

Vaccination with a Live Attenuated Cytomegalovirus Devoid of a Protein Kinase R Inhibitory Gene Results in Reduced Maternal Viremia and Improved Pregnancy Outcome in a Guinea Pig Congenital Infection Model

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ABSTRACT

Development of a vaccine to prevent congenital cytomegalovirus infection is a major public health priority. Live vaccines attenuated through mutations targeting viral mechanisms responsible for evasion of host defense may be both safe and efficacious. Safety and vaccine efficacy were evaluated using a guinea pig cytomegalovirus (GPCMV) model. Recombinant GPCMV with a targeted deletion of *gp145* (designated $\Delta 145$), a viral protein kinase R (PKR) inhibitor, was generated. Attenuation was evaluated following inoculation of 10^7 PFU of $\Delta 145$ or parental virus into guinea pigs immunosuppressed with cyclophosphamide. Efficacy was evaluated by immunizing GPCMV-naïve guinea pigs twice with either 10^5 or 10^6 PFU of $\Delta 145$, establishing pregnancy, and challenging the guinea pigs with salivary gland-adapted GPCMV. The immune response, maternal viral load, pup mortality, and congenital infection rates in the vaccine and control groups were compared. $\Delta 145$ was substantially attenuated for replication in immunocompromised guinea pigs. Vaccination with $\Delta 145$ induced enzyme-linked immunosorbent assay (ELISA) and neutralizing antibody levels comparable to those achieved in natural infection. In the higher- and lower-dose vaccine groups, pup mortality was reduced to 1/24 (4%) and 4/29 (14%) pups, respectively, whereas it was 26/31 (81%) in unvaccinated control pups ($P < 0.0001$ for both groups versus the control group). Congenital infection occurred in 20/31 (65%) control pups but only 8/24 (33%) pups in the group vaccinated with 10^6 PFU ($P < 0.05$). Significant reductions in the magnitude of maternal DNAemia and pup viral load were noted in the vaccine groups compared to those in the controls. Deletion of a GPCMV genome-encoded PKR inhibitor results in a highly attenuated virus that is immunogenic and protective as a vaccine against transplacental infection.

IMPORTANCE

Previous attempts to develop successful immunization against cytomegalovirus have largely centered on subunit vaccination against virion proteins but have yielded disappointing results. The advent of bacterial artificial chromosome technologies has enabled engineering of recombinant cytomegaloviruses (CMVs) from which virus genome-encoded immune modulation genes have been deleted, toward the goal of developing a safe and potentially more efficacious live attenuated vaccine. Here we report the findings of studies of such a vaccine against congenital CMV infection based on a virus with a targeted deletion in *gp145*, a virus genome-encoded inhibitor of protein kinase R, using the guinea pig model of vertical CMV transmission. The deletion virus was attenuated for dissemination in immunocompromised guinea pigs but elicited ELISA and neutralizing responses. The vaccine conferred protection against maternal DNAemia and congenital transmission and resulted in reduced viral loads in newborn guinea pigs. These results provide support for future studies of attenuated CMV vaccines.

Infection with human cytomegalovirus (HCMV) causes considerable morbidity and occasional mortality in immunocompromised solid organ transplant and hematopoietic stem cell transplant patients, HIV-infected individuals, and newborns that acquire infection *in utero* (1, 2). Due to the lifelong morbidity associated with congenital CMV infection, a preconception vaccine capable of preventing virus transmission to the fetus would provide a highly cost-effective public health advance (3). Unfortunately, the lack of clear immunological correlates of protective immunity has hampered development of an HCMV vaccine. In spite of this uncertainty, there is evidence that virus-neutralizing antibody responses targeting viral envelope glycoproteins, as well as cellular immune responses ($CD4^+$ and $CD8^+$) targeting multiple structural and regulatory proteins, play important roles in protection against acquisition and reactivation of infection (4–7).

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Recombinant vaccines employing a variety of expression strategies have focused on the immunodominant glycoprotein B (gB), as well as the major CD8⁺ T-cell target pp65, in clinical trials. These vaccines have demonstrated various levels of protection against HCMV infection and/or disease in both immunocompetent women (8) and immunocompromised solid organ and hematopoietic stem cell transplant recipients (9, 10). To date, no clinical trials have been powered to assess the efficacy of any vaccine for protection against congenital infection.

In addition to subunit vaccine studies, live attenuated vaccine approaches have also been pursued. The Towne vaccine, attenuated by extensive passage (>125 times) in tissue culture, is the best-studied live HCMV vaccine. In studies with a total of nearly 1,000 human subjects, the Towne vaccine has been evaluated for safety and efficacy in renal transplant recipients (11, 12) and HCMV-seronegative mothers of young children who were actively shedding virus (13). Because of inadequate efficacy in clinical trials, efforts to improve the immunogenicity of the Towne vaccine have included the generation of chimeric viruses containing both Towne sequences and sequences from the less attenuated Toledo strain (14), the evaluation of prime-boost strategies (15), and the coadministration of Towne vaccine with recombinant interleukin-12 (16). The molecular basis of attenuation of the Towne vaccine is uncertain, although a mutation in the Towne *UL130* gene coding sequence that abrogates the synthesis of a functional UL130 protein (17), a component of the pentameric complex (PC) of HCMV proteins that have recently received considerable attention as potential HCMV subunit vaccine candidates, has been described (18). Other mutations in the Towne strain likely contribute to its attenuation when used as a vaccine, although the molecular basis of attenuation remains poorly understood. Given this uncertainty about the basis of attenuation, a live HCMV vaccine based on the targeted deletion of specific viral genes important for pathogenesis and/or immune modulation would be desirable. Such an approach to vaccine design could help assuage some of the regulatory and safety issues inherent in live virus vaccination (19, 20).

Because of the species specificity of cytomegaloviruses, experimental HCMV vaccines cannot be evaluated for efficacy against congenital infection in animal models. However, guinea pig cytomegalovirus (GPCMV) is able to cross the placenta to establish congenital infection and in many respects recapitulates the pathogenesis observed for HCMV infection in infants. Several GPCMV proteins have been identified to be immunologically important targets for modeling vaccine development, including gB and pp65 homologs (21, 22). The live attenuated vaccine approach has also been modeled in the guinea pig, in which GPCMV vaccines generated using bacterial artificial chromosome (BAC)-based mutagenesis strategies have been evaluated (23, 24). One limitation of these studies is that the BAC containing the GPCMV genome was found on sequence analysis to contain a 4-bp deletion that frameshifts the *gp129* open reading frame (ORF) (25), hence rendering viruses reconstituted from the BAC unable to assemble the GPCMV homolog of the HCMV pentameric complex (PC), composed of GPCMV proteins gH, gL, gp129, gp131, and gp133. In this respect, live GPCMV vaccines derived from the N13R10 BAC have an attenuated parental genetic background similar to that of the Towne vaccine. In spite of this limitation, BAC-based mutagenesis of the GPCMV genome has allowed elucidation of the functional significance of deletion of several GPCMV immune

modulation genes, and these engineered viruses have in turn been evaluated as potential vaccine candidates offering protection against congenital infection in this model.

Among the virus genome-encoded immune evasion genes with potential application for vaccine development are those that encode inhibitors of protein kinase R (PKR). PKR is an intracellular host factor that senses viral infection through binding to double-stranded RNA (dsRNA) and subsequently inhibits viral replication via global repression of protein synthesis. HCMV encodes two well-characterized PKR antagonists, IRS1 and TRS1, which inhibit PKR activation by binding to both dsRNA and PKR (26–28). Murine cytomegalovirus also encodes two proteins, M142 and M143, which form a complex that binds dsRNA and prevents activation of the PKR pathway (29–32). Recently, a similar function was attributed to gp145, a protein encoded by the *gp145* ORF of GPCMV. In a proteomic screen, gp145 was found to bind dsRNA. That it functions as a PKR antagonist was subsequently suggested by its ability both to rescue the replication of a mutant vaccinia virus that lacks the vaccinia PKR inhibitor and to counteract the effects of guinea pig PKR in cotransfection assays (33).

For many pathogenic viruses, deletion of PKR antagonists attenuates viral replication by permitting PKR to shut down protein synthesis at late stages of the infectious cycle when dsRNA has accumulated. In herpesviruses, immediate early or early viral proteins are still synthesized, although their levels are somewhat reduced (27, 34). Because live vaccines comprised of viruses lacking PKR antagonists alone (35) or in combination with other viral gene deletions (36) have been shown to be safe and efficacious in other systems, we hypothesized that targeted deletion of PKR antagonists from a live cytomegalovirus vaccine could be an effective strategy for attenuation that would provide the safety of an inactivated virus vaccine yet retain the immunogenicity of a live vaccine. We therefore generated a recombinant GPCMV deleted of *gp145* and evaluated its safety, immunogenicity, and efficacy as a live vaccine in the context of the congenital infection challenge model.

MATERIALS AND METHODS

Guinea pigs. Outbred Hartley guinea pigs were purchased from Elm Hill Laboratories (Chelmsford, MA). All animals used for challenge studies were confirmed to be GPCMV seronegative by enzyme-linked immunosorbent assay (ELISA) (37), and approval for the study was obtained from the Institutional Animal Care and Use Committee at the University of Minnesota, Minneapolis, MN. GPCMV-seropositive animals obtained from a commercial supplier were bled, and sera were used as a source for ELISA and neutralization antibody studies of naturally seropositive animals, as described below.

Virus and cells. A salivary gland (SG)-passaged GPCMV stock used in animal studies was prepared by sequential passage of GPCMV (strain 22122; American Type Culture Collection [ATCC]) in strain 2 guinea pigs as previously described (38). Cell culture propagation of virus was carried out in guinea pig lung (GPL) fibroblast cells (ATCC CCL158) maintained in F-12 medium supplemented with 10% fetal calf serum (FCS; Fisher Scientific), 10,000 IU/liter penicillin, 10 mg/liter streptomycin (Gibco-BRL), and 0.075% NaHCO₃ (Gibco-BRL) or as described previously (33). Growth curves and viral titers were determined as described previously (39).

For neutralization assays, a green fluorescent protein (GFP)-expressing virus, vJZ848, was generated. Briefly, plasmid pKTS839 containing nucleotides 3059 to 4836 of GPCMV strain 22122 (GenBank accession number [KC503762](#)) was digested with BglII and ligated to a 2.3-kb BamHI fragment from plasmid pQ106 (40) containing *gpt*/GFP expression cas-

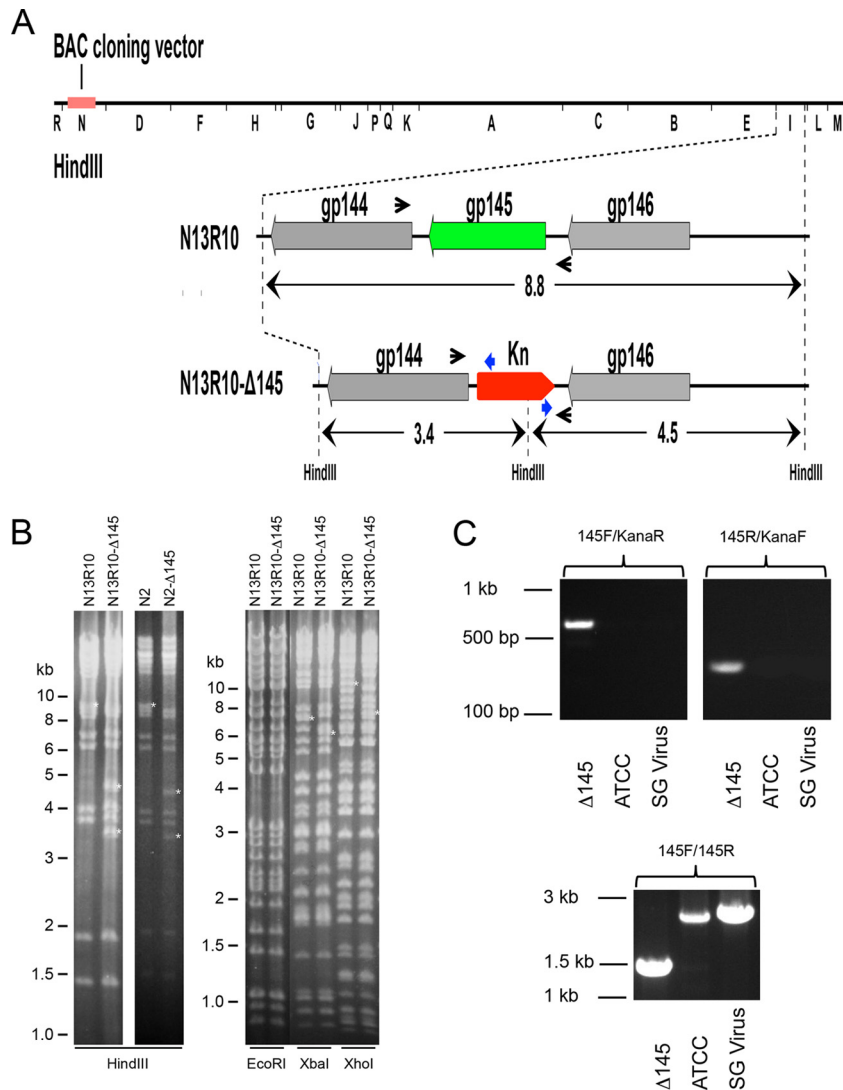


FIG 1 Construction and *in vitro* characterization of recombinant viruses lacking *gp145*. (A) HindIII restriction map of BAC N13R10 (top) with the HindIII-I fragment expanded below. In BACs N13R10-Δ145 and N2-Δ145, replacement of *gp145* with a *Kn* marker cassette results in the loss of the 8.8-kb HindIII-I fragment and creation of novel 3.4- and 4.5-kb HindIII fragments. Arrows indicate the positions of PCR primers gp145F/gp145R (black) and KanaF/KanaR (blue), used for confirmation of the genome structure in panel C. (B) Confirmation of recombinant BAC-derived constructs by restriction pattern analysis. DNA from parental and Δ145 mutant BACs was subjected to restriction digestion with HindIII, EcoRI, XbaI, or XhoI and analyzed by agarose gel electrophoresis. White asterisks indicate restriction fragments that are diagnostic for the Δ145 mutation: for HindIII, 8.8 kb lost and 3.4 and 4.5 kb gained; for EcoRI, 38.9 kb lost and 38.0 kb gained (differences are not resolvable under the gel conditions used in the present study); for XbaI, 6.9 kb lost and 6.0 kb gained; and for XhoI, 10.5 kb lost and 7.2 kb gained. (C) PCR was used to confirm the genome structure of Δ145 in the stock used for *in vivo* studies. An aliquot of the Δ145 stock was used to infect GPL cells, and infected cell DNA was isolated and PCR amplified. Primer pairs gp145F/KanaR (left) and gp145R/KanaF (right) were predicted to amplify 647-bp and 320-bp products, respectively, from Δ145 DNA and should not amplify wild-type DNA from ATCC or SG-passaged stocks. Primer pair gp145F/gp145R (bottom) was predicted to amplify a 1,446-bp product from Δ145 DNA and a 2,370-bp product from both wild-type DNAs.

settes to generate plasmid pKTS848. Plasmid DNA was linearized with XbaI and then transfected into GPL cells, which were subsequently infected with SG-passaged GPCMV and subjected to selection with xanthine and mycophenolic acid as previously described (41). Clonal recombinant virus was generated by multiple rounds of selection and limiting dilution as described elsewhere (41). The genome structure of vJZ848 was confirmed by restriction endonuclease digestion, PCR, and targeted sequencing. Previous GFP-tagged GPCMV strains contain 4-bp or 1.6-kb deletions that impact the *gp129/gp133* locus and that confer an *in vivo* attenuation phenotype (25, 42). Virus vJZ848 retained the 1.6-kb locus, demonstrated no loss of the 1.6-kb locus after over 10 passages in GPL cells (as assessed by PCR), and maintained the wild-type sequence, as

demonstrated by sequence analysis of PCR-amplified DNA from multiple serial passages for the *gp129*, *gp131*, and *gp133* ORFs encoding components of the GPCMV pentameric complex (43, 44) (data not shown).

Deletion of *gp145*, assessment of genome structure, and generation of the Δ145 vaccine. BAC construct N13R10 contains the complete GPCMV strain 22122 genome, while the BAC N2 contains a 17.9-kb deletion (39). BAC N2 with a targeted deletion of *gp145* (N2-Δ145) and N13R10 with a targeted deletion of *gp145* (N13R10-Δ145 or Δ145) were constructed using a linear recombination approach to result in the substitution of a kanamycin resistance cassette (*Kn*) for nucleotides 224206 to 226104 (GenBank accession number [KM384022](https://www.ncbi.nlm.nih.gov/nuccore/KM384022) [25]) comprising the *gp145* open reading frame (Fig. 1A). A primer pair was next utilized in

order to amplify by PCR *Kn* from plasmid pEPKans, producing a PCR product comprised of *Kn* flanked by GPCMV homology sequences (which are underlined below). The primers (and their sequences) employed were as follows: GP145 forward (5'-CACCCGGATACAGTCCGCGTCACCGCGCGCGCCCGCCCGCGCGACTCCCGCTCCCGGATCGCGGCCCGACGTTAGGGATAACAGGGTAATCGATTT-3') and GP145 rev (5'-CGCCGCGCCAGAATCGAACGATATAGTCCGGTGATCTCACGGATCGCATCGACCGATTCCAGACAGATACGGAAAGGCCAGTGTACAAACCAATTAACC-3'). Linear recombination between the PCR product and BAC N2 or N13R10 was conducted as previously described (45). Candidate clones were screened by PCR to identify clones with the proper insertion of *Kn* using primers gp145F (ACAGCAAGGTCCCTCTGAGC), gp145R (TATAGTCGGTGATCTCACGG), KanaF (CGGATTCAGTCGTCATCAT), and KanaR (GCGAGCCATTATACCCAT). PCRs were performed, using 1 ng of purified BAC DNA with GoTaq Long PCR master mix (Promega), for 35 cycles of denaturation for 30 s at 94°C, primer annealing for 15 s at 57°C, and extension for 2.5 min at 72°C, followed by a final extension at 72°C for 7 min.

BAC clones were analyzed by restriction endonuclease digestion with HindIII, EcoRI, XbaI, and XhoI to confirm that only the predicted restriction polymorphisms were present (Fig. 1B). A clone with the predicted *gp145* deletion identified by these analyses was designated N13R10-Δ145. A similar recombinant, constructed in the N2 BAC (46), was also generated and designated N2-Δ145. GFP-positive viruses N2 and N2-Δ145 were reconstituted by transfection of their respective BAC DNAs into GPL cells without *cre*-mediated excision of the BAC origin, while viruses N13R10 and Δ145 were reconstituted by cotransfection of BAC N13R10 or N13R10-Δ145 DNA, respectively, with plasmid pCre, followed by limiting dilution isolation of GFP-negative viruses (from which the BAC origin was excised) as described previously (46).

The Δ145 virus was expanded in GPL cells to produce a large stock for vaccine and pathogenesis studies. Given the reduced replication capacity of the Δ145 virus (see Fig. 3), viral stocks were concentrated by centrifugation using 10-fold more material from tissue culture flasks in order to achieve an adequately concentrated stock of virus for vaccine studies. An aliquot from the final stock was used to infect GPL cells, and the Δ145 genome structure was again confirmed by PCR as described above using 1 ng purified infected cell DNA.

Northern and reverse transcriptase (RT)-PCR analyses. Transcription of the *gp145* gene in N13R10- and Δ145-infected cells was examined by Northern blotting. GPL cells were infected at a multiplicity of infection (MOI) of 5, and total RNA was harvested from these and mock-infected cells at 72 h postinfection (hpi) using a Direct-zol RNA MiniPrep kit (Zymo Research). For Northern analysis, equal quantities of total RNA were resolved on a denaturing formaldehyde agarose gel and transferred to a positively charged nylon membrane (Roche). To generate a double-stranded DNA (dsDNA) probe specific to *gp145*, pKTS786 (33) was digested with HindIII and XbaI, and the resulting 1,995-bp fragment, which included the entire *gp145* ORF, was gel purified and labeled using an Amersham ECL direct nucleic acid labeling and detection system (GE Healthcare). For analysis of the *gp147* transcript, primers 5'-GGTCTCGTCGGTTGAAGTT-3' and 5'-GGCAACGGAACGTACTTAGAA-3' were used to amplify wild-type GPCMV DNA using *Taq* polymerase, and the resulting 299-nucleotide *gp147*-specific fragment was gel purified for use as a probe in Northern blot assays. The hybridization of each specific probe to the nylon membranes was detected using an Amersham ECL Prime Western blotting detection reagent (GE Healthcare).

To examine the impact of the *gp145* deletion on the transcription of adjacent ORFs, RT-PCR of RNA purified from Δ145- and N13R10-infected cells was performed (see Fig. 2B). RNA purified from mock-infected cells was used as a control. Nucleic acid was extracted from cells infected with N13R10 or N13R10-Δ145 or mock-infected GPL cells at 72 hpi using the Direct-zol RNA MiniPrep kit (Zymo Research) according to the manufacturer's instructions. cDNA was synthesized from 1,000 ng of

TABLE 1 Viral loads in cyclophosphamide-treated guinea pigs challenged with N13R10 or Δ145 virus

Challenge virus	Mean ± SEM level of DNAemia (no. of genome copies/ml)		Mean ± SEM tissue viral load (no. of genome copies/mg)	
	Day 7 ^a	Day 14	Liver ^b	Spleen ^c
N13R10	6,328 ± 985	573 ± 473	92 ± 26	430 ± 371
Δ145	<100 ^d	<100 ^d	<1.0 ^d	<1.0 ^d

^a *P* < 0.0001 versus Δ145.

^b *P* = 0.002 versus Δ145.

^c *P* = 0.03 versus Δ145.

^d Limit of detection of PCR assay.

total RNA using a QuantiTect reverse transcription kit (Qiagen) with oligo(dT). Conventional PCR was carried out using cDNA as the template and GoTaq (Promega). The primer pairs used for these PCRs were as follows: for *gp144*, CB94 (5'-GACTGCTGAAGTGGTACG-3') and CB95 (5'-GGGTACAGCGAGAAGACC-3'); for *gp145*, CB96 (5'-ATTCCGGCCTGACGTTTC-3') and CB97 (5'-CGGTCCATGATCTTCAGC-3'); and for *gp146*, CB98 (5'-GTTCGTTTCATACGAGACC-3') and CB99 (5'-GCTCCGGCAGATCCAG-3'). The expected sizes of the PCR amplification products for *gp144*, *gp145*, and *gp146* were 537 bp, 511 bp, and 486 bp, respectively. The conditions for the *gp145* PCR were initial denaturation at 95°C for 10 min, followed by 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s for a total of 35 cycles. The same conditions were used for *gp144* and *gp146*, but 40 cycles were used. All reactions employed a final elongation step at 72°C for 7 min. The PCR product was run in a 1.0% agarose gel and visualized by ethidium bromide staining.

Animal pathogenicity studies and vaccination. Viral challenge studies were performed in cyclophosphamide-treated immunocompromised guinea pigs as previously described (47). Nonpregnant Hartley guinea pigs were treated with 100 mg of cyclophosphamide per kg of body weight by intraperitoneal (i.p.) injection 1 day prior to viral challenge and again 14 days after challenge. Three groups of four animals each were challenged with the Δ145 or N13R10 virus or sham infected with phosphate-buffered saline (PBS). Viruses were administered subcutaneously (s.c.) at a dose of 1×10^7 PFU. Animal weights were monitored every 3 to 5 days, and blood samples were obtained at days 7 and 14 postinoculation for viral load determination by quantitative real-time PCR (qPCR). Liver and spleen samples for qPCR were collected upon necropsy on day 35 (Table 1).

For vaccination/challenge studies, young weanling female Hartley guinea pigs were divided into 3 groups of 8 animals each. Animals in group 1 were immunized with two s.c. injections of 10^6 PFU Δ145 virus separated by 1 month. Group 2 animals were immunized with two s.c. injections of 10^5 PFU of Δ145 virus separated by 1 month. The control group (*n* = 8) was unimmunized. Blood was obtained 21 days after each vaccine dose. Study animals were placed with seronegative breeder males 1 month after completion of the immunization series and examined weekly for evidence of pregnancy. At mid- to late gestation (40 to 45 days), dams were challenged with 10^5 PFU of SG-passaged GPCMV and observed daily until delivery. Animals that failed to become pregnant (one animal each in groups 1 and 2 and two animals in group 3) were included in the immunogenicity comparisons but not in the pregnancy outcome analyses. Following delivery, tissue was immediately harvested from still-born pups or within 72 h postdelivery from live-born pups. There were 7, 7, and 6 evaluable pregnancies in groups 1, 2, and 3, respectively.

ELISA and neutralization and Western blot assays. GPCMV-specific serum IgG titers were determined by ELISA using protocols that have been previously described (21). ELISA titers were defined as the reciprocal of the highest dilution that produced an absorbance of at least 0.10 and twice the absorbance of a negative-control antigen prepared from uninfected GPL cells. Titers of <20 were assigned a value of 10 for statistical comparison (21). The GFP-tagged recombinant vJZ848 virus was used for

neutralization assays, using previously published protocols (37). Neutralizing titers were defined as the dilution resulting in a $\geq 50\%$ reduction in GFP-positive foci. ELISA and neutralization assays using six serum samples from GPCMV-seropositive animals obtained from the commercial supplier were also performed in a separate experiment to compare the vaccine-induced responses with those observed in naturally infected animals. All neutralizing titers were determined in assays with guinea pig lung cells (ATCC CCL158). Analyses of phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF2 α) were conducted using immunoblot methods and antibodies as previously described (24, 30) following spin inoculation ($700 \times g$, 1 h, room temperature) of GPL cells pretreated with mouse beta interferon (10 units/ml).

qPCR analysis. Maternal blood was obtained on days 7, 14, and 21 postchallenge with SG-passaged GPCMV and analyzed for viral load by qPCR as described previously (41). Briefly, DNA was extracted either from 100 μ l citrated maternal blood (MagNA Pure LC DNA isolation kit I; Roche) or from tissues, using 0.1 g of homogenized samples of liver, lung, or spleen tissue (MagNA Pure LC DNA isolation kit II; Roche). Amplification primers GP83TM_F1 (5'-CGTCCTCCTGTGCGGTCAAAA C-3') and GP83TM_R1 (5'-CTCCGCCTTGAACACCTGAA-3') were used at a final concentration of 0.4 μ M, while the GP83 hydrolysis probe (FAM-CGCCTGCATGACTCACGTCGA-BHQ1, where FAM is 6-carboxyfluorescein and BHQ1 is black hole quencher 1) was used at 0.1 μ M. PCR was performed using a LightCycler 480 real-time PCR system (Roche) under the following conditions: 40°C for 10 s, 95°C for 15 min, and 45 amplification cycles of 95°C for 10 s, 56°C for 15 s, and 72°C for 10 s. Data were analyzed with LightCycler data analysis software (version 1.5; Roche) using standard curves generated from known copy numbers of modified plasmid pCR 2.1 containing GP83 sequences. DNAemia was expressed as the total number of genome copies per milliliter of blood. The limit of detection was approximately 200 copies/ml. For the purpose of statistical comparisons, a level of 100 copies/ml was assigned to negative samples. Tissue viral loads were expressed as the number of genome copies per milligram of tissue. The limit of detection was approximately 2 genome copies/mg of tissue. For the purpose of statistical comparisons, a value of 1 copy/mg was assigned to negative samples. For the purpose of ascertaining congenital infection rates, any pup that had qPCR values falling below the limit of detection of the assay was considered uninfected.

Statistical analyses. GraphPad Prism software (version 6.0) was used for statistical analyses. Pup mortality and transmission were compared using Fisher's exact test with one-sided comparisons. Pup weights in pregnancy/challenge studies were compared by use of the Kruskal-Wallis followed by Dunn's multiple-comparison test. Antibody titers were compared using the Mann-Whitney test. Parametric data included viral load measurements in blood and tissue and weight loss in cyclophosphamide challenge studies. These were compared using 2-way analysis of variance (ANOVA) followed by Bonferroni's multiple-comparison posttest.

RESULTS

Generation of $\Delta 145$ virus. A *gp145*-null mutant was constructed using BAC mutagenesis in *Escherichia coli*. The BAC clone N13R10 contains the complete GPCMV genome derived from a tissue culture-passaged stock of GPCMV strain 22122 (39). A *gp145*-null mutant was constructed by insertion of a *Kn* marker cassette concomitant with deletion of the entire *gp145* ORF using bacteriophage lambda Red-mediated recombination. Candidate clones were first screened by PCR and then confirmed by HindIII restriction fragment length polymorphism analyses, as indicated in Fig. 1B. The mutant BAC was predicted to lose the 8.8-kb HindIII-I fragment and to gain two novel HindIII fragments of 3.4 and 4.5 kb (Fig. 1B). A clone that demonstrated the expected HindIII pattern was selected and designated N13R10- $\Delta 145$ ($\Delta 145$). To determine if N13R10- $\Delta 145$ contained deletions or rearrangements elsewhere in the genome, N13R10- $\Delta 145$ was com-

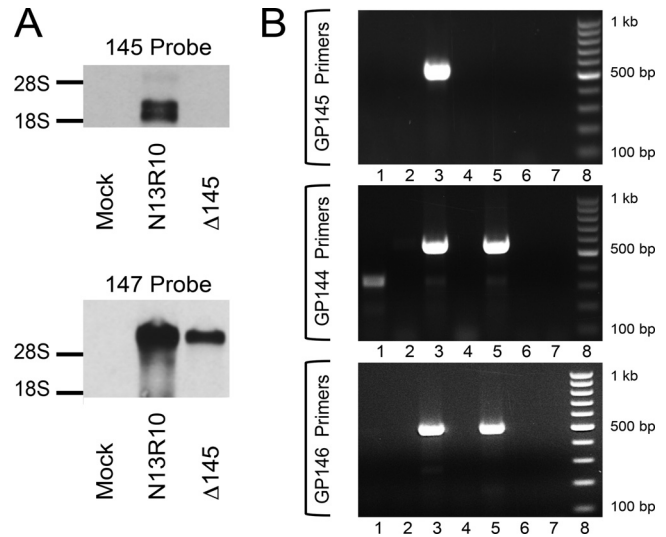


FIG 2 Transcriptional analyses of N13R10 and $\Delta 145$ viruses. (A) Northern blot showing a dsDNA probe specific to *gp145* (top) or *gp147* (bottom) hybridized to RNA purified from mock-, N13R10-, or $\Delta 145$ -infected GPL cells at 72 hpi. The locations of rRNAs (visualized by ethidium bromide staining) are indicated. (B) RT-PCR analysis of RNA from N13R10- or $\Delta 145$ -infected cells. Oligo(dT)-primed RNA from mock-infected and virus-infected cells was harvested at 72 hpi and subjected to PCR using primers specific to *gp145* (top), *gp144* (middle), or *gp146* (bottom). Lanes 1, RNA from mock-infected cells plus RT; lanes 2, RNA from mock-infected cells with no RT; lanes 3, RNA from N13R10-infected cells plus RT; lanes 4, RNA from N13R10-infected cells with no RT; lanes 5, RNA from $\Delta 145$ -infected cells plus RT; lanes 6, RNA from $\Delta 145$ -infected cells with no RT; lanes 7, no-template PCR control; lanes 8, molecular size markers (100-bp ladder).

pared to N13R10 using three additional restriction enzymes. For each enzyme, the observed pattern changes were attributable to the mutation (Fig. 1B), suggesting that other regions of the genome were unaffected by deletions or rearrangements. Viruses designated N13R10 and $\Delta 145$ were reconstituted from the parental BAC N13R10 and mutant BAC N13R10- $\Delta 145$, respectively.

Evaluation of $\Delta 145$ genome structure and transcriptional analyses. Large stocks of $\Delta 145$ were produced for *in vivo* studies (see below). To ensure that the $\Delta 145$ virus used *in vivo* retained the expected genome structure, the final stock was assessed by PCR analysis as described above. Two PCRs specific for regions that spanned the junctions between the *Kn* sequence and flanking GPCMV sequences to the left or right of the *Kn* insertion were performed. Both reactions produced products of the expected size when DNA from the $\Delta 145$ stock was amplified and produced no products from wild-type virus stocks (Fig. 1C). Amplification with GPCMV-specific primers spanning the *Kn* insertion in $\Delta 145$ or *gp145* in wild-type virus produced the predicted ~ 2.4 -kb product from wild-type DNA and a predicted ~ 1.5 -kb product from $\Delta 145$ DNA, consistent with the smaller size of the *Kn* insertion than *gp145* (Fig. 1C).

To confirm the knockout of *gp145* at the transcriptional level, Northern blot studies were performed using probes designed to hybridize to the *gp145* sequence. RNA was purified from $\Delta 145$ - and N13R10-infected GPL cells at a late time point (72 h) after infection at an MOI of 5. A dsDNA probe corresponding to the *gp145* ORF detected 2.2- and 2.5-kb RNAs in N13R10-infected GPL cells but not in $\Delta 145$ -infected GPL cells (Fig. 2A, top). This

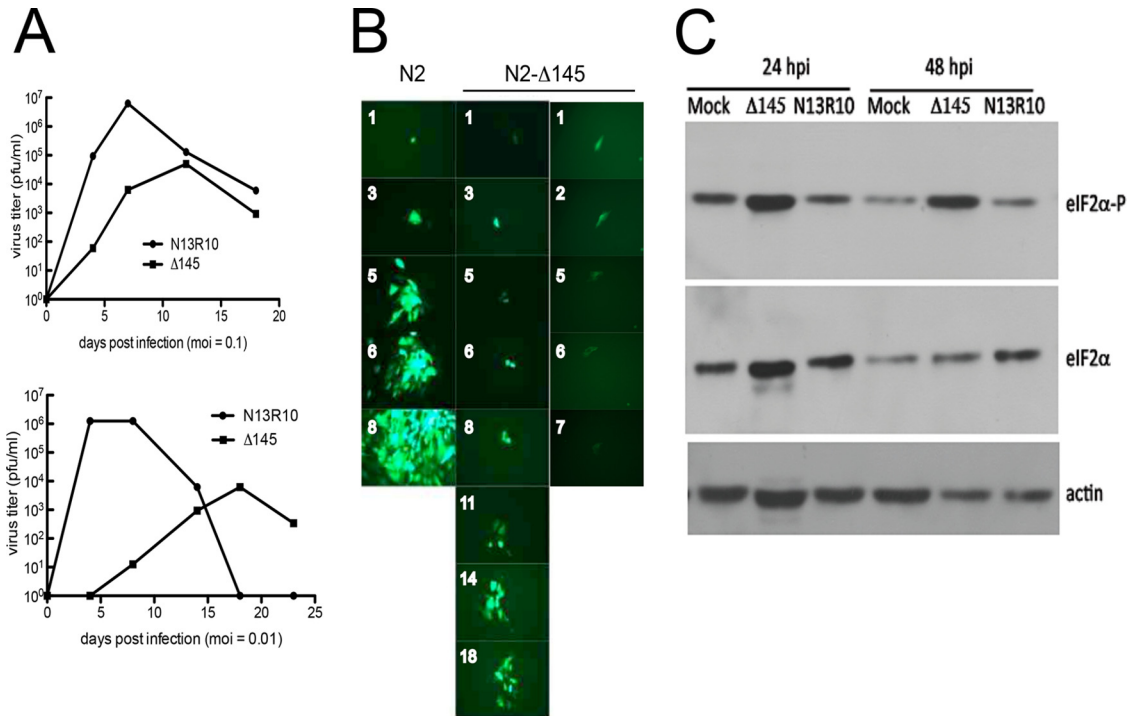


FIG 3 Growth characteristics of $\Delta 145$ virus and effects of deleting *gp145* on eIF2 α phosphorylation. (A) GPL cells were infected with *gp145*-null virus $\Delta 145$ or parental virus N13R10 at MOIs of 0.1 or 0.01, and virus titers in the culture supernatants were determined up to 23 days postinfection. (B) GPL cell monolayers were infected with GFP-tagged *gp145*-null virus N2- $\Delta 145$ or parental virus N2 at an MOI of 0.001. One day later, six GFP-positive cells infected with each virus were marked and photographed. Markings were used to locate and photograph the same foci on subsequent days (the numbers of days are indicated in white). One representative series of photographs is shown for N2 (left). One N2- $\Delta 145$ -infected cell spread to form a focus of GFP-positive cells (center). The other five N2- $\Delta 145$ -infected cells failed to spread and GFP regressed to undetectable levels by 6 to 21 days postinfection; the regression of one representative N2- $\Delta 145$ -infected cell is shown on the right. (C) GPLs were treated with 10 units/ml of murine beta interferon overnight and then mock treated or infected (MOI = 3) with $\Delta 145$ or N13R10. After 24 and 48 h, eIF2 α phosphate (eIF2 α -P), total eIF2 α , and actin levels were detected by immunoblot assays.

result suggests that two distinct transcription start sites may drive *gp145* transcription. As a control, infected cell RNAs were examined by Northern analysis using a probe specific for the *gp147* ORF. Although the amount of the ~ 8 -kb *gp147*-specific transcript synthesized in cells infected with $\Delta 145$ was less than that in cells infected with N13R10 (Fig. 2A, bottom), compatible with the decreased replication efficiency of the $\Delta 145$ virus, the synthesis of the *gp147* transcript in $\Delta 145$ -infected cells confirmed that the absence of the *gp145* transcript was not due to a generalized absence of viral gene expression.

To further compare the transcription of the N13R10 and $\Delta 145$ viruses and to evaluate whether the knockout of *gp145* unexpectedly perturbed the synthesis of the adjacent *gp144* and *gp146* ORFs, RT-PCR was performed (Fig. 2B). Transcripts corresponding to *gp144* and *gp146* were readily detected in both viruses (Fig. 2B, middle and bottom), but a *gp145*-specific transcript could be identified only in cells infected with the N13R10 virus and not the $\Delta 145$ *gp145*-knockout virus (Fig. 2B, top). These results confirmed the knockout of the *gp145* transcript and suggest that deletion of *gp145* did not result in the alteration of transcription of nearby genes.

Characterization of $\Delta 145$ virus in cell culture. The growth of the $\Delta 145$ virus was attenuated compared to that of parental virus N13R10, as seen in growth curves conducted at MOIs of 0.1 and 0.01 (Fig. 3A). $\Delta 145$ achieved peak titers roughly 100-fold lower than those of N13R10, and the times to the peak titer were delayed

by 7 to 14 days (Fig. 3A). While titrating virus stocks, we observed that the rate of focus formation (assessed by the cytopathic effect) was substantially slower for $\Delta 145$ (data not shown). In order to utilize GFP to visualize viral spread, we used two additional recombinant viruses, N2 and N2- $\Delta 145$, that express GFP and either retain or lack *gp145*, respectively (Fig. 3B). Although the BAC origin in N13R10 includes a GFP expression cassette, when viruses are reconstituted from N13R10, it is necessary to use *cre* recombinase to excise the 8.8-kb LoxP-flanked BAC origin in order to prevent the overlong genome from spontaneously acquiring compensatory deletions (46). However, since BAC N2 has an 18-kb deletion in the HindIII-D region (39), viruses reconstituted from N2 can accommodate retention of the BAC origin (46). Thus, GFP-positive N2 BAC-derived viruses (reconstituted without *cre*-mediated excision of the BAC origin cassette) provided an expedient means to visualize the impact of deleting *gp145*.

To compare the ability of these two viruses to infect and spread within fibroblast cultures, GPL cells were infected with N2 or N2- $\Delta 145$ at an MOI of 0.001. One day after infection, six individual GFP-positive cells infected by each virus were identified, photographed, and marked. Daily thereafter the markings were used to locate and photograph the same cells or foci originating from those cells. All six of the N2-infected cells rapidly expanded to form large GFP-positive foci by 8 days postinfection. A series of micrographs showing focus expansion from one representative cell is shown in Fig. 3B. In contrast, only one of the six N2- $\Delta 145$ -infected cells formed a

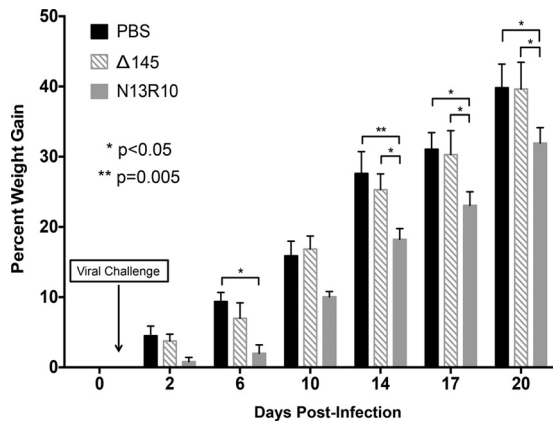


FIG 4 Deletion of *gp145* results in attenuation of disease following challenge of cyclophosphamide-immunosuppressed guinea pigs. Adult Hartley guinea pigs were treated with cyclophosphamide 1 day before and 14 days after s.c. challenge with 10^7 PFU $\Delta 145$, 10^7 PFU N13R10, or PBS ($n = 4$ per treatment group). The timing of viral challenge (day 1) is indicated by an arrow. Animals were weighed on the indicated days after challenge, and the percent weight gain is plotted versus time; P values were determined by ANOVA.

focus, and its rate of formation was significantly lower than that of the N2 foci (Fig. 3B). Surprisingly, the remaining five N2- $\Delta 145$ -infected cells that were initially GFP positive lost their GFP expression within a few days. These results suggest that the N2- $\Delta 145$ virus is far more likely than the N2 virus to enter an abortive pathway in which the cell initially expresses GFP (and, presumably, other viral proteins) but subsequently either dies or otherwise fails to progress to the production of viral progeny that spread to infect neighboring cells. Moreover, on the uncommon occasion that productive infection is achieved, the rate of N2- $\Delta 145$ spread to neighboring cells is low compared to that of the N2 virus.

Because *gp145* was shown to bind to dsRNA and inhibit PKR in transfection assays (33), we evaluated its effect on the phosphorylation of eIF2 α , the primary substrate of PKR, during infection. By 2 days postinfection, the abundance of phosphorylated eIF2 α in cells infected with $\Delta 145$ was increased compared to that in cells that were mock infected and infected with the N13R10 virus (Fig. 3C). In this experiment, we pretreated the cells with interferon, but similar changes were also observed in the absence of interferon (data not shown). These results strengthen the hypothesis that antagonism of the PKR pathway by *gp145* is necessary for efficient GPCMV replication.

Attenuation of $\Delta 145$ virus in immunocompromised guinea pigs. Nonpregnant adult Hartley guinea pigs ($n = 4$ /group) were treated with cyclophosphamide (100 mg/kg, i.p.) and 1 day later were challenged with 10^7 PFU of $\Delta 145$ or N13R10 inoculated s.c. or were sham inoculated with PBS. Animals were weighed every 3 to 5 days, and additional cyclophosphamide (100 mg/kg, i.p.) was administered on day 14 postchallenge. Patterns of weight gain (measured as the amount of weight gained over time as a percentage of the total body weight) were very similar in the $\Delta 145$ -challenged animals and the sham (PBS)-inoculated group, but the patterns of weight gain were different in the N13R10-challenged group. Statistically significant differences in the patterns of weight gain were observed on days 6, 14, 17, and 20 postchallenge (Fig. 4). For example, on day 20 postchallenge, N13R10-challenged animals had a cumulative weight gain of 32%, whereas both the PBS

group and the $\Delta 145$ -challenged group had cumulative weight gains of 40% ($P < 0.05$ for the PBS group versus the N13R10 group and for the $\Delta 145$ group versus the N13R10 group; Fig. 4).

Blood samples were collected on days 7 and 14 postinfection, and lung and spleen tissue samples were harvested upon termination of the experiment on day 35 postinfection. DNA PCR was used to measure viral loads in blood and end organs as a measure of viral dissemination. As expected, blood and tissue viral loads were below the limits of detection for all sham-infected animals (data not shown). Surprisingly, all blood and tissue viral loads for $\Delta 145$ -challenged animals were also below the limits of detection. In contrast, all four N13R10-challenged animals had detectable DNAemia on day 7 postinfection, while half (2/4) remained DNAemic on day 14 postinfection. On day 7 the mean viral load in the N13R10 group was 6.3×10^3 genome copies/ml, whereas it was <100 genome copies/ml (the limit of detection) in $\Delta 145$ -challenged animals (Table 1; $P < 0.0001$, 2-way ANOVA with Bonferroni's correction).

At necropsy, 4/4 N13R10-challenged animals had viral DNA present in liver homogenate and 2/4 had viral DNA present in spleen homogenate. In contrast, 0/4 cyclophosphamide-treated animals challenged with the $\Delta 145$ virus had any demonstrable viral DNA in organ homogenates (Table 1). Animals challenged with N13R10 had a mean viral load in the liver of 92 copies/mg. In contrast, the amount of viral DNA in $\Delta 145$ -challenged animals was below the detection limit (1 genome copy/mg; $P = 0.002$, 2-way ANOVA with Tukey's posttest). Similarly, the spleens of N13R10-challenged animals had a significantly higher mean viral load of 430 copies/mg than the spleens of animals challenged with $\Delta 145$, in which the amount of viral DNA was below the limit of detection (<1 genome copy/mg; $P = 0.03$). Thus, even in highly immunosuppressed animals challenged with the $\Delta 145$ virus, no evidence of viremia or dissemination of virus to visceral organs was found, indicating that it was dramatically attenuated compared to the parental virus, N13R10.

Immune response to $\Delta 145$ vaccination. The immunogenicity and efficacy of the $\Delta 145$ virus used as a live attenuated vaccine were next evaluated in immunocompetent guinea pigs. Young, weanling female guinea pigs were immunized using two regimens: the low-dose group ($n = 8$) received two s.c. injections of 10^5 PFU $\Delta 145$ 1 month apart, while the high-dose group ($n = 8$) received two s.c. injections of 10^6 PFU $\Delta 145$ 1 month apart. In contrast to cyclophosphamide-treated adult animals (see the previous section), some animals exhibited low-grade DNAemia at 7, 14, or 21 days after immunization with the first dose of the $\Delta 145$ vaccine. A total of 3/16 animals had DNAemia at day 7 postvaccination (mean viral load, 2×10^3 genomes/ml), 1/16 animals had DNAemia at day 14 (mean viral load, 1.8×10^3 genomes/ml), and 5/16 animals had DNAemia at day 21 (mean viral load, 3.2×10^3 genomes/ml). In total, 8/16 animals had at least one positive value by qPCR postimmunization. This biphasic pattern of low-grade DNAemia in immunized animals suggested that some limited but measurable viral replication took place *in vivo*.

Antibody responses to GPCMV antigens measured by ELISA and neutralizing responses in sera obtained 21 days after each dose revealed that both regimens were highly immunogenic. ELISA responses were higher in the group immunized with 10^6 PFU than the group immunized with 10^5 PFU following both doses, although the differences were statistically significant only when the titers following the second dose were compared (Fig. 5A). Surpris-

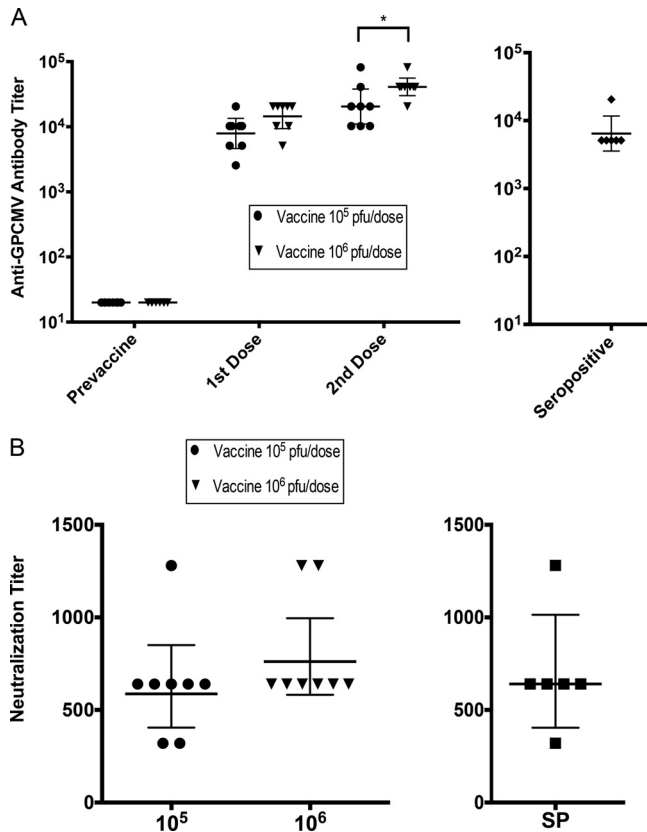


FIG 5 Immune responses to vaccination with $\Delta 145$ with comparisons to naturally seropositive animals. Young weanling female Hartley guinea pigs were vaccinated with two s.c. inoculations separated by 1 month, with each inoculum containing either 10^5 PFU or 10^6 PFU of $\Delta 145$ ($n = 8$ each group). (A) Anti-GPCMV antibody responses (geometric mean titers) were measured by ELISA using serum obtained 21 days after each inoculation (left). For comparison, the anti-GPCMV antibody titers for a group of six seropositive animals were determined in a separate experiment (right). Statistical analyses were conducted by the Kruskal-Wallis test, and the P values of the statistical significance of the differences between vaccine groups are provided in the Results. *, $P < 0.01$. (B) Neutralization titers were determined using serum obtained 21 days after the second vaccination, and neutralization assays were performed on guinea pig lung cells (as described in Materials and Methods) (left). The neutralization titers from a group of seropositive animals (SP; $n = 6$) were also determined in a separate experiment (right). Bars indicate the geometric mean titers \pm 95% confidence intervals.

ingly, these differences were not reflected in statistically significant differences in neutralizing antibody titers (Fig. 5B). In a separate experiment, the immune responses to vaccination were compared to the ELISA and neutralizing antibody responses in six naturally GPCMV-seropositive guinea pigs obtained from the same commercial vivarium that supplied the seronegative animals used in the vaccination study. Overall, the ELISA titers in the seropositive animals were significantly lower than the titers achieved after two doses of the $\Delta 145$ vaccine ($P < 0.01$ compared to the group immunized with 10^5 PFU and $P < 0.001$ compared to the group immunized with 10^6 PFU) and were comparable to those observed after a single dose of the $\Delta 145$ vaccine (Fig. 5A). These results indicate that one dose of $\Delta 145$ induced antibody responses comparable to those induced by natural infection, while two doses induced antibody responses superior to those induced by natural infection. The neutralizing responses following two doses of the

TABLE 2 Impact of $\Delta 145$ vaccination on pup mortality

Group	Total no. of litters	Pup mortality ^a	No. (%) of litters with dead pups
Control	6	26/31 (81)	6 (100)
$\Delta 145$ (10^5 PFU)	7	4/29 (14) ^b	2 (29)
$\Delta 145$ (10^6 PFU)	7	1/24 (4) ^b	1 (14)

^a The data represent the number of dead pups/total number of pups delivered to the vaccine or control groups (percent).

^b $P < 0.0001$ versus the control group.

$\Delta 145$ vaccine were statistically significantly equivalent to those induced by natural infection (Fig. 5B).

Pregnancy outcomes after challenge. Of the animals described in the previous section, one animal in each vaccine group and two animals in the unvaccinated control group did not become pregnant. Hence, pup outcome data were available for 7 dams from each vaccine group and 6 unvaccinated controls. All pregnant dams were challenged with SG-passaged GPCMV in the third trimester of pregnancy. Vaccination resulted in a significant reduction in pup mortality from 26/31 (81%) pups in the unvaccinated control group to 4/29 (14%) pups in the group vaccinated with 10^5 PFU of $\Delta 145$ ($P < 0.0001$) and 1/24 (4%) pups in the group vaccinated with 10^6 PFU of $\Delta 145$ ($P < 0.0001$; Table 2). All 6 litters in the control group had at least one dead pup; in contrast, 1/7 litters in the group vaccinated with 10^6 PFU and 2/7 litters in the group vaccinated with 10^5 PFU had at least one dead pup ($P < 0.005$ for the vaccine groups combined versus the control group, Fisher's exact test).

We also compared the weights of the pups in the vaccine and control groups as an indicator of the impact of vaccination on pup health. The weights of the pups from vaccinated dams were significantly improved, from a mean of 63.5 g for the control group to means of 96 g for the group vaccinated with 10^5 PFU and 95.6 g for the group vaccinated with 10^6 PFU (for both groups, $P < 0.0001$ versus the control group, Kruskal-Wallis and Dunn's multiple-comparison tests; Fig. 6).

Congenital infection and viral loads in dams and pups. The viral loads in dams were examined by qPCR on days 7 and 14 after SG-passaged GPCMV challenge. Preconception vaccination had a significant impact on the magnitude and duration of DNAemia compared to those in the unvaccinated control dams (Fig. 7A). All dams in the control group and the group vaccinated with 10^5 PFU

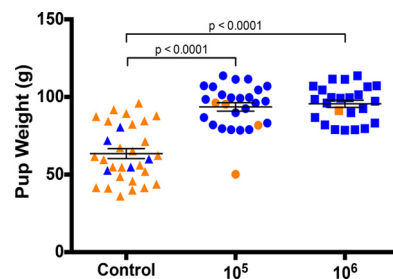


FIG 6 Vaccination with $\Delta 145$ improves pup weights after GPCMV challenge. Unvaccinated (control) guinea pigs or guinea pigs vaccinated with two inoculations of 10^5 PFU or 10^6 PFU of $\Delta 145$ were mated and challenged with SG-passaged GPCMV in the third trimester of pregnancy. The weights of pups born live (blue) or dead (orange) are shown; the P values were determined by the Kruskal-Wallis test and Dunn's multiple-comparison test.

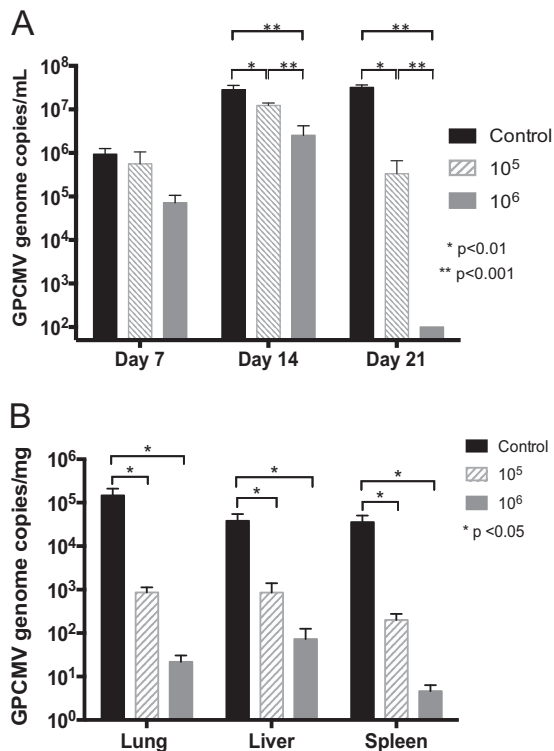


FIG 7 Vaccination with $\Delta 145$ reduces maternal and fetal viral loads. Unvaccinated (control) or $\Delta 145$ -vaccinated guinea pigs were challenged during pregnancy, and qPCR was used to determine blood viral loads in dams and tissue viral loads in pups. (A) Blood viral loads in dams on days 7, 14, and 21 postchallenge. No dams in the group immunized with 10^6 PFU had detectable DNAemia at day 21, and the dams were therefore assigned a value of 100 genomes/ml (the threshold of detection of DNAemia by the qPCR assay). Significant reductions in DNAemia in both vaccine groups were noted at day 14, and significant differences were observed when the results for the groups vaccinated with 10^5 and 10^6 PFU at days 14 and 21 were compared; P values were determined by ANOVA. (B) Viral loads in pup tissues determined by qPCR. Negative samples were assigned a value of 1 genome/mg (the threshold of detection for the qPCR assay) for the purposes of statistical comparison. Comparisons were performed using ANOVA with Bonferroni's multiple comparisons; P values are indicated.

of $\Delta 145$ and 6/7 dams in the group vaccinated with 10^6 PFU of $\Delta 145$ were DNAemic on day 7. Three dams in the control group died after delivering pups but prior to the day 14 viremia time point (two at day 8 after SG-passaged virus challenge and one at day 10 postchallenge). On day 14, only 2/7 dams in the group vaccinated with 10^6 PFU were DNAemic, whereas 7/7 dams in the group vaccinated with 10^5 PFU and 3/3 surviving dams in the control group were DNAemic. On day 21, 0/7 dams in the group vaccinated with 10^6 PFU and 5/7 in the group vaccinated with 10^5 PFU remained DNAemic, while 3/3 surviving dams in the control group were DNAemic. The magnitude of DNAemia in dams vaccinated with the 10^5 -PFU dose at both the day 14 and day 21 time points (1.2×10^7 and 3.3×10^5 genomes/ml, respectively) was statistically significantly lower than that in the unvaccinated controls (2.8×10^7 and 3.2×10^7 genomes/ml, respectively; $P < 0.01$). Vaccination with the 10^6 -PFU dose similarly reduced the mean blood viral loads compared to those in the controls ($P < 0.001$) at the day 14 and 21 time points (Fig. 7A). There were also statistically significant differences in viral loads at days 14 and 21

when the groups vaccinated with 10^6 and 10^5 PFU were compared ($P < 0.001$).

To compare the rates of congenital GPCMV transmission and the magnitude of the viral load in infected pups between the vaccine and control groups, real-time qPCR of liver, lung, and spleen tissue extracted from live-born and stillborn pups was performed. In total, 20/31 (65%) of pups from the control group had congenital GPCMV transmission, as evidenced by the fact that one or more samples were positive for GPCMV DNA by PCR. In comparison, there was a significant reduction in the rate of congenital transmission in pups born to dams from the group vaccinated with 10^6 PFU (8/24 [33%] pups were infected; $P < 0.05$ compared to the control group, Fisher's exact test). In the group vaccinated with 10^5 PFU, congenital transmission was observed in 19/29 (66%) pups (P was not significant when the results were compared to those for the controls; Table 3).

Viral loads in pup lung, liver, and spleen tissues were also reduced by vaccination. Viral loads in infected pups born to vaccinated dams that received the 10^6 -PFU dose were reduced by 500- to 7,000-fold compared to those in pups born to the control dams (Fig. 7B). In the control group, mean viral loads were 1.5×10^5 , 3.8×10^4 , and 3.6×10^4 genome copies/mg tissue in lung, liver, and spleen tissue, respectively, while the mean viral loads in the 10^6 -PFU-dose group were 22, 73, and 5 genome copies/mg tissue in lung, liver, and spleen tissue, respectively ($P < 0.05$ for all tissues compared to the control group). For the group vaccinated with 10^5 PFU of $\Delta 145$, the mean viral loads were 8.6×10^2 , 8.6×10^2 , and 2×10^2 genomes/mg tissue in lung, liver, and spleen tissue, respectively ($P < 0.05$ compared to the control group).

DISCUSSION

The strategy of attenuating live viral vaccines by deletion of PKR antagonists is founded on the idea that these viruses replicate poorly or not at all *in vivo* yet express a large set of viral proteins prior to the shutoff of protein synthesis that typically occurs at later stages of infection. Unchecked accumulation of dsRNA may also enhance the immune response through the activation of PKR and other dsRNA-sensing pathways. This approach has had success in the vaccinia virus (VACV) system, where a VACV lacking E3L (48, 49) provides a useful comparison. Similar to the PKR-inhibitory genes of cytomegaloviruses (26–33, 50), vaccinia virus E3L binds to and sequesters dsRNA and thereby prevents activation of type I interferon-induced PKR and oligoadenylate synthetase (51). In a primate study, an E3L gene deletion was engineered into the New York City Board of Health (NYCBH) strain of vaccinia, and the resulting virus (NYCBH Δ E3L) was examined for

TABLE 3 Impact of $\Delta 145$ vaccination on congenital GPCMV transmission

Group	Transmission ^a		Overall transmission rate ^b
	Live born	Stillborn	
Control	3/5	17/26	20/31 (65)
$\Delta 145$ (10^5 PFU)	17/25	2/4	19/29 (66)
$\Delta 145$ (10^6 PFU)	8/23	0/1	8/24 (33) ^c

^a The data represent the number of pups to which virus was transmitted/number of pups in the live-born and stillborn groups.

^b The data represent the number of pups to which virus was transmitted/total number of pups, whether stillborn or live-born (percent).

^c $P < 0.05$ versus the control group.

its ability to protect cynomolgus macaques from heterologous challenge with monkeypox virus (35). Vaccination with the NYCBHΔE3L vaccine provided some protection of macaques from death, although it was unable to completely protect from the morbidity associated with monkeypox disease. It was proposed in this study that a minimum threshold of antigen was likely required to obtain an effective repertoire of neutralizing antibodies following NYCBHΔE3L immunization and that this antigen threshold may not have been met due to the severe attenuation for replication induced by the E3L mutation (35).

In the current report, we describe the construction of a recombinant GPCMV with a deletion of the PKR inhibitor *gp145* for use as a live attenuated vaccine. GPCMV lacking *gp145* replicated poorly in cell culture, particularly when inoculated at a low MOI. Peak titers were reduced by more than 2 log units compared to those of the parental *gp145*-positive virus, and the time required to reach the peak titer was delayed by 1 to 2 weeks. Using GFP-tagged viruses, it was further revealed that deletion of *gp145* results in a high incidence of abortive infection and a reduced rate of focus expansion. Infection of cells with Δ145 led to increased levels of eIF2α phosphate. These results are consistent with a presumed mechanism in which *gp145*-null viruses are unable to block the PKR-mediated shutdown of protein translation at later stages of infection. However, while *gp145*-null viruses retained some limited replication capability, human and murine cytomegaloviruses deleted of their known PKR antagonists are fully defective for replication in cell culture (27, 32). This difference raises the possibility that, like HCMV, GPCMV may encode a second, as yet unidentified PKR antagonist that enables Δ145 to replicate to a limited extent. It is also possible that guinea pig GPL cells have some inherent deficiency in the PKR pathway compared to cells used for studying the replication of human or mouse cytomegaloviruses.

In vivo the *gp145*-null virus Δ145 was highly attenuated for replication and dissemination in adult immunocompromised guinea pigs, as evidenced by improved weight gain and the absence of DNAemia or detectable viral DNA in end organ tissues, in marked contrast to the results for the parental N13R10 virus. Although there was no evidence of DNAemia in immunocompromised guinea pigs, there was evidence of transient DNAemia following the first vaccination of 8/16 young female weanling guinea pigs. The reason for the discrepancy is unclear, but it may be related to differences in the age or sex of the animals used in the two experiments. Irrespective of these differences, the Δ145 virus was clearly highly attenuated, although it was not completely disabled for replication *in vivo* following immunization of young female weanling guinea pigs.

Despite a high level of attenuation, Δ145 administered as a preconception vaccine was highly immunogenic, inducing robust ELISA and neutralizing antibody responses and resulting in improved pup outcomes following the third-trimester challenge of pregnant dams. Pups born to dams that received the 10⁶-PFU-dose vaccine had substantially reduced mortality relative to that of the unvaccinated controls (4% and 81%, respectively), and the rate of congenital GPCMV transmission was reduced (33% and 65%, respectively). Viral loads were also substantially reduced in pups born to Δ145-immunized dams, particularly in the 10⁶-PFU-dose group (reductions of 525-fold in liver tissue, 6,700-fold in lung tissue, and 7,700-fold in spleen tissue compared to the loads in the controls). However, there was clearly a dose depen-

dence in the magnitude of protection, with the 10⁶-PFU dose consistently being associated with significantly improved reductions in mortality, transmission, and viral load compared to the values obtained with the 10⁵-PFU dose. The difference in efficacy was apparently not due to the higher neutralizing antibody responses conferred by the 10⁶-PFU dose, although total antibody responses, measured by ELISA, were significantly higher after the second dose of vaccine in the group immunized with the 10⁶-PFU dose than in the group immunized with the 10⁵-PFU dose (Fig. 5A). It is possible that the 10⁶-PFU dose resulted in a stronger antibody-dependent cytotoxicity response, a more robust cytokine response, or a better cell-mediated immune response.

The protection conferred following immunization with the Δ145 vaccine was comparable to that conferred by natural GPCMV seropositivity (23) or by preconception vaccination with adjuvanted, purified recombinant gB (22, 37), on the basis of comparisons of our results with those of other studies. Mortality data were previously evaluated in a study in pups born to naturally GPCMV-seropositive dams challenged with SG-passaged virus, using the same experimental design used in this study (23). In that study, five pregnant dams were challenged with the same dose of SG-passaged virus used in this study, and pup mortality was 29% (4/14), which is statistically significantly comparable to the mortality rates that we observed in Δ145-immunized dams. In studies from the CIDMTR laboratory of purified, recombinant gB vaccine adjuvanted with Freund's adjuvant, pup mortality rates have ranged from 14 to 36% and vertical transmission rates have ranged from 22 to 73% (22, 37). Thus, the metrics of protection conferred by the Δ145 vaccine compare very favorably to those associated with natural seropositivity or adjuvanted protein subunit immunization reported previously.

The results for the Δ145 vaccine also compare favorably with those for other live attenuated vaccine approaches tested in the GPCMV model. A study of a recombinant GPCMV vaccine deleted of three putative major histocompatibility complex class I homologs, 3DX (23), demonstrated that preconception vaccination conferred a reduction in pup mortality (22%) of approximately 2-fold compared to that for unimmunized controls (44%). Another study of a live attenuated GPCMV vaccine, v545 (engineered to have a deletion of a viral chemokine homolog of macrophage inflammatory protein 1α), demonstrated a reduction in the rate of pup mortality from 70% to 15% (41).

Both N13R10 and Δ145 have a 4-bp deletion that frameshifts the *gp129* ORF (25) and renders these viruses unable to assemble a pentameric complex (PC) composed of gH, gL, *gp129*, *gp131*, and *gp133* (42–44, 52). In this respect, they resemble the HCMV Towne strain variants that comprise the Towne vaccine, which also contains a mutation that disrupts expression of a PC subunit (17). This inability of the Towne virus to assemble a functional PC may contribute to the Towne vaccine's attenuation (53) and may limit its efficacy by impairing the vaccine's ability to induce epithelial entry-neutralizing antibodies directed against the PC (54). The potential to enhance the immunogenicity and protective efficacy of the Δ145 vaccine by restoring a wild-type *gp129* sequence will require further study.

In summary, deletion of the GPCMV PKR antagonist *gp145* resulted in a virus that was highly attenuated in nonpregnant immunosuppressed guinea pigs yet, when given as a preconception vaccine, protected against maternal viremia, congenital transmission, and pup mortality. In previous studies, live tissue culture

attenuated GPCMV vaccines were able to cause congenital infection (23). Notably, the $\Delta 145$ virus demonstrated no evidence of end organ replication but nonetheless served as an effective vaccine in a dose-dependent fashion. This suggests that $\Delta 145$ is unlikely to be transmitted *in utero*, an important safety consideration for any analogously engineered live HCMV vaccine, particularly if inadvertent administration occurred in a pregnant woman. The antibody titers observed following $\Delta 145$ immunization were comparable to those observed following natural infection or immunization with other live attenuated GPCMV vaccine candidates (23, 41). Interestingly, we observed a dose-response effect on the magnitude of protection favoring the group vaccinated with 10^6 PFU, although neutralizing antibody titers were not statistically significantly different between the two groups. The magnitude of the cell-mediated immune response may have favored the group vaccinated with 10^6 PFU, and this will be an important area for future study. Although the $\Delta 145$ vaccine protected against GPCMV mortality in pups and reduced the viral load in both pups and dams postchallenge, sterilizing immunity was not observed. However, the inoculum of SG-passaged GPCMV used for this study was high (10^5 PFU) and delivered by a nonphysiologic route of challenge. Challenge by a more physiologic route, such as the intravaginal (55) or intranasal route, might demonstrate improved protection against maternal or fetal infection. Efforts to enhance the antibody response by employing $\Delta 145$ immunization in the context of a prime-boost approach that includes an adjuvanted recombinant glycoprotein(s) may help confer enhanced protection in the GPCMV model (56) and may be a strategy relevant to future human clinical trials. Although the gp145 mutant was able to replicate sufficiently to generate virus for vaccination, for ideal production strategies, it may be necessary for stocks to be propagated on complementing cells, particularly for an HCMV vaccine deleted of all PKR antagonists. Additionally, the fact that sterilizing immunity protecting against congenital GPCMV transmission was not conferred by the $\Delta 145$ vaccine may relate to the inability of the virus to form a functional PC, based on the known mutation in the *gp129* gene in the parent N13R10 virus (25). Generation of a *gp145* deletion against the background of a *gp129*-repaired BAC will help clarify the potential for the development and optimal design of HCMV vaccines based on knockout of PKR-inhibitory functions.

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