or the parental strain, each with a defined equalized infectivity, were mixed with 1:100 dilutions of seven anti-MV serum samples ranging in microneutralization titers from 1:33 to 1:1,067, and the samples were incubated at 37°C for 1 h. Residual infectivity was then determined by an endpoint dilution assay.

As shown in Fig. 4, for serum dilutions with a relatively lower capacity to neutralize MV (neutralizing titer of \( \sim 1:213 \)), all three viruses maintained their titer in an approximately equivalent manner. When incubated with the more strongly neutralizing preparations obtained from dilutions of neutralizing sera with titers of >1:427, however, MVvac2 precipitously lost most of its infectivity so that the aliquot incubated with the most strongly neutralizing sera retained <0.1% of its original titer, while MVvac2-H2 retained \( \sim 10\% \) of its original titer. Indeed, while MVvac2 infectivity declined almost 3 logs in titer over the assayed range of neutralization, MVvac2-H2 lost only 1 log in titer over the same series. This suggests that the presence of additional H...
MV with Enhanced Hemagglutinin Expression

FIG 4 Kinetics of neutralization of recombinant MVs by anti-MV polyclonal sera. Standardized titers of MVvac2, MVvac2-H2, or MVvac2-Hsol were incubated at 37°C for 1 h with 100-fold dilutions of immune sera with a known anti-MV titer from HuCD46Ge-IFNarKO mice vaccinated with MVvac2. The anti-MV neutralizing potency ranged in titer from 1:33 to 1:1,067, as indicated. Following incubation, the remaining infectivity in each sample was determined in triplicate by an endpoint dilution assay and graphed.

protein on the envelope of MVvac2-H2 renders this virus relatively more resistant than MVvac2 to neutralizing antibodies. Since MVvac2-Hsol and MVvac2 preparations had a theoretically equivalent virus surface, they showed similar patterns of reduction in infectivity in response to serum dilutions with increased neutralization potential.

MVVs expressing additional H are more immunogenic. Since H is the major antigenic target of neutralizing immunity, we hypothesized that the enhanced expression of H protein by our recombinant MVs correlated with increased immunogenicity relative to that of the parental vaccine strain. To test this hypothesis, we initially inoculated non-MV-susceptible CD1 mice with the three viruses. This animal host does not replicate measles to the best of our knowledge, but we reasoned that their immune system would detect differences in virus structure such as the enhanced display of H epitopes in the absence of the potentially confounding effects of viral replication. In this host, we expected MVvac2-H2 to generate stronger neutralizing immunity, given the enhanced display of envelope-bound H by particles of this virus. Three experimental groups of eight mice (MVvac2, MVvac2-H2, or MVvac2-Hsol) received two intraperitoneal doses of purified particles with an infectivity of 10^3 TCID_{50} at a 28-day time interval. Two weeks after the second dose, we assessed the neutralizing immunity of individual mice against MV by a microneutralization assay. In support of our hypothesis, animals that received MVvac2-H2 developed significantly higher neutralizing titers (1:733 on average) than those inoculated with MVvac2 (1:297 on average) (Fig. 5A). This 2.46-fold difference reached statistical significance (P = 0.0229, as determined by an unpaired two-tailed t test). Unexpectedly, MVvac2-Hsol also induced higher neutralizing titers on average than did MVvac2 in these mice, which was also statistically significant (1:861 and 1:297, respectively; P = 0.0473). Furthermore, serous IgG reactivity against MV was determined by using a quantitative ELISA. As shown in Fig. 5B, animals from the three experimental groups were indistinguishably reactive against MV, with readings from 181 to 468 AEU/ml. To corroborate the antigenic nature of the inocula in terms of viral protein composition, we analyzed the presence of N, F, and H proteins in viral particles with an infectivity of 2.5 × 10^3 TCID_{50} by immunoblotting (Fig. 5C). We used the same viral particle preparations for immunoblots as the ones used for animal experiments. As expected, MV H was incorporated at a higher rate in MVvac2-H2 than in MVvac2, while the viral surface of MVvac2-Hsol more closely resembled the virion surface composition of MVvac2 although with a relatively lower signal for MV N. Since we were not able to detect the His tag signal in MVvac2-Hsol virion preparations, the secreted Hsol may not have been passively adsorbed in virions. Thus, by increasing the incorporation of the MV H glycoprotein or inducing its secretion, we demonstrated an increased neutralizing immune response in CD1 mice.

To further validate the enhanced immunogenic potential of our recombinant MVs, we inoculated HuCD46Ge-IFNarKO mice with the viruses. These mice express the human CD46 receptor for vaccine strains of MV with human-like specificity in a type I interferon knockout background and are therefore susceptible to measles (36), allowing evaluation of our vaccine candidates’ immunogenicity in a more human-like model than the CD1 mouse. In consequence, we inoculated animals with a single dose of 10^3 TCID_{50}. Four weeks after inoculation, we documented neutralizing titers that were significantly higher in mice immunized with MVvac2-H2 than in those inoculated with MVvac2 (1:79 and 1:214 on average, respectively; P = 0.0436) (Fig. 5D). Contrary to our expectations, mice immunized with a single dose of MVvac2-Hsol generated neutralizing titers almost three times lower than but not significantly different from those documented in mice immunized with MVvac2 (1:79 and 1:27 on average, respectively; P = 0.1117); MVvac2-Hsol provoked detectable neutralizing immunity in five out of six recipients, while the other two viruses elicited measurable titers in all of the animals tested. As expected, Ig reactivity against MV did not differ among the three experimental groups. Quantitative readings from 7 to 23 AEU/ml were documented (Fig. 5E). Again, the relative content of MV H, F, and N was documented by immunoblot analysis of virus preparations used in the animal experiment and with the same infective potential (10^3 TCID_{50}) as that of the preparations inoculated into the mice (Fig. 5F). These results demonstrated that the increase of measles immunogenicity per infective unit was not caused merely by the initial antigenic input. In sum, the 2.79-fold enhanced immunogenicity documented for MVvac2-H2 confirms our hypothesis that increased display of H targets on the virion surface improves stimulation of neutralizing immunity against MV in two small-animal models of measles vaccination, with similar rates of increased immunogenicity compared to the parental strain in both hosts.

DISCUSSION

Eradication of MV remains a theoretically feasible but elusive goal. The variable presence of maternal antibodies is the hurdle to overcome to reduce measles mortality among the very young. The use of DNA vaccines (38–50); non-MV vectors expressing H, F, and/or N (51–57); immune-stimulating complexes incorporating the H and F proteins (57); or a combination of these strategies (58) has been proposed. Vaccination by these methods presumably avoids inhibition by maternal antibodies, and some of these vaccines have indeed proven protective when administered to infant
macaques with circulating maternal antibodies. Still, for the most part, the strength, durability, and safety of the immunity induced by the current MV vaccine have been difficult to recapitulate in these experimental vaccines. Our approach is appealing because it takes advantage of the time-tested efficiency of the current vaccine, industrial production and distribution operative procedures are already in place, and safety studies would be arguably more straightforward and experience based. More appealing is the fact that our optimization of the current vaccine by genetically modifying its antigenic potency is cost-effective, if safety and efficacy as assessed in the macaque model support its further development.

We show here that recombinant MV can express and incorporate additional copies of its hemagglutinin into its viral envelope by doubling the H cistron. In vitro, we have corroborated that passage 6 of MVvac2-H2 still expresses the additional copy of H. The genetic stability of the insertion of an additional copy of the H gene in MV after in vivo passage remains to be explored. However, in other studies, the stability of genetic modification of the MVvac2 vector platform after an in vivo passage in nonhuman primates has been documented. Inactivation of the interferon control viral proteins V and C or expression of HBsAg (34, 59) was genetically stable. The genetic stability of MVvac2-H2 could be promoted, if necessary, by deletion of the native H cistron, forcing selection on the added copy of H at the high-expression locus.

Despite a careful clonal selection process, our MVvac2-Hsol vector showed a heterogeneous array of plaque sizes in Vero/hSLAM cells. This observation might be caused by a subtle interference of the soluble H protein with viral fusion. The two recombinants showed a conserved replicative profile in both Vero/hSLAM cells and primary chicken embryo fibroblasts grown at 32°C, the latter of which are suitable for vaccine production. For our vector expressing an additional full-length copy of the H cistron, Western blotting demonstrated a much greater increase in the cellular expression level of H than in its incorporation into

**FIG 5** Humoral immune responses in outbred and genetically modified mice vaccinated with MVs expressing additional H. (A) Groups of eight CD1 mice received two doses of 10^5 TCID₅₀ of purified particles of MVvac2, MVvac2-H2, or MVvac2-Hsol at a 28-day interval. Sera obtained 14 days after the boosting dose were assayed for anti-MV neutralizing immunity. (B) Anti-MV IgG reactivity measured by an ELISA. Readings were obtained from individual mice; group averages and standard deviations from each animal reading are shown. (C) Analysis of the antigenic composition of the viral inocula used for the experiment described above. Viral particles with the indicated infectivity were subjected to SDS-PAGE and analyzed by immunoblotting using antibodies with the indicated specificity. (D) Groups of six HuCD46Ge-IFNar-KO mice received a single dose of 10^3 TCID₅₀ MVvac2, MVvac2-H2, or MVvac2-Hsol. Anti-MV microneutralization titers were assessed in sera obtained at 28 days postimmunization. (E) Reactivity against MV determined by an ELISA as described above for panel B. (F) Analysis of expression of key MV proteins in virus preparations used for the animal experiment described above. Antigenic material with the same titer as that used in the animal experiment was subjected to SDS-PAGE, membrane transferred, and probed against the indicated antibody. In panels A and D, the statistical significance of differences in neutralizing immunity was assessed by a two-tailed, unpaired t test. In panels B and E, AEU (arbitrary ELISA units) were quantitatively determined by comparison with an internal MV standard.
vion. The excess H expression relative to its incorporation sug-
gests a structural limitation preventing further inclusion of H into
vion. This result is interesting given the generally accepted
pleiomorphic structure of measles virus vion (60–62). Others
have proposed the notion of a more highly structured particle for
members of the Mononegavirales, such as Newcastle disease virus
(NDV) (63). Such a rigid model of envelope structure for MV
would seem to not be permissive to the up to 3-fold incorporation
enhancement of H per infectious unit documented here. Non-
evertheless, even the presumably more rigidly structured rabies virus
(RV) can incorporate additional glycoprotein units in its bullet-
shaped envelope (19). It remains to be tested if further H expres-
sion intensifies H incorporation in a functional viral vector.

The enhancement of H immunogenic stimulus increased neu-
tralizing responses in both nonsusceptible and MV-susceptible
mice. Inoculation of viral particles purified by ultracentrifugation
resulted in improved induction of neutralizing immunity by our
modified H expression vectors in nonsusceptible, outbred mice.
We expected these hosts to provide only a limited number of rep-
licative cycles so that the antigenic stimulus that these animals
received would be majorly represented by the composition of the
viral envelope. Purified viral particles were used to eliminate the
presence of unincorporated H or soluble H present in crude virus
preparations as a confounding factor, as shown in the correspond-
ing immunoblot. The MVvac2-Hsol envelope did not differ ma-
nerly from the parental strain’s, indicating that the initial antigenic
stimulus presented to the CD1 mice was the same for animals
receiving either MVvac2 or MVvac2-Hsol. As expected, MVvac2-H2
was significantly more immunogenic than the parental vaccine
MVvac2 in this host. Surprisingly, MVvac2-Hsol also induced sig-
ificantly more immunogenicity than the parental strain, further supporting the notion that

ACKNOWLEDGMENTS
We thank Roberto Cattaneo for sharing valuable reagents. We also thank
Ivonne Ceballos-Olvera and Amanda Huff for excellent technical assis-
tance and Rowida Abdelgalel for her comments on the manuscript.
Emily Julik is an ARCS Phoenix Chapter scholar.

FUNDING INFORMATION
This work was supported by funds appropriated from the Arizona State
Board of Regents on behalf of Arizona State University.

REFERENCES
537–552. In Kaslow RA, Stanberry LR, Le Duc JW (ed), Viral infections of
2. Gastanaduy PA, Redd SB, Fiebelkorn AP, Rota J, Rota P, Bellini WJ,
Seward JF, Wallace GS. 2014. Measles—United States, January 1-May 23,
Measles outbreak—California, December 2014-February 2015. MMWR Morb
4. Perry RT, Gacic-Dobo M, Dabbagh A, Mulders MN, Strebel PM,
Okwo-Bele JM, Rota PA, Goodson JL. 2014. Progress toward regional
measles elimination—worldwide, 2000–2013. MMWR Morb Mortal
Estimates of measles case fatality ratios: a comprehensive review of com-
1093/ije/dyn224.
6. Crowe JE, Jr. 2001. Influence of maternal antibodies on neonatal immu-
//dx.doi.org/10.1086/322971.
7. Cutts FT, Grabowsky M, Markowitz LE. 1995. The effect of dose and
strain of live attenuated measles vaccines on serological responses in
for immunization series. WHO, Geneva, Switzerland.
9. Markowitz LE, Sepulveda J, Diaz-Ortega JL, Valdespino JL, Albrecht P,
Zell ER, Stewart J, Zarate ML, Bernier RH. 1990. Immunization of
six-month-old infants with different doses of Edmonston-Zagreb and
10.1056/NEJM199003123200903.
10. Aaby P, Samb B, Simondon F, Whittle H, Seck AM, Knudsen K,
15. Cenna
18. Tao
21. Tatsuo
5278
jvi.asm.org June 2016 Volume 90 Number 11Journal of Virology
Julik and Reyes-del Valle
12. Li
24. Liu
Wu X, Rupprecht CE.
23. Zhai
27. Ball
Tatsuo
Garenne
11. Krempl
25. Krempl
2011. Generation of a recombinant rabies Flury LEP virus car-
3. DNA vaccination against measles: neutralizing antibody against either
50: .1038/77506.
27: 665–
73: http://dx.doi.org/10.1128/CVI.00120-08.
73: http://dx.doi.org/10.1128/CVI.00120-08.
73: http://dx.doi.org/10.1128/CVI.00120-08.
73: http://dx.doi.org/10.1128/CVI.00120-08.
73: http://dx.doi.org/10.1128/CVI.00120-08.


